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Peroxynitrite and Opiate Antinociceptive Tolerance: A Painful Reality

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Chronic severe pain is a significant global health problem [1]. In the US alone, one third of Americans suffer some form of chronic pain, and in these individuals over 30% of reported pain is resistant to analgesic therapy [1]. The economic impact of pain is equally large at approximately $100 billion annually [1]. While selective cyclooxygenase-2 (COX-2) inhibitors are effective for several forms of chronic pain, their occasional side-effects including increased risks of heart attack and stroke [2] prompted the precipitous withdrawal of some of them (i.e. Vioxx) from the market in 2004.

Morphine sulfate and other opiate/narcotic analgesics are the most effective treatments for acute and chronic severe pain. However, their clinical utility is often hampered by the development of analgesic tolerance as well as by *de novo* painful hypersensitivity to innocuous and noxious stimuli with such phenomena observed in both animal and human studies [3;4;5]. For morphine in particular, development of tolerance necessitates escalating doses to achieve equivalent pain relief [6], even as the onset of morphine-induced hypersensitivity subverts the therapeutic impact of such dose increases [3;4;5]. This complex pathophysiological cycle contributes significantly to decreased quality of life in the growing population of subjects with chronic pain due to oversedation, reduced physical activity, respiratory depression, constipation, potential for addiction, and other side-effects [6]. Accordingly, there is growing interest in new approaches that would maintain opiate efficacy during repetitive dosing without engendering tolerance or unacceptable side-effects. Considerable evidence implicates nitroxidative stress in the development of pain of several etiologies and importantly in opiate antinociceptive tolerance, caused by the presence of superoxide, $O_2^{\cdot -}$, nitric oxide, ·NO and more recently peroxynitrite ($ONOO^-$ or its protonated counterpart ONOOH) that is the product of their interaction (Figure 1). In addition to the 3 routes of reducing $ONOO^-$ toxicity depicted in Figure 1, there is a fourth: scavenging of the radicals from ONOOH (urate, methionine and tyrosine peptides are examples in this category) [7].

The objectives of this first mini-review written on peroxynitrite and morphine antinociceptive tolerance are to discuss the importance of nitroxidative stress in this process and argue that peroxynitrite is a rational target for therapeutic intervention in pain management. These concepts provide a pharmacological basis for developing inhibitors of peroxynitrite biosynthesis as novel non-narcotic analgesics, thus addressing a large and currently unmet medical need with major socioeconomic consequences.

Morphine-induced antinociceptive tolerance: Is there a role for peroxynitrite?

Prolonged use of opiates results in antinociceptive tolerance, such that higher doses are required to achieve equivalent analgesia [6] or antinociception [5;8;9]. Adaptative modifications in cellular responsiveness, particularly desensitization and downregulation of opioid receptors, underlie this phenomenon [10]. By contrast, a competing hypothesis is that stimulation of opioid receptors over time triggers activation of anti-opioid systems that reduce sensory thresholds, thus causing hypersensitivity to tactile stimulation (allodynia) and noxious thermal stimulation (hyperalgesia) [8;11;12]. As a corollary, such opioid-induced hypersensitivity paradoxically diminishes the net analgesic effect of the opioid agonist [8;11;12]. *In vivo* support for this alternative hypothesis has been found in animals [3;13;14] and in humans [4;15;16]. Thus, analgesic tolerance likely arises when pain facilitatory systems become sensitized or hyperactive after repeated opioid use.

The mechanisms by which prolonged opiate exposure induce tolerance and hypersensitivity remain unclear, although a role for peroxynitrite-mediated nitroxidative stress has been identified [17]. Peroxynitrite is a potent pro-inflammatory and pro-apoptotic reactive species [18;19;20] and a potent inducer of hyperalgesia (defined as augmented pain intensity in response to painful stimuli) [21]. Besides its role in the development of morphine-induced antinociceptive tolerance that will be reviewed herein, peroxynitrite is also implicated in the development of hyperalgesia associated with acute and chronic inflammation and in response to spinal activation of the N-methyl-D-aspartate (NMDA) receptor [22;23;24] (Figure 1). We reasoned that since inhibiting formation of peroxynitrite precursors ($O_2\cdot^-$ or ·NO) blocks the development of morphine antinociceptive tolerance, then peroxynitrite is most likely the common and final signaling mediator of nitroxidative stress accompanying antinociceptive tolerance [17]. In support, it has been repeatedly shown that non-selective inhibitors, as well as those selective for iNOS and nNOS, prevent development of morphine-induced antinociceptive tolerance [25;26;27;28;29;30;31;32;33;34]. These beneficial effects of NOS inhibition were associated with attenuation of spinal neuroimmune activation and reduced release of pro-inflammatory and pro-nociceptive cytokines, achieved at least in part by blocking redox-sensitive transcription factors such as p38 MAPK [17;35;36;37;38]. While links among morphine hypersensitivity, tolerance and ·NO production clearly exist, the contributions of different isoforms by pharmacological, antisense and genetic approaches remain controversial. In general nNOS is considered the primary source, although evidence also implicates iNOS [32;39]. A defined role of eNOS must await development of selective inhibitors of this isoform; one study using eNOS knockout mice indicated that these animals develop tolerance in a manner similar to wild types [32]. Inhibition of $O_2\cdot^-$ formation with superoxide dismutase mimetics blocks tolerance events and is associated with suppressed spinal formation of TNF-α, IL-1βand IL-6, and reduced apoptosis [17]. Repeated administration of morphine in rodents promotes the nitration and thus the enzymatic inactivation of spinal manganese superoxide dismutase (MnSOD). Consequently, morphine may provide a critical source of spinal peroxynitrite that contributes to the development of morphine antinociceptive tolerance through three well-defined biochemical pathways within the dorsal horn of the spinal cord: (1) post-translational nitration of proteins involved in glutamate homeostasis (2) neuroimmune activation (release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin (IL)-1β, and IL-6) and (3) neuronal apoptosis [17]. Thus, reducing $ONOO^-$ formation either *indirectly* (with nitric oxide synthase inhibitors or superoxide dismutase inhibitors) or *directly* (using pharmacological approaches to catalytically decompose $ONOO^-$) inhibits these three events [17]. Collectively then, experimental evidence points to peroxynitrite as a canonical signaling molecule in morphine antinociceptive tolerance. The mechanisms leading to nitroxidative stress upon repeated administration of morphine during the development of antinociceptive tolerance are not known to date but are the subject of current investigation in my laboratories. However a link between

morphine and oxidative stress has been documented. For example, morphine-induced $O_2\cdot^-$ production seems to occur as a result of activation of μreceptors, leading to the activation of the phospholipase D pathway, and an increase in Ca^{2+}, leading to the activation of NADPH oxidase; generation of superoxide through this pathway evokes apoptosis in macrophages [40;41]. Furthermore, morphine has been shown to exert oxidative stress in various cells including cells that we know play a critical role in antinociceptive tolerance namely neurons, microglial cells and astrocytes [42;43]. Another potential source for peroxynitrite in response to repeated administration of morphine and the development of antinociceptive tolerance includes the activation of NMDA receptors and glial cells. *Why?* Substantial evidence has been gathered over the last decade to demonstrate that NMDA receptor activation as well as the activation of glial cells play a key role in the development of morphine tolerance since NMDA receptor antagonists, inhibitors of glial cell activation and anti-cytokine therapies block morphine antinociceptive tolerance [26;35;44;45;46;47;48;49;50;51;52]. NMDA receptor activation and glial cell activation release the precursors in the formation of $ONOO^-$ namely $O_2\cdot^-$ and ·NO [53;54;55;56;57;58;59;60]. We therefore propose that repeated administration favors the formation of $ONOO^-$ as a result of at least in part μ receptor activation, NMDAR activation and glial cell activation (Figure 2). In this paradigm the enzymatic sources in $ONOO^-$ formation include NOS (already discussed), nitration and enzymatic inactivation of MnSOD (*vide infra*) and activation of the NADPH oxidase. The $O_2\cdot^-$ -generating enzyme, NADPH-oxidase, is dormant in resting cells and produces superoxide only upon activation. Unlike the regulation of NOS, the principal regulation of NADPH oxidase is post-translational and depends on assembly of several membrane-bound and cytosolic components to form an active enzyme complex. In resting cells, the enzyme consists of two membrane-bound components, gp91phox and p22phox, and several cytosolic components, including p47phox, p40phox, p67phox, and rac1-2 [61]. Gp91phox is a flavocytochrome and the catalytic core of the enzyme. Upon activation, the cytosolic components translocate to the membrane and associate with membrane components to form an assembled, activated, and superoxide-producing enzyme complex. Although this enzyme is best characterized in immune cells and leukocytes for its involvement in superoxide production, it is now known that various protein components of NADPH oxidase are expressed in neurons, astrocytes and microglia [61;62;63;64]. These include the following NADPH oxidase subunits: gp91phox, p22phox, p40phox, p47phox, and p67phox [64;65]. Furthermore, a recent study with hippocampal slices has demonstrated a link between NMDA and production of superoxide through NADPH oxidase [66]. Cytokines such as TNF-α and IL-1β activate this enzyme and activated glial cells generate $ONOO^-$ by iNOS and NADPH oxidase leading to neuronal death [58;59;67;68]. Importantly, superoxide autoaugments superoxide formation by upregulating gp91phox creating a self-perpetuating cascade [67]. The role of this enzyme in superoxide formation during pathological settings is supported by the following observations. First, apocynin, a well-known inhibitor of the NADPH-oxidase prevents serine phosphorylation of p47phox, and blocks its association with gp91phox [69;70]. This blunts NADPH oxidase activation leading to beneficial effects in animal models of oxidative stress including rheumatoid arthritis, diabetes, atherosclerosis, neurodegeneration, stroke and ischemia-reperfusion injuries [71;72;73;74;75;76;77;78]. Second, these pharmacological observations are supported by genetic approaches demonstrating that mice lacking a functional NADPH oxidase subunit (gp91phox) show substantial decrease in $O_2\cdot^-$ and $ONOO^-$ formation and reduced oxidative stress in animal models [79]. Our preliminary results have shown that besides nitration and enzymatic inactivation MnSOD, the NADPH oxidase is also an important target source in the generation of $ONOO^-$ via $O_2\cdot^-$. Thus, co-administration of morphine with apocynin, a well-characterized specific inhibitor of this enzyme blocked antinociceptive tolerance (Salvemini, manuscript in preparation). As discussed above NOS activation will provide ·NO, the second precursor in $ONOO^-$ formation.

Role of peroxynitrite in the development of morphine antinociceptive tolerance: Proposed molecular and biochemical pathways

A. Post-translational nitration and protein modification

Considerable evidence supports the notion that a key biologically relevant feature of peroxynitrite is post-translational tyrosine nitration and consequent modification of protein function [80;81;82]. The biological importance of post-translational nitration is thus underscored by compelling evidence linking this phenomenon to diseases driven by overt production of peroxynitrite including sepsis, ischemia/reperfusion injury, cancer, neurodegenerative disorders [22;23;83;84;85;86;87;88], and more recently for pain and opiate antinociceptive tolerance [17;21;23;24;89].

A1: Protein nitration and superoxide/peroxynitrite homeostasis—Several proteins are nitrated, a modification associated with loss, gain or change of function [90;91;92]. A key example of lost enzyme activity due to nitration *in vivo* is mitochondrial MnSOD that normally keeps concentrations of superoxide under tight control [93]. The MnSOD protein is nitrated by peroxynitrite on Tyr-34 by a Mn-catalysed process which leads to enzyme inactivation [94]. Nitration of MnSOD, and its subsequent enzymatic inactivation, favor the accumulation of peroxynitrite which then nitrates and alters additional proteins and receptors, thereby perpetuating and extending the initial damage [80;81;82;95]. To determine likely sources of sustained production of peroxynitrite during antinociceptive tolerance, we asked whether nitration/inactivation of MnSOD was a possibility. Our studies revealed that repeated administration of morphine leads to spinal nitration and enzymatic inactivation of MnSOD and that inhibition of peroxynitrite blocks nitration, restores the enzymatic activity of the enzyme and blocks tolerance suggesting the key role of nitrated MnSOD as a source of peroxynitrite in tolerance [17]. Interestingly, St. Clair and colleagues reported that when activated glial cells release cytokines such as TNF-α, iNOS is induced in neighbouring neurons; as a consequence formation of ·NO-derived peroxynitrite in such neurons nitrates MnSOD causing neuronal cell death [96]. Their results led us to postulate that nitration and inactivation of MnSOD contributes to the neuronal death often accompanying antinociceptive tolerance, and this hypothesis is being evaluated in our laboratory.

A2: Protein nitration and glutamate homeostasis—Dysfunction of the glutamatergic pathway is a key component of nociception [3;35;36;46;56]. Peroxynitrite alters glutamate homeostasis through post-translational nitration and modification of key proteins involved in maintaining a normal glutamate balance. Indeed research in diverse fields including amyotrophic lateral sclerosis and septic shock have demonstrated that peroxynitrite nitrates and inactivates 1) NMDA receptors [97;98;99], 2) the transport activity of sodium-dependent high-affinity glutamate transporters (GTs) [100;101] and 3) glutamine synthase [102;103; 104]. While these excitatory amino acid transporters also transport cysteine, for simplicity we shall refer to them as glutamate transporters GTs, and not excitatory amino acid transporters, EAATs.

We will next discuss why these observations are critically important in the context of morphine-induced antinociceptive tolerance and associated hyperalgesia: Glutamate neurotransmission, in particular that mediated via NMDA receptors under chronic pain conditions, is fundamentally involved in the development of opioid tolerance, especially tolerance arising from μ-opioid receptor stimulation [26;44]. cDNA cloning has revealed that the NMDA receptor is formed by several NMDA receptor subunits. The coexpression of NR1 with various NR2 subunits is required for a fully functional ion channel receptor and the combined expression of NR1 with different NR2 subunits results in channel with distinct pharmacological and physiological properties that define NMDA receptor heterogeneity

[105]. Peroxynitrite interacts with the NMDA receptor leading to nitration of the tyrosine residues present on the NMDA receptor subunits. This nitration is an irreversible reaction that leads to a constant potentiation of synaptic currents, calcium influx, and ultimately excitotoxicity [97;98;99].

Glutamate, as the primary endogenous ligand for the NMDA receptor, is not metabolized by extracellular enzymes but must be removed from the synaptic cleft by cellular uptake. Thus, homeostasis of extracellular glutamate is tightly regulated by GTs in the plasma membranes of both neurons and glia [106;107;108;109]. There are five membrane GTs, termed GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5 [110]. Of these, GLAST and GLT-1 are localized primarily to astrocytes and EAAC1, EAAT4 and EAAT5 to neurons. EAAT4 and EAAT5 are restricted to cerebellar Purkinje cells and the retina, respectively, whereas EAAC1 is widely expressed in the CNS [111]. Astrocyte glutamate transporters are limited to glutaminergic synapses, whereas EAAC1 is detected diffusely over cell bodies and processes [112]. Three glutamate transport protein subtypes isolated in the spinal cord [GLAST and GLT-1 associated with glial cells, and EAAC1 associated with neurons [113;114;115; 116;117;118]], are considered essential to maintain low resting levels of glutamate (< 1μM), and to prevent overstimulation of GTs [108;119;120;121;122]. Knockdown expression of GLAST or GLT-1 in rats using antisense oligonucleotides increased the extracellular glutamate concentration [123]. Notably, these glutamate transport proteins are concentrated in the superficial dorsal horn of the spinal cord and are responsible for > 80% of total glutamate transport [110]. In elucidating potential mechanisms of morphine-induced antinociceptive tolerance and hypersensitivity, activation of NMDA receptors can lead to neurotoxicity under many circumstances [124;125;126;127]. Thus, peripheral nerve injury has been shown to activate spinal cord NMDA receptors, causing intractable neuropathic pain and neuronal apoptosis [128;129;130;131]. Furthermore, crosstalk between the pathways underlying opioid tolerance and neuropathic pain has been proposed, suggesting that a common cellular mechanism may be causal in both conditions [3;132]. Extending this reasoning, it is possible that the cellular process leading to the development of opioid tolerance may also cause neurotoxic changes in response to prolonged opioid administration [133]. Thus, a number of studies indicate that functional glutamate transporters prevent glutamate neurotoxicity under both physiological and pathological conditions [101;108;109;121;134]. In brain tissue, decreases in GLT-1 mRNAs have been observed after naloxone-precipitated morphine withdrawal [135]. Of note, the activity of glutamate transporters decreases during morphine tolerance and is associated with spinal apoptosis [136]. Glutamate transporter inhibitors, or GT activators such as MS-135, increase and decrease respectively the development of spinal apoptosis, hyperalgesia and tolerance [136;137]. In addition, agents such as dexamethasone or amitryptiline attenuate analgesic tolerance to morphine in part by preventing the downregulation of glutamate transporters, with consequent reduction in synaptic levels of glutamate [138;139]. Not unexpectedly, nitration of GLT-1 by peroxynitrite inhibits its glutamate transport capacity and causes excitotoxicity [103].

Besides regulating synaptic levels of glutamate, these GTs play a crucial role in the uptake of cysteine, and thus contribute to the overall thiol redox state of cells that is regulated by intracellular levels of glutathione (GSH). GSH plays a critical role in protecting cells from oxidative stress as well as maintaining the thiol redox state. GSH depletion enhances oxidative stress leading to neuronal degeneration as shown in several studies [140;141]. GSH is a tripeptide composed of glutamate, cysteine and glycine. In neurons, cysteine is the rate-limiting substrate for GSH synthesis [142] and in neurons approximately 90% of total cysteine uptake is mediated by EAATs [143;144;145]. Thus, EAAC1 transports cysteine at a rate comparable to that of glutamate, with an affinity 10- to 20-fold higher than that of GLAST or GLT-1 [146]. Recent studies have shown that peroxynitrite-mediated nitration of EAAC1 in neurons reduces the uptake capacity of cysteine leading to a depletion of intracellular GSH and neuronal

cell death [100]. Integrating these findings, tolerance could develop due to excitotoxicity from increased synaptic concentrations of glutamate and a decrease in neuronal thiol redox state due to decreased intracellular levels of cysteine and thus GSH. We are currently evaluating such a concept.

In contradistinction to the central role of GTs in regulating the homeostasis of extracellular glutamate, glutamine synthase (GS) plays a pivotal role in glutamate's intracellular metabolic fate. Once taken up into glial cells, glutamate is converted into nontoxic glutamine by endogenous GS [147]. In the brain, GS is located mainly in astrocytes; a primary roles of these cells is to protect neurons against excitotoxicity by taking up excess ammonia and glutamate, converting them into glutamine [detoxification of ammonia by GS will not be discussed here for simplicity]. Studies have shown that in glutamatergic brain areas, the distribution of both glial glutamate receptors and glial transporters parallels the location of GS suggesting a functional coupling between the two systems to prevent damage [148;149;150]. Furthermore, through feedback regulation, a decrease in GS activity can reduce the activity of GTs [148]. Thus, dysfunctional glutamate metabolism likely contributes to antinociceptive tolerance [133;137;138;139]. These observations prompted us to show that post-translational tyrosine nitration of spinal glutamate transporters (GLT-1) and GS by peroxynitrite contributes to the development of antinociceptive tolerance to morphine [17]. Increased levels of glutamate can be decreased by reducing the production of cytokines such as TNF-αand IL-6 that have been shown to inhibit glutamate uptake [151]. Since peroxynitrite increases cytokine production (*vide infra*) it is likely that peroxynitrite modulates glutamate homeostasis via the cytokine signaling pathway.

B: Inflammation

Peroxynitrite is a potent pro-inflammatory nitroxidative species with an established role in "neuronal inflammation" (defined here as neuroimmune activation which includes activation of glial cells and release of proinflammatory *cytokines)* [35;45;46;47;48;49;50;51;52]. Chronic administration of morphine promotes activation of spinal cord glial cells, as well as production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and spinal sensitization [35; 36;52]. Thus, inhibitors of glial cell metabolism and/or anti-proinflammatory cytokine approaches block morphine-induced antinociceptive tolerance and hyperalgesia [35;36;52]. In addition, other anti-inflammatory agents including dexamethasone [138] [152], non-steroidal anti-inflammatory drugs [45;51], IL-10 [48], NOS inhibitors [27;28;45], p38 kinase inhibitors [38] and superoxide dismutase mimetics [153]have been shown to inhibit morphine-induced antinociceptive tolerance and hyperalgesia. The possible mechanisms for chronic morphine-induced glial cell activation are not known with certainty. Although μ-opiate receptors are present on microglia and astrocytes [154], acute administration of morphine does not activate these cells [52]. On the other hand, morphine primes glial cells for enhanced production of pro-inflammatory cytokines [155]. In inflammation, peroxynitrite induces endothelial cell damage and increased microvascular permeability [156;157], activates redox-sensitive transcription factors including NF-κ B and AP-1 that in turn regulate genes encoding various pro-inflammatory and pronociceptive cytokines genes such as interleukin-1β, tumor necrosis factor-α and interleukin-6 (IL-1β, TNF-α and IL-6 respectively [158;159;160;161;162;163; 164]. Peroxynitrite also up-regulates adhesion molecules such as ICAM-1 and P-selectin to recruit neutrophils at sites of inflammation [163;165], auto-catalyzes the destruction of neurotransmitters and hormones such as norepinephrine and epinephrine [166;167], lipid peroxidation and oxidation [20]. In the development of morphine antinociceptive tolerance, inhibition of peroxynitrite formation with NOS inhibitors, superoxide dismutase mimetics or decomposition of peroxynitrite with peroxynitrite decomposition catalysts, block spinal formation of IL-1β, TNF-α and IL-6 [17]. The cyclooxygenase (COX) pathway has also been implicated in tolerance. In animals, a number of studies have confirmed that neuronal

cyclooxygenase (COX) activity contributes to the expression of opioid tolerance and that certain COX inhibitors can be used for the prevention, and even the reversal of morphine tolerance [168;169] As discussed previously in this review article, it has also been established that NOS inhibitors can effectively attenuate opioid tolerance [25;26;27;28;29;30;31;32;33;34;39] In this setting, another potential molecular pathway by which peroxynitrite may influence the development of antinociceptive tolerance is through the constitutive (COX-1) and inducible (COX-2) enzymes. A significant body of experimental evidence suggests a relationship between NO biosynthesis and PG generation [170;171;172]. As originally reported by our group [173] and subsequently extended by several other investigators [170;172;174;175;176;177] the COX enzymes (constitutive COX-1 and inducible COX-2) are "receptor targets" for the multifaceted action of ·NO and as such are regulated in its presence. Although the mechanisms by which ·NO activates COX enzymes remain undefined, we now know that $ONOO^-$ is involved in this activation through the oxidative inactivation and/or modification of key amino acids residues in the COX polypeptide backbone [178;179]. Other possibilities in this complex reaction biochemistry have been raised and discussed in detail [172;176;180]. In addition to effects on COX-2 enzyme activity, ·NO and $ONOO^-$ increase the production of PGs from macrophages by acting post-transcriptionally or translationally to increase COX-2 protein levels or to increase its mRNA stability, at least in part through $O_2{}^{\cdot-}$ and the p38 MAPK pathway [174;175;181;182;183;184]. Furthermore, iNOS binds COX-2, and iNOS-derived ·NO increases the catalytic activity of COX-2 through S-nitrosylation in a macrophage cell line [185]. Furthermore, and as discussed nitroxidative species activate transcription factors such as AP-1 and NF-*k*B as well as mitogen activated protein kinases (MAPK) such as p38 MAP kinase, which is known to induce COX-2 protein expression during inflammation [159;161;162;186]. Substantial evidence supports the conclusion that the activation or induction of COX enzymes by nitro-oxidative stress augments the production of pro-inflammatory and pro-nociceptive prostaglandin PGE_2 (PGE_2) at sites of inflammation [170;173]. It is therefore likely that the beneficial effects of peroxynitrite decomposition catalysts are due to suppressed production of local and spinal pro-inflammatory and pronociceptive cytokines and prostaglandins.

C: Apoptosis

Peroxynitrite is a potent pro-apototic and cytotoxic molecule and a role for spinal neuronal apoptosis in morphine antinociceptive tolerance is well established [136;187;188]. Peroxynitrite is considered the major oxidant responsible for DNA strand breakage which then activates the nuclear enzyme poly(ADP-ribose) polymerase (PARP). Rapid activation of PARP depletes the intracellular concentration of its substrate, nicotinamide adenine dinucleotide, thus slowing the rates of glycolysis, electron transport, and subsequent ATP formation [189]. Exposure of neurons to high concentrations of peroxynitrite more often leads to rapid necrosis due to acute, severe cellular energetic derangements [18;190]. In contrast, lower concentrations of peroxynitrite can lead to delayed, apoptotic neuronal death [191]. Peroxynitrite-induced apoptosis is similar to other forms of oxidant/free radical mediated apoptosis in being dependent on activation of caspases-2, -3, -8 and -9 [192;193;194]. Mitochondria are key sites of cellular death and constitute a primary locus for the intracellular formation and reactions of peroxynitrite [80]. Peroxynitrite-mediated inactivation of mitochondrial MnSOD favors more peroxynitrite formation, resulting in positive feedback processes that promote mitochondrial dysfunction and the triggering of apoptotic signaling of cell death, including activation of PARP and caspases [195;196;197;198]. As discussed previously, peroxynitrite also causes neuronal death via nitration of MnSOD following activation of neurons by glial cell-derived cytokines [96].

Previous reports have implicated apoptosis in antinociceptive tolerance and associated hypersensitivity. Indeed, chronic morphine exposure causes apoptosis in the spinal cord dorsal

horn as determined by *in situ* terminal deoxynucleotidyl transferase (TdT)-mediated dUPT-biotin nick-end labeling (TUNEL) staining, upregulation of the pro-apoptotic caspase-3 and Bax proteins, and downregulation of the anti-apoptotic Bcl-2 protein [136;187;188]. Caspase-3 inhibitors that block apoptosis prevent the development of morphine hyperalgesia and antinociceptive tolerance [136;187;188]. Interestingly in these studies, apoptosis was found in neurons but not glial cells, although morphine can cause glial cell apoptosis [199]. The mechanisms of this morphine-induced apoptosis remain unclear. However, a role for peroxynitrite exists since its spinal inhibition during the development of antinociceptive tolerance to morphine, blocks oxidative DNA damage and PARP activation [17]. Taken together, these results provide a likely link between peroxynitrite, apoptosis and tolerance. Spinal PARP activation is seen during neuropathic pain and morphine tolerance where it induces excitotoxic transynaptic morphological changes in superficial dorsal horn “dark neurons” [129;132]. Preventing PARP activation with PARP inhibitors or with peroxynitrite decomposition catalysts inhibits the development of morphine antinociceptive tolerance [136;187]. It is therefore likely that the beneficial effects of peroxynitrite decomposition catalysts occur by attenuating neuronal and/or glial apoptosis during opiate-induced tolerance driven by PARP and caspase activation and nitration of MnSOD.

Concluding remarks and looking ahead—Considerable evidence over the years has supported the roles of ·NO and O_2·$^-$ as precursors of peroxynitrite, in the development of morphine antinociceptive tolerance. Since the rate of interaction between ·NO and O_2·$^-$ to form peroxynitrite is faster than the dismutation of O_2·$^-$ by superoxide dismutase, peroxynitrite formation from O_2·$^-$ and ·NO is the likely signaling molecule involved in antinociceptive tolerance [17] as in pain of several etiologies [23;24;89;200;201] (Figure 1). Because studies have only recently begun to unravel the role of peroxynitrite in antinociceptive tolerance and pain, few data are available to help understand the molecular and biochemical pathways engaged by this nitro-oxidative species. To date we know that peroxynitrite contributes to peripheral and central sensitization by increasing production of pro-inflammatory cytokines, by activating PARP, and modulating the cyclooxygenase pathway to increase the production of proinflammatory and pronociceptive PGE_2 (activation of COX-1 and COX-2 and induction of COX-2) [21]. Peroxynitrite is also involved in neuroimmune activation, apoptosis and post-translational nitration and modification of key proteins known to be implicated in central and peripheral sensitization [17;23;89] (Figure 2). Additionally, nitroxidative species may be involved more subtly in central sensitization at least in part by sensitizing wide dynamic range neurons in the dorsal horn [202]. Importantly for eventual clinical management, the peroxynitrite decomposition catalysts evaluated to date apparently synergize with non-selective COX-1/COX-2 inhibitors and selective COX-2 inhibitors, and do so at greatly reduced doses. This synergism should minimize the obvious side effects of either drug class when coadministered [21]. Considering the many molecular, biochemical, and pharmacological similarities between opiate-mediated antinociceptive tolerance and the hypersensitivity associated with chronic neuropathic pain, the broader implication of our proposed studies is that peroxynitrite is a viable therapeutic target in both disease states (Figure 1). We believe that continued research in this field will soon provide a valid pharmacological basis for developing peroxynitrite-based therapeutic targets as adjuncts or alternatives to opiates (or other analgesics such as NSAIDs) in the management of pain and in particular chronic pain.

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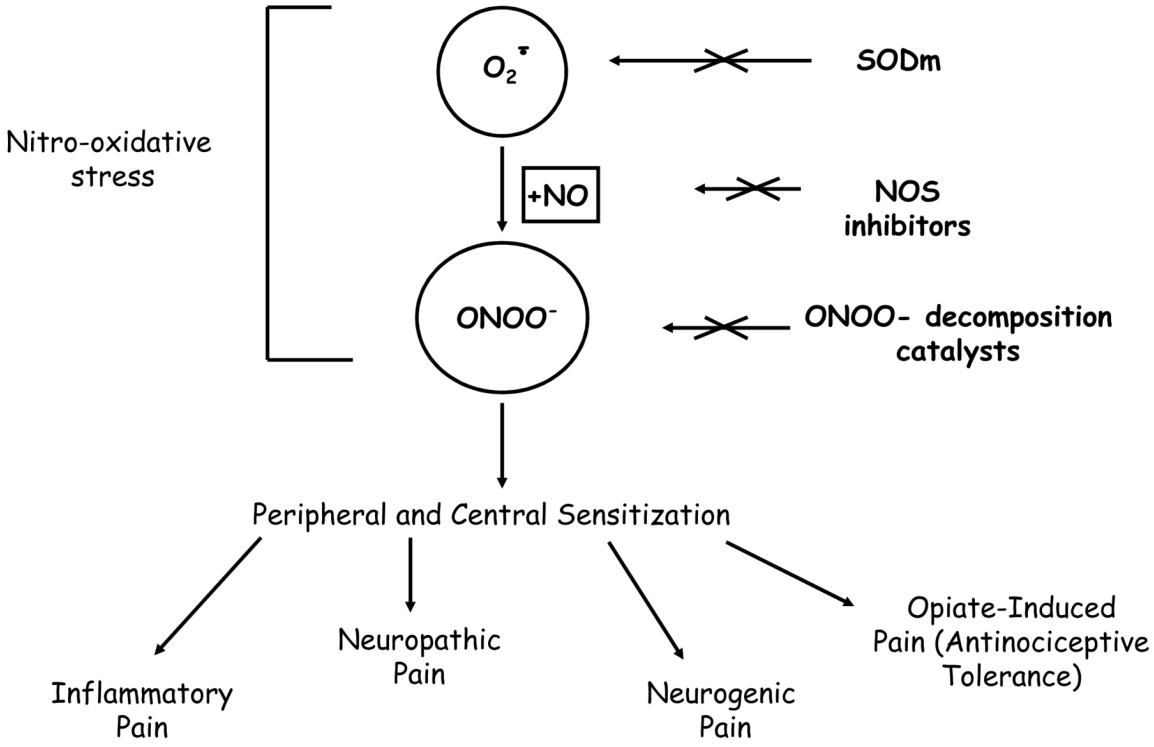

Figure 1.
Peroxynitrite (ONOO-) Mediated Nitro-Oxidative Stress in Pain

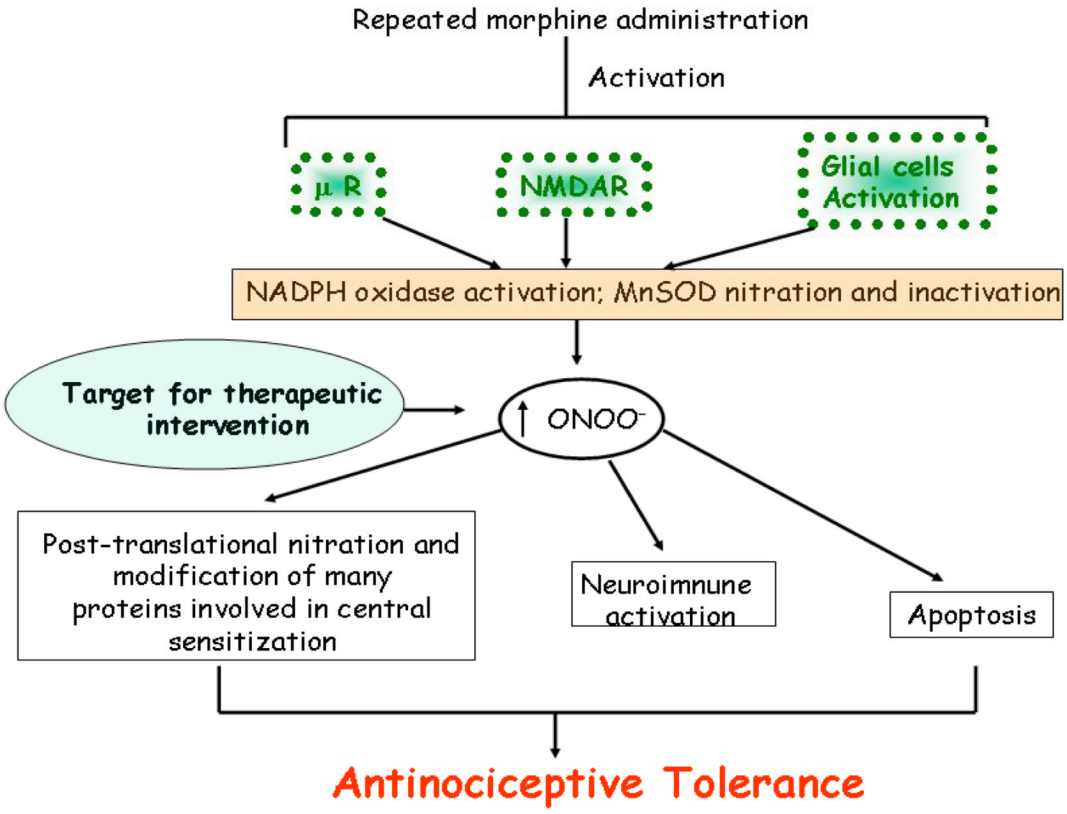

Figure 2.
Peroxynitrite a Viable Target for Novel Therapeutic Intervention in Pain