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ÁLVARO JOSÉ CHÁVEZ SILVA

POTENCIAL TERAPÊUTICO DE PORFIRINAS DE MANGANÊS EM MODELO EXPERIMENTAL DE NEUROPATIA INDUZIDA POR CONSTRIÇÃO DO NERVO ISQUIÁTICO

ILHÉUS, BAHIA
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Tese apresentada à Universidade Estadual de Santa Cruz, como parte das exigências para obtenção do título de Doutor em Ciência Animal.

Linha de pesquisa: Biotecnologia e experimentação animal

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Orientador: Prof. Dr. Mário Sérgio Lima de Lavor

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Mário Sérgio Lima de Lavor – DSc.
UESC/DCAA
Orientador/Presidente da banca examinadora

_____

Juneo Freitas Silva – DSc.
UESC/DCB

_____

Rosana Maria de Oliveira Clark– DSc.
UESC/DCAA

_____

Graciane Aiello – DSc.
UFSM/CCR/HVU

_____

Juliana Filgueira da Silva - – DSc.
UFOP/DEFAR

ILHÉUS – BAHIA
2025

Dedico este trabalho as pessoas com dor crónica, que coabitam o avesso da sua pele com a constante sombra da dor. Quando pensei em desistir, pensei em vocês, e segui, buscando razões benévolas nos pequenos detalhes para chegar ao fim.

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“*Como uma intrusa, sequestra os neurônios;*

transformando uma proteção, num augúrio;

uma promessa, numa sentença,

e uma dracma, numa adaga.

Mas também uma pele, num agasalho;

e nos coloca frente ao espelho do mundo.

Uma lembrança da frágil humanidade,

e uma lição de resiliência em mim

e compaixão pelo outro...”

Álvaro Chávez S.

POTENCIAL TERAPÊUTICO DE PORFIRINAS DE MANGANÊS EM MODELO EXPERIMENTAL DE NEUROPATIA INDUZIDA POR CONSTRIÇÃO DO NERVO ISQUIÁTICO

RESUMO

A dor neuropática é caracterizada por alterações estruturais e funcionais nas membranas celulares, mitocondriais e no retículo endoplasmáticos, decorrentes de desequilíbrios dos sistemas redox, levando a disfunções neurais. As abordagens terapêuticas convencionais frequentemente apresentam eficácia limitada e efeitos adversos, tornando urgente a busca por novas estratégias. As porfirinas de manganês (MnP) possuem propriedades antioxidantes promissoras e podem representar uma alternativa para doenças neuroinflamatórias. Este estudo avaliou o potencial terapêutico das porfirinas de manganês, MnP I $[MnIIITE\text{-}2\text{-}PyP]^{5+}$ e MnP II $[MnIIIT5B3EPyP]^{5+}$ em modelo de dor neuropática induzida por lesão constritiva isquiática (LCI) em ratos. Foram determinadas a ED50 e LD50 pela via intratecal, analisados os mecanismos moleculares das MnP I e MnP II nas vias intraperitoneal e intratecal, além da avaliação do limiar mecânico nociceptivo (LMN) por meio do analgesímetro digital. Também foram dosadas as espécies reativas de oxigênio (ROS) e peroxinitritos (PRN), e expressão gênica de diversos biomarcadores moleculares de estresse oxidativo (*Cat, Sod1, Sod2, Gpx1* e *Ho-1*), inflamação *(Il-1b, Il-6, CX3CL1* e *CX3CR1*), apoptose (*Caspase-3, Caspase-9* e *Chop)*, estresse oxidativo do retículo endoplasmático (*Grp78, Atf6, Perk* e *Nrf2*) e fatores neuroproterores (*Hif-1a, e Gdnf*). Os animais tratados com MnP I e MnP II pela via intratecal apresentaram um aumento significativo do LMN em comparação ao grupo controle positivo (PCG). A DL50 de MnP I pela via intratecal foi de 0,08 mg/kg e a ED50 foi de 0,004 mg/kg. Os animais tratados por via intraperitoneal com MnP I demonstraram um aumento significativo do LMN em comparação ao PCG. Além disso, a redução na produção de ROS e PRN, aliada à modulação da expressão de genes associados ao estresse oxidativo, inflamação, apoptose e neuroproteção, reforça o potencial terapêutico dessas moléculas. Assim, as porfirinas de manganês representam uma abordagem inovadora no tratamento da dor neuropática, destacando-se como potenciais candidatas para intervenções clínicas futuras.

Palavras-chave: dor crônica; estresse oxidativo; MnP; neuropatia; inflamação.

THERAPEUTIC POTENTIAL OF MANGANESE PORPHYRINS IN AN EXPERIMENTAL MODEL OF NEUROPATHY INDUCED BY SCIATIC NERVE CONSTRICTION

ABSTRACT

Neuropathic pain is characterized by structural and functional alterations in cellular, mitochondrial and endoplasmic reticulum membranes, resulting from redox system imbalances, leading to neural dysfunctions. Conventional therapeutic approaches often present limited efficacy and adverse effects, making the search for new strategies urgent. Manganese porphyrins (MnP) have promising antioxidant properties and may represent an alternative for neuroinflammatory diseases. This study evaluated the therapeutic potential of manganese porphyrins, MnP I $[MnIIITE\text{-}2\text{-}PyP]^{5+}$ and MnP II $[MnIIIT5B3EPyP]^{5+}$ in a model of neuropathic pain induced by ischial constrictive injury (SCI) in rats. The ED50 and LD50 were determined by the intrathecal route, the molecular mechanisms of MnP I and MnP II were analyzed in the intraperitoneal and intrathecal routes, in addition to the evaluation of the mechanical nociceptive threshold (MNT) by means of the digital analgesia. Reactive oxygen species (ROS) and peroxynitrites (PRN) were also measured, as well as gene expression of several molecular biomarkers of oxidative stress (*Cat*, *Sod1*, *Sod2*, *Gpx1* and *Ho-1*), inflammation (*Il-1b*, *Il-6*, *CX3CL1* and *CX3CR1*), apoptosis (*Caspase-3*, *Caspase-9* and *Chop*), endoplasmic reticulum oxidative stress (*Grp78*, *Atf6*, *Perk* and *Nrf2*) and neuroprotective factors (*Hif-1a*, and *Gdnf*). Animals treated with MnP I and MnP II intrathecally showed a significant increase in LMN compared to the positive control group (PCG). The LD50 of MnP I intrathecally was 0.08 mg/kg and the ED50 was 0.004 mg/kg. Animals treated intraperitoneally with MnP I demonstrated a significant increase in LMN compared to PCG. Furthermore, the reduction in ROS and PRN production, combined with the modulation of gene expression associated with oxidative stress, inflammation, apoptosis and neuroprotection, reinforces the therapeutic potential of these molecules. Thus, manganese porphyrins represent an innovative approach in the treatment of neuropathic pain, standing out as potential candidates for future clinical interventions.

Keywords: chronic pain; oxidative stress, MnP; neuropathy; inflammation.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

$_1O^2$	Oxigênio singlete
4-HNE	4 – Hidroxynonena
8-OHdG	8-hidroxi-2'-deoxiguanosine
8-OxodG	8-oxo-7,8-dihidro-2'-deoxiguanosine
AD	Doença de Alzheimer
ALS	Esclerose lateral amiotrófica
AP-1	Proteína ativadora 1
AS	Aterosclerose
ATF6	Fator de transcrição ativador 6
BDNF	*Brain-derived neurotrophic factor* (Fator neurotrófico derivado do cérebro)
CAT	Catalase
CHOP	Proteína homologada ao C/EBP
$CO_3^{\bullet-}$	Ânion radical carbonato
Cys	Cisteína
EGF	*Epidermal growth fator* (fator de crescimento epidérmico)
eNOS	NO sintase endotelial
ER	Retículo endoplasmático
ERK	Quinase regulada extracelularmente
ERN	Espécie reativa de nitrogênio
ERO	Espécie reativa de oxigênio
ERO-1	ER-Oxidorreductina 1
ETC	*Electron transfer chain*
ETC	Cadeia de transporte de elétrons
FAD	Flavina adenina dinucleotídeo
GH	Glutationa
GLUT1	Transportador de glucose 1
GPx	Glutationa peroxidase
GR	Glutationa redutase
GRP78/BIP	*Immunoglobulin heavy chain binding protein*
GSH	Glutationa reducida
GSSG	Glutationa oxidada
H_2O_2	Peróxido de hidrogênio
HIF-1α	Fator induzível por hipoxia 1α
$HO^{\bullet}$	Radical hidroxila
HOCL	Ácido hipocloroso
$HOO^{\bullet}$	Radical hidroperoxil
IL	Interleucina
iNOS	NO sintase induzível
IRE-1α	*Inositol – requiring kinase 1*
IRI	*Ischemic reperfusion injury* (lesão de isquemia – reperfusão)
JNK	c-Jun NH_2 quinase terminal
KEAP1	proteína 1 associada a ECH do tipo Kelch

LCI	Lesão constritiva isquiática
LOOH	Peróxidos lipídicos
M	Molar
MAPK	Proteína quinase ativada por mitógeno
MCU	Canal uniporter mitocondrial
MDA	malondialdeído
Met	Metionina
MnP	Manganese porphyrin
mtNOS	NO sintase mitocondrial
NAD	Nicotinamida adenina dinucleotídeo
NCX	Trocadores de Na^{+}/Ca^{2+}
NF-kB	Factor nuclear kB
NGF	*Nerve growth fator* (fator de crescimento neural)
nNOS	NO sintase neuronal
NO	Óxido nítrico
NOX	NADPH oxidase
NRF2	Fator 2 relacionado ao fator nuclear eritróide 2
$O_2^{\bullet-}$	Radical ânion superóxido
$ONOO^{-}$	Peroxinitrito
PDI	Proteína dissulfeto isomerase
PERK	*Protein kinase (PKR) - like endoplasmic reticulum kinase*
PG	Prostaglandina
PI3K	Fosfatidilinositol 3 quinase
PIP	Fosfatidilinositol fosfato
PIP2	Fosfatidilinositol bifosfato
PIP3	Fosfatidilinositol trifosfato
PMCA	Ca^{2+} ATPase da membrana plasmática
PPP	*Pentose phosphate pathway* (via das pentoses – fosfato)
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Radical peroxil
ROS	Reactive oxygen species
$RS^{\bullet}$	Radical tiil
S	Segundo
SERCA	Ca^{2+} ATPase do retículo sarcoplasmático
SOD	Superóxido dismutase
Tyr	Tirosina
VGF	*Vascular growth fator* (fator de crescimento vascular)
XO	Xantina oxidase

SUMARIO

1 INTRODUÇÃO

A dor crônica é uma das principais fontes de sofrimento e incapacidade humana (BURMA et al., 2017). Embora a própria dor e muitas doenças associadas à dor crônica não são imediatamente fatais, elas diminuem significativamente a qualidade de vida de pessoas adultas e animais não humanos em todo o mundo. Esse impacto é especialmente grave em pacientes com dor crônica, nos quais distúrbios do sono, ansiedade e depressão são frequentes e intensos, constituindo-se como fator de risco associado a idealização de suicídio, tentativas e morte por suicídio, em quem o risco é duplamente agravado. (GOLDBERG; MCGEE, 2011; RACINE, 2018). A prevalência de vida de idealização de suicídio e tentativas de suicídio é de 28.9% e 10.8%, respectivamente, em pessoas com dor crônica (DIBLASI et al., 2024)**.** Já o risco de morte por suicídio é três vezes maior pacientes diagnosticados com dor crônica (THEMELIS et al., 2025).

A dor neuropática, definida pela Associação Internacional para o Estudo da Dor (IASP – siglas em inglês) como dor causada por lesão ou doença do sistema nervoso somatossensorial, é descrita como ardor, queimação, agulhadas, dor congelante ou compressivo. Associa-se à necessidade de maior prescrição de medicamentos para obter efeitos desejados, o que pode acarretar efeitos adversos devido ao aumento nas doses e visitas frequentes a profissionais de saúde. (COLLOCA et al., 2017; “Terminology, IASP,” [s.d.]). Na inflamação crônica, a hiperalgesia é o resultado de um estado persistente de sensibilização periférica aferente, que conduz à sensibilização espinhal por liberação do glutamato em interneurônios GABAérgicos o que leva à inibição pré-sináptica e produção de radicais livres e espécies reativas de oxigênio e nitrogênio (ROS/PRN) (ILARI et al., 2020c).

O estresse oxidativo é conhecido como o desequilíbrio entre os sistemas oxidativo e antioxidante celular, que envolve lesão celular e interrupção da sinalização redox. Isso conduz a efeitos pro-inflamatórios, como dano celular endotelial e aumento da permeabilidade microvascular, recrutamento de neutrófilos nos locais de inflamação (SALVEMINI et al., 1999), destruição autolítica de neurotransmissores e hormônios, peroxidação lipídica e oxidação, dano de DNA e ativação de poli-ADP-ribose polimerase (PARP) (MUSCOLI et al., 2003, 2010; XU et al., 2008).

O papel do estresse oxidativo é amplamente reconhecido em diversos distúrbios do SNC, uma vez que a produção excessiva de ROS, ultrapassando a capacidade dos sistemas

antioxidantes celulares, pode resultar na disfunção dos principais sistemas celulares mitocondriais e do retículo endoplasmático (RE), aumentando a concentração ROS/PRN durante as condições de estresse (CHENG et al., 2015; FIGUEIRA et al., 2013; ILARI et al., 2020c). Entre os danos causados por ROS estão a função mitocondrial alterada e estresse do RE, ambos desempenhando papéis importantes nesses distúrbios neurais (ROUSSEL et al., 2013).

A remoção de ROS/PRN por antioxidantes pode prevenir os achados característicos associados à dor, inativando o desenvolvimento de hiperalgesia, redução da formação de malondialdeído (MDA) e 4-hidroxynonena (4-HNE), bem como a modificação pós-translacional das proteínas celulares (ILARI et al., 2020a; NISTICÒ et al., 2013), restaurando assim a atividade das enzimas endógenas (ILARI et al., 2020a).

Diversas metaloporfirinas têm sido identificadas como catalizadores potentes de numerosas reações redox; particularmente porfirinas de manganês (MnP), que foram encontradas como catalizadores antioxidantes eficientes (BATINIC-HABERLE; SPASOJEVIC, 2014; CARBALLAL et al., 2018). Estas substâncias dismutam íons superóxidos com algumas das maiores taxa conhecidas por catalisadores sintéticos (RAUSARIA et al., 2011b) e têm demonstrado efeito terapêutico em condições em que o aumento da produção de ROS/PRN é um fator patológico chave (ALI et al., 2013).

A pesquisa do potencial terapêutico de $[Mn^{III}TE\text{-}2P\text{-}PyP]^{5+}$ (MnP I) com propriedades antioxidantes estudadas em modelos de acidente vascular cerebral e câncer (BATINIC-HABERLE et al., 2012; RATCLIFFE et al., 2017) e de $[Mn^{III}T(5\text{-}Br\text{-}3\text{-}E\text{-}Py)P]^{5+}$ (MnP II), ainda não testada em modelos experimentais de doenças associadas a dor neuropática, é necessária para a elucidação dos potenciais usos clínicos das MnP, a partir da compreensão das vias e mecanismos que subjazem a dor crônica de origem neuropática, considerando as suas diferenças estruturais e químicas.

Estudos sugerem que um aumento nas ROS está associado ao desenvolvimento da dor neuropática (JANG; LEE, 2011; TEIXEIRA-SANTOS; ALBINO-TEIXEIRA; PINHO, 2020; TREVISAN et al., 2016). Este estudo visa testar a hipótese de que a administração de Porfirinas de Manganês diariamente levaria uma redução de ROS e uma modulação no sistema antioxidante endógeno por essas MnP, e uma redução na neuroinflamação, com consequente redução da nocicepção nos pacientes acometidos por dor neuropática.

2 OBJETIVO GERAL

Avaliar o potencial terapêutico de Porfirinas de Manganês, MnP I [Mn^{III}TE-2P-PyP]$^{5+}$ e MnPII [Mn^{III}T(5-Br-3-E-Py)P]$^{5+}$ na dor neuropática induzida por constrição do nervo isquiático em ratos.

3 OBJETIVOS ESPECÍFICOS

1. Avaliar o efeito de MnP I e MnP II sobre o limiar mecânico nociceptivo em modelo de dor neuropática induzida por constrição do nervo isquiático em ratos.

2. Avaliar curva dose-resposta da administração intratecal de MnP I e MnP II em modelo de dor neuropática induzida por constrição do nervo isquiático em ratos.

3. Comparar o efeito de MnP I e MnP II sobre a produção de espécies reativas de oxigênio e peroxinitritos, administradas pelas vias intraperitoneal e intratecal, em modelo de dor neuropática induzida por constrição do nervo isquiático em ratos.

4. Avaliar o efeito de MnP I e MnP II sobre marcadores moleculares de inflamação nas vias de administração intraperitoneal e intratecal em modelo de dor neuropática induzida por constrição do nervo isquiático em ratos.

5. Investigar os possíveis mecanismos de ação das MnP I e MnP II na modulação do estresse de retículo endoplasmático na dor neuropática.

4 REVISÃO DE LITERATURA

1 INTRODUCTION

Silva, Á.J.C.; de Lavor, M.S.L. Nitroxidative Stress, Cell—Signaling Pathways, and Manganese Porphyrins: Therapeutic Potential in Neuropathic Pain. *Int. J. Mol. Sci.* **2025**, *26*, 2050. https://doi.org/10.3390/ijms26052050

Chronic pain is a major source of human suffering and disability (BURMA et al., 2017). Although pain itself and many diseases associated with chronic pain are not immediately fatal, they significantly reduce the quality of life of adults and animals worldwide. This impact is especially severe in patients with neuropathy, in whom sleep disorders, anxiety and depression are frequent and intense, constituting a risk factor in suicide attempts (GOLDBERG; MCGEE, 2011; RACINE, 2018). Neuropathic pain is a specific type of chronic pain, described by persistent or intermittent spontaneous pain, increased response to mechanical and thermal stimuli, either innocuous or noxious (allodynia or hyperalgesia, respectively), that arises from lesions to the somatosensory nervous system of peripheral or central origin (FINNERUP et al., 2015), ectopic activity in sensorial fibers, and imbalance of inhibitory and excitatory neurotransmitters relation, with involvement of neuroinflammation and nitroxidative stress playing a pivotal role in its instauration and maintenance (MOBASHERI et al., 2018; TEIXEIRA-SANTOS; ALBINO-TEIXEIRA; PINHO, 2020). It is associated with the need for more prescription medications to obtain desired effects, which can lead to adverse effects due to increased doses and frequent visits to health professionals (COLLOCA et al., 2017; “IASP Terminology - IASP”, [s.d.]).

Although neuropathic pain is a multifaceted condition driven by several cellular and molecular mechanisms, those considered key to its development and perpetuation are neuroinflammation, glial cell activation, release of proinflammatory cytokines, and oxidative stress (TEIXEIRA-SANTOS; ALBINO-TEIXEIRA; PINHO, 2020), contributing to altered signaling pathways, which facilitates the expression of further inflammatory mediators and enhanced sensitivity to pain, ultimately, maintaining the cycle of pain where neuroinflammation and oxidative stress influence each other (JI et al., 2018; MOBASHERI et al., 2018).

Oxidative stress is known as the imbalance between the cellular oxidative and antioxidant systems, and is widely recognized in chronic pain conditions, since excessive ROS production, exceeding the capacity of cellular antioxidant systems, can result in dysfunction of

the main cellular mitochondrial and endoplasmic reticulum (ER) systems, increasing ROS/PRN concentration during stress conditions (HASHEMI et al., 2024; ILARI et al., 2020b; SINGH et al., 2020; ZAMANI et al., 2025).

Initial research on free radicals primarily focused only on oxygen, with its poisonous characteristics being noted. These toxic properties were theorized and explained due to partially reduced forms of oxygen, known as free radicals (GERSCHMAN et al., 1954). Also, through evolution, living organisms have not only adapted to the coexistence with free radicals, but have developed strategies to use these molecules to its own advantages in metabolic relevant physiological processes (DI MEO; VENDITTI, 2020). Free radicals are molecules highly reactive with unpaired electrons in the outer valence, which makes them inherently chemically unstable and prone to react with surrounding molecules to achieve stabilization (DI MEO; VENDITTI, 2020). Free radicals can be produced exogenously from ionizing radiation, ozone, cigarette smoking, or by-products of environmental metabolisms. Endogenous sources, as those derived from oxygen (reactive oxygen species, ROS) or nitrogen (reactive nitrogen species, RNS), are produced through enzymatical or non-enzymatical processes, for instance, in the electron transfer chain (ETC), phagocytosis, cytochrome P-450 system, and prostaglandin (PG) synthesis during normal neuronal functioning or during neuroinflammation processes associated to neuropathic pain, either accepting an electron from or donating an electron to other molecules, therefore acting as oxidants or (LOBO et al., 2010; TELEANU et al., 2022). Such chemical behavior, when excessive, generates an environment suitable for damage on molecules as DNA, carbohydrates, lipids, and proteins, leading to homeostasis disruption and cellular damage, and ultimately conducting to apoptosis and lowering enzymatic and non – enzymatic antioxidants and cell viability in (KIM et al., 2010; OSMANLIOĞLU; NAZIROĞLU, 2025).

A great amount of information has been developed around free radical chemistry, leading to better understandings of their biological significance, beyond the concept of harmful uncontrolled molecules. Importantly, it is known that free radicals originate in normal physiological conditions, and have several physiological functions in cellular growth, differentiation, migration, apoptosis, and necrosis, as well as defense against pathogens and species evolution (LOBO et al., 2010; SIES; JONES, 2020) Additionally, increasing information sustains the role of reactive species in normal synaptic plasticity and cognitive functions such as memory and learning (MASSAAD; KLANN, 2011; MOLDOGAZIEVA et

al., 2018), and transducing agonistic modulation during neuropathic pain (TREVISAN et al., 2016).

Consequently, understanding the complex dynamics of free radical production, and their role on even more complex oxidative stress and neuroinflammation processes, as well as enzymatical antioxidants systems, as a target to alleviate such pathophysiological states, can lead to the development of researches regarding the treatment of chronic pain states, reducing associated symptoms like, anxiety, depression, self-mutilation and suicide that can manifest in these conditions (BARNHAM; MASTERS; BUSH, 2004), through the myriad of therapeutic targets intrinsic of the pro-oxidants and antioxidants mechanisms.

Several metalloporphyrin have been identified as potent catalysts for numerous redox reactions; particularly manganese porphyrins (MnP), which have been found to be efficient antioxidant catalysts (BATINIC-HABERLE; SPASOJEVIC, 2014; CARBALLAL et al., 2018). These substances dismutate superoxide ions at some of the highest rates known for synthetic catalysts and have demonstrated therapeutic effects in conditions where increased ROS/PRN production is a key pathological factor (ALI et al., 2013), overcoming major conventional monotherapy approaches, usually unsuccessful and accompanied with cumulative adverse effects.

Research into the therapeutic potential of MnP with antioxidant properties studied in disease models involving oxidative stress (BATINIC-HABERLE et al., 2012; RATCLIFFE et al., 2017) and in models of chronic pain (RAUSARIA et al., 2011b) considering their structural, chemical, pharmacokinetic and pharmacodynamic properties, is necessary to elucidate the clinical uses of MnP, based on understanding the pathways and mechanisms that underlie chronic pain of neuropathic origin.

This review examines the nature of ROS/RNS, endogenous antioxidant systems, nitroxidative signaling in neuropathic pain, and the potential of synthetics MnP in the restitution of neuronal redox homeostasis, which is crucial for understanding scavengers' therapeutic strategies, considering that neuropathic pain conditions still face significant limitation towards effective treatments on human and non – human animals.

2. REACTIVE OXYGEN AND NITROGEN SPECIES

Molecular oxygen and nitrogen are undeniably significant in biology, being essential for proper metabolism, cellular function, and the physiological state of eustress in living organisms. While these molecules play critical roles in structural and functional processes such as signal transduction, gene transcription and expression, and other cellular activities, they can also exert harmful effects on biomolecules in the form of reactive oxygen and nitrogen species, thereby illustrating their dual physiological and pathological nature(SINGH et al., 2019).

It is important to note that reactive species and free radicals are not always the same. Besides free radicals, certain non-radical molecules, like lipid peroxides (LOOHs), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCL), also have significant oxidizing potential. (NAKAI; TSURUTA, 2021). Broadly speaking, oxygen- and nitrogen-containing molecules with oxidizing power are termed "reactive oxygen species" (ROS) and "reactive nitrogen species" (RNS) (ALI et al., 2020; DI MEO; VENDITTI, 2020).

Since most of reactive species' effects relies on its compartmental concentration and its chemical nature, it is of relevant concern understand their main subcellular sources, allowing the compartmentalization of the reactive species gradients, redox-mediated regulation and aiding in cell-signaling from several membrane receptors (BEDARD; KRAUSE, 2007; MURPHY, 2009; SIES; JONES, 2020). In this section, we discuss chemistry and sources of ROS and RNS in the context of neuropathic pain.

2.1. Reactive oxygen species: chemistry and sources

Oxygen stands by far as the most vital element, for it bears the basis of biological oxidations, where most of the available energy for aerobic organisms is produced (MILLER; BUETTNER; AUST, 1990). Yet in excessive amounts, rends toxic effects, at both cellular and systemic levels. Over the course of evolution, organisms have evolved intricate mechanisms to regulate the intake and utilization of this crucial element (ZHENG; STORZ, 2000). The well-known toxic effect of oxygen is attributed to its chemical structure. Molecular oxygen in its simplest form has a unique electronic configuration, which makes it a radical itself. When an electron is added to the primary structure, the superoxide anion ($O_2^{\bullet-}$), also known as the primary ROS, is formed. $O_2^{\bullet-}$ will interact with other available molecules to generate secondary ROS (VALKO; MORRIS; CRONIN, 2005). Given that ROS are electrically charged, they will tend to neutralize themselves, reacting with other molecules, causing its oxidation, either reversibly or irreversibly, intrinsically increasing the phosphorylation (activity) of various

proteins kinases and reducing the phosphatase activity of other proteins; the overall effect appears to enhance neuronal responsivity to sensorial inputs, although, high sustained levels of ROS might excessively oxidize proteins and membrane lipids, causing abnormal responsiveness of neurons to afferent inputs, inducing central sensitization and pain (PARK et al., 2006; SALVEMINI et al., 2011). These deleterious effects occur due to structural alteration of organic biomolecules, such as, protein oxidation, DNA strand breakage through guanine residues oxidation, RNA oxidation, polyunsaturated fatty acids in bilipid membranes oxidation, mitochondrial depolarization causing, consequently, apoptosis, and oxidative stress (GRACE et al., 2016; MOBASHERI et al., 2018; SIES; JONES, 2020). Among the most relevant reactive species containing oxygen, exist hydroxyl radical (HO•), superoxide radical anion ($O_2^{\bullet -}$), hydroperoxyl radical (HOO•), and peroxyl radicals (ROO•); and non-radicals hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which in a series of transformation, involving gain or loss of electrons, leads to the changes in the charge and reactivity of the molecules **(figure 1)** (DRÖGE, 2002; HALLIWELL, 2006).

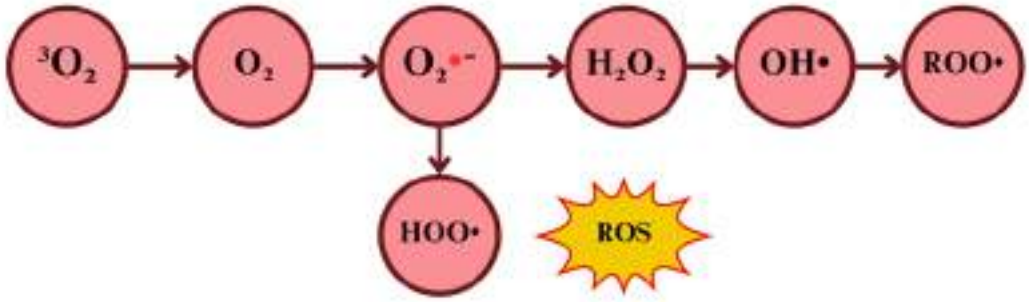
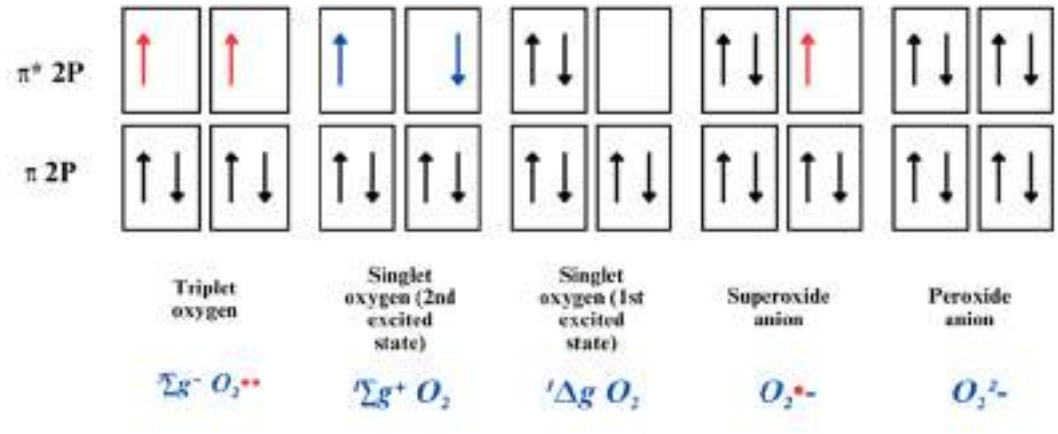


Figure 1 – Reactive oxygen species transformation from ; triplet oxygen ($^3O_2$), singlet oxygen (atmospheric) ($^1O_2$), superoxide anion ($O_2^{\bullet -}$), hydroperoxyl radical (HOO•), hydrogen peroxide (H_2O_2), hydroxyl radical (HO•), and peroxyl radicals (ROO•). ROS: reactive oxygen species.

2.1.1. Singlet oxygen

O_2 is considered a radical molecule due to the possession of two unpaired electrons with identical spin quantum numbers. The most stable form is triplet oxygen ($^3\sum g^- O_2$) also written $^3O_2$, as commonly present in the atmosphere **(Figure 2)**. It acts as a potent oxidant, and its spin arrangement enables it to readily interact with other molecules by accepting electrons individually, rather than releasing them. This specific characteristic clarifies why O_2 tends to react more outstandingly with radicals than non-radicals (KLOTZ; BRIVIBA; SIES, 2000).

The induction of an energy input can lead to the restructuration of electrons' arrangement; once one of the unpaired electrons gains energy, the electron is excited and changes its spin, resulting in the creation of a more reactive dioxygen molecule, known as singlet oxygen (AL-SHEHRI, 2021; TURRENS, 2003). This process eliminates unpaired electron spin restrictions, thereby enhancing its oxidative capacity, enabling interactions with proteins, DNA, and lipids (HALLIWELL, 2006). The predominant form observed in living organisms is typically the singlet oxygen in its first excited state ($^1\Delta gO_2$), as singlet oxygen in its second excited state ($^1\sum g^+O_2$) swiftly transitions to the former. Notably, due to the absence of unpaired electrons in its final spin, the first excited form naturally lacks a radical state (KLOTZ; BRIVIBA; SIES, 2000). Several membrane oxidases are able to perform one-electron



reduction of molecular oxygen, this results in the formation of superoxide radical anion ($O_2^{\bullet-}$) (FRIDOVICH, 1997).

Figure 2 – Outer valence and spin distribution of molecular oxygen in different electronic states. π*: antibonding orbital, π: bonding orbital, 2P: second level main energy of oxygen atomic orbital.

2.1.2. Superoxide radical anion

When an electron is added to O_2 outer orbital containing an impaired electron, a less radical molecules is formed, superoxide radical anion ($O_2^{\bullet-}$). This molecule possesses a single unpaired electron; therefore, it is less reactive than its preceding singlet oxygen (HALLIWELL, 2006). $O_2^{\bullet-}$ is considered a "primary" ROS, produced during the ETC in mitochondria, by electron leakage towards molecular oxygen prematurely, in the process of oxidative phosphorylation which is known to be implicated in a myriad of pathophysiology of diseases (KOVACIC et al., 2005). It can readily continue interacting with other molecules to produce "secondary" ROS, by means of direct interaction or via enzyme or metal-catalyzed processes (VALKO et al., 2004; VALKO; MORRIS; CRONIN, 2005).

In physiological conditions, most of the superoxide radical is present in its anionic form ($O_2^{\bullet-}$), and only a 0,6% is present in its protonated form, hydroperoxyl radical ($HOO^{\bullet}$); nonetheless, the catalyzed reaction with $HOO^{\bullet}$ is a lot faster ($k = 7 \times 10^9\ M^{-1}\ S^{-1}$) that the uncatalyzed reaction involving two $O_2^{\bullet-}$ molecules ($k < 0{,}3\ M^{-1}\ S^{-1}$) to produce the dismutation of both radical forms, to H_2O_2 and O_2, serving as a great source of H_2O_2 (DE GREY, 2004), which is of pronounced importance in the context of DNA damage, once $O_2^{\bullet-}$ does not react directly with DNA (JOMOVA et al., 2023; VALKO et al., 2004). Much of the nuclear DNA damage is restored by the cell, yet, mitochondrial DNA (mtDNA) cannot be fixed in the same manner, which conduces to accumulated damage over time, mitochondrial damage and cell death (VALKO et al., 2004). Additionally, the protonated form ($HOO^{\bullet}$) is uncharged and can easily migrate through lipidic membranes and induce the production of carbon-centered lipidic polyunsaturated radicals (SIES; JONES, 2020).

Increased levels of $O_2^{\bullet-}$ have been largely observed in neuropathic painful conditions in dorsal horn neurons of rats' spinal cord after nerve ligation (PARK et al., 2006; SINISCALCO et al., 2007), capsaicin – induced hyperalgesia (LEE et al., 2007; SCHWARTZ et al., 2008), phenyl N-*tert*-butylnitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPOL) (KIM et al., 2004; LEE et al., 2007; YOWTAK et al., 2011), intraplantar injection of $O_2^{\bullet-}$ (YOWTAK et al., 2011), and found to mediate the development and maintenance of central and peripheral sensitization, either increasing nociceptive neurons' excitability, activating calcium/calmodulin – dependent kinase II in glutamatergic spinal neurons, which leads to presynaptic inhibition of GABAergic interneurons, causing disinhibition, enhancing neuroexcitability in pain signaling pathways and possible excitotoxicity (GWAK; HASSLER; HULSEBOSCH, 2013; KALLENBORN-GERHARDT; SCHRÖDER; SCHMIDTKO, 2022).

2.1.3. Hydrogen peroxide

H_2O_2 is a very unstable and slowly decomposing molecule. It comprises an oxygen-oxygen bond uncharged peroxide, with a pKa ± 10.8 at neutral pH, making it able to cross through cellular membranes. It has a high oxidizing power, yet it holds a rather slow rate of reaction with surrounding molecules; such characteristics enables it to exert its radical properties distant from the place it was formed, and confers an elevated capability to accumulate in cells, in relative excessive concentrations (ANDRÉS et al., 2022).

The main source of H_2O_2 is formed by the dismutation of $O_2^{\bullet-}$ through the SOD-catalyzed reaction. Also, NADPH-oxidase system is responsible for H_2O_2 production, since it performs the catalysis of NAPDH oxidation by O_2, which yields NAD^+ and H_2O_2 (SINGH et al., 2019), as well as other enzymatic sources, such as glucose oxidase, amino acid oxidase, urate oxidase, and others (JOMOVA et al., 2023).

Given that H_2O_2 is a by-product of oxidative stress in cell metabolism, for a long time it was considered a strictly harmful and undesired molecule; but it was until a few decades ago, that its role in several biological processes, such as cell differentiation, proliferation, inflammation, wound healing, and more, proved its value as a key redox signaling molecule (LENNICKE et al., 2015; SIES, 2017). Additionally, H_2O_2 plays a pivotal role in growth factor – induced signal transduction, thiol redox homeostasis and mitochondrial function (JOMOVA et al., 2023).

This "enemy-ally" duality will drastically depend upon the cellular type, subcellular compartment, specific metabolic context, rate of production and clearance, once at micromolar concentrations appears reactive, whilst at high concentrations can trigger serious damage, by inactivating the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (ANDRÉS et al., 2022; HYSLOP; CHANEY, 2022). H_2O_2, is capable of directly oxidizing a significant number of molecules and inactivate enzymes containing thiol groups (RSH) or methionine (Met) residues in their active sites (DAY et al., 2012). Moreover, H_2O_2 reacts efficiently with many other radicals, markedly NO, inducing the formation of peroxynitrite ($ONOO^-$) (SIES; JONES, 2020).

H_2O_2 can increase the action potentials frequency and amplitude in neurons of the DRG in neuropathic models (SÖZBIR; NAZIROĞLU, 2016) and in neurons from within the subnucleus caudalis of the spinal trigeminal nucleus, causing facial pain induced by formalin injection in the lips of rats (VIGGIANO et al., 2005). Additionally, H_2O_2 can trigger cGMP – dependent kinase Iα (cGKI), causing an increased neurotransmitter release from sensory neurons in the dorsal horn of spinal cord of neuropathic rats (LORENZ et al., 2014), thus, contribute to glutamate homeostasis disturbance, which can potentially cause exacerbated synaptic conductance and amplified Ca+ influx in neuronal terminals, through phosphorylation of subunit receptors of NMDA, inhibition of glutamate transporter (GLT-1) and glutamine synthetase, instigating excitotoxicity advance, key in the development of neuropathic pain (CHEN et al., 2010).

After SOD-catalyzed dismutation reaction of H_2O_2, and under unhinged redox conditions, unbound metal ions, i.e. Fe^{+3}, can catalyze heterolytic cleavage of H_2O_2, resulting in the formation of hydroxyl radicals ($OH^{\bullet}$) and anion hydroxide (OH^{-}), through a well-known reaction referred to as Fenton reaction ($k = 7{,}6 \times 10^{1}\ M^{-1}\ S^{-1}$) (SCHIEBER; CHANDEL, 2014; VALKO; MORRIS; CRONIN, 2005). When reducing agents as $O_2^{\bullet-}$ is present, the oxidized metal ions can be transformed back to their active reduced form; its efficiency in producing hydroxyl radicals is directly proportional to the amount of ion metal available ($k = 3{,}1 \times 10^{5}\ M^{-1}\ S^{-1}$) (JOMOVA et al., 2023).

2.1.4. Hydroxyl radical

Hydroxyl radical ($OH^{\bullet}$) is one of the most reactive radicals in biological systems, with a lifespan of 10^{-8} – 10^{-9} s. It can be produced through homolytic cleavage of H_2O using high-energy irradiation, which yields $OH^{\bullet}$ and H^{+}, by photolytic decomposition of alkyl-hydroperoxides, and by means of redox metal – catalyzed decomposition of H_2O_2. The latter is the main source of $OH^{\bullet}$ in mammals, in the presence of transition metal ions, especially when these metal ions are iron or copper (HALLIWELL et al., 2000; KADIISKA; MASON, 2002).

$OH^{\bullet}$ may interact with other surrounding molecules by abstraction of H^{+}, transfer of electrons or addition reactions, generating other radicals, although less reactive. Due to this high reactivity, $OH^{\bullet}$ affects molecules non-selectively, by extracting carbon-bound H^{+} on relatively simple molecules, whether abundant or not, by attacking lipidic membranes that triggers oxidizing radical reactions, and by addition to nitrogenous bases, such as deoxyribosyl backbone of DNA, that leads to oxidation, damaged bases, or strand break (VALKO et al., 2004; ZASTAWNY et al., 1995). A direct link was established between the hydroxyl radical and DNA strand damage to neurons in the context of neuropathic pain induced by CCI in rats, where an increase of cellular dysfunction and apoptosis in neuropathic animals was observed and promptly reduced in animals treated with an aqueous extract of *Luehea divaricate* containing ROS scavengers. DNA strand break causes activation of pain pathways, that are also directly activated by hydroxyl radicals, that can further release proinflammatory cytokines, increasing pain sensitization (KROTH et al., 2020). Hydroxyl radical is relevant for the attenuation of long-term potentiation and facilitation of long-term depression in GABAergic inhibitory interneurons, primarily contributing to pain through GABAergic disinhibition in the dorsal horn conducing to enhanced excitatory transmission, hence deriving in neuropathic pain (BITTAR et al., 2017).

2.2. Reactive nitrogen species: chemistry and sources

2.2.1. Nitric oxide and peroxynitrite

Nitric oxide ($NO^{\bullet}$) is a small abundant reactive molecule that holds an odd number of electrons (fifteen), seven in its last orbital, and specifically one unpaired electron on the antibonding orbital $2\pi^*$, consequently considered a radical. Is endogenously synthetized through a 5-electron oxidative reaction involving L-arginine metabolization to L-citrulline, by specific nitric oxide synthases (NOSs). $NO^{\bullet}$ has a participation in a variety of physiological processes related to neurotransmission, synaptic plasticity in the CNS, smooth muscle relaxation, vascular tone, platelet aggregation inhibition, leukocyte adhesion inhibition, and antimicrobial immune response (BERGENDI et al., 1999; GHAFOURIFAR; CADENAS, 2005). Due to its hydrophobicity, $NO^{\bullet}$ can freely migrate through the cytoplasm and cellular membranes, and react distantly from its site of formation, and a quite long lifespan (1 – 10 s), compared to other molecules (IGNARRO, 1990).

Four main isoforms of NOSs have been recognized and located. A mitochondrial NOS (mtNOS) (GHAFOURIFAR; CADENAS, 2005); a neuronal NOS (nNOS), expressed in the postsynaptic terminal of neurons and Schwann cells; an endothelial NOS (eNOS), highly expressed in DRG consistently with allodynia, constitutively expressed and regulated by Ca^{2+}/Calmodulin interaction; and an inducible NOS (iNOS), induced as a response to inflammation, trauma, or infection, which is not regulated by Ca^{2+}, and highly expressed in the cytosol of glial cells (CURY et al., 2011; DI MEO et al., 2016; NATHAN; XIE, 1994). The $NO^{\bullet}$ produced by each isoform will perform different and specific functions, depending on the place where they are produced, e.g. regulation of mitochondrial oxygen consumption and transmembrane potential via a reversible reaction with cytochrome c oxidase (mtNOS) , communication among neurons (nNOS), relaxation of blood vessels and maintenance of blood pressure in endothelium (eNOS), and contribution to defensive mechanisms of macrophages (iNOS) (NATHAN; XIE, 1994).

Even if $NO^{\bullet}$ is not a strong oxidant nor reductant, despite its radical nature, excessive production, surpassing cellular capacity to counter act, reduce and regulate it, may lead to protein structural alterations, hence, affecting cellular normal functions. $NO^{\bullet}$ can readily

interact with metal-centered proteins for instance, heme – iron, iron – sulfur cluster, zinc – sulfur cluster, and copper; this reaction can lead to nitrosyl-metal complexes that are quite stable, or redox reactions. In both circumstances, some extent of modifications of the protein functions is reasonable to appear (RADI, 1996). All of those interactions and outcomes directly depends on the $NO^{\bullet}$ concentration and the redox environment (BARTESAGHI; RADI, 2018; VALKO et al., 2007). The end products nitrites and nitrates are shown to increase bilaterally after five minutes of cerebral contusion, and to persist for six hours, only ipsilaterally to the affected side (LEWÉN; MATZ; CHAN, 2000).

In the context of oxidative stress, the cytotoxic potential of $NO^{\bullet}$ is due to the secondary production of $NO^{\bullet}$- derived oxidants, that are by far more reactive, than by the oxidative potential itself (RADI, 2004) Additionally, a rather slow autoxidation, temperature-dependent process can occur ($k = 2.8 \times 10^6\ M^{-2}\ S^{-1}$), that leads to nitrogen dioxide production ($NO_2^{\bullet}$); this species is more oxidatively reactive and nitrating than its preceding. $NO_2^{\bullet}$ can further interact with $NO^{\bullet}$ to yield dinitrogen trioxide (N_2O_3), which is even more reactive than the priors, with a high ability to cause protein nitrosation and deamination reactions (BARTESAGHI; RADI, 2018). Therefore, $NO^{\bullet}$ oxidizing potential is mainly related to its ability to interact with $O_2^{-\bullet}$ once they react in to producing significant amounts of a much more oxidatively molecule, peroxynitrite anion ($ONOO^-$) (CARR; MCCALL; FREI, 2000).

Regarding $ONOO^-$, it is a short-lived (10 ms) one- or two-electron oxidant radical formed as a result of oxygen and $NO^{\bullet}$ powerful and efficient interaction, serving as the main $NO^{\bullet}$ derived oxidant. This process occurs in a competitive environment of $O_2^{-\bullet}$ against the enzymatic SOD-catalyzed dismutation to H_2O_2, $NO^{\bullet}$ migration across the cell, $NO^{\bullet}$ preferential reaction with guanylate cyclase, and finally, the reaction of $O_2^{-\bullet}$ with $NO^{\bullet}$, to ultimately yield $ONOO^-$ in a potentially faster reaction when $NO^{\bullet}$ concentrations increase **(Figure 3)** (BARTESAGHI; RADI, 2018).

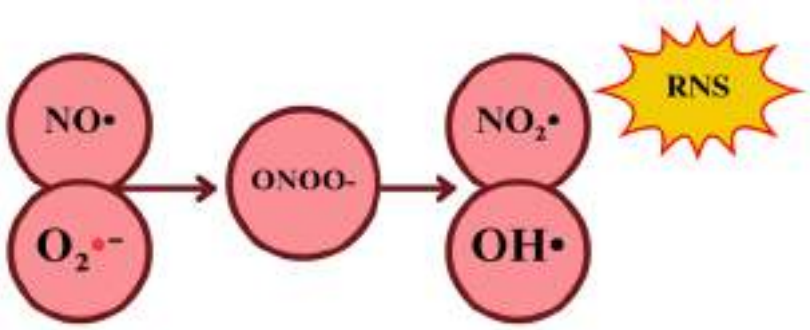


Figure 3 – Peroxynitrite (ONOO-) formation from nitric oxide (NO•) and anion superoxide (O2-•), and subsequent formation of hydroxil radical (OH•) nitrogen dioxide (NO2•) from peroxynitrite. RNS: Reactive Nitrogen Species.

The ratio of the competitive environment determines the switch from physiological functions to pathological pathways signaling, once $ONOO^-$ can act either as a cytotoxic effector molecule against pathogens or as an endogenous cytotoxic molecule towards host cells, based on the kinetics setting (FERRER-SUETA; RADI, 2009).

$ONOO^-$ is able to engage with biomolecules like DNA, proteins, and lipids that lead to structural modifications in biomolecules, resulting in dysfunction such as interference with signaling pathway through protein amino acid nitration. The two direct major ways of $ONOO^-$ actions over biomolecules are through a one-electron (with transition metals) or two-electron (with thiols and CO_2) oxidation reactions in an aqueous milieu (RADI et al., 2001).

On the other hand, $ONOO^-$ can indirectly induce oxidation or nitration in amino acids including cysteine (Cys), tyrosine (Tyr), and methionine (Met) (BARTESAGHI; RADI, 2018), as well as freely diffuse though lipidic membranes (ONOOH), and react via its secondary radicals with proteins and lipids in the hydrophobic milieu; thus, negatively impacting protein structural and functional roles, or induce lipid peroxidation (SZABÓ; ISCHIROPOULOS; RADI, 2007)

$ONOO^-$ has the capacity to slowly decompose to $OH^{\bullet}$ and $NO_2^{\bullet}$ by proton – catalyzed homolysis, that can subsequently act in nitration and oxidation reactions. Additionally, another substantially important reaction in biological systems, occurs when CO_2 and HCO_3^-, in equilibrium, reacts with $ONOO^-$, yielding $NO_2^{\bullet}$ and $CO_3^{\bullet-}$ radicals, that can cause oxidative damage to biomolecules, (RADI et al., 2000).

Both $ONOO^-$ and $NO^{\bullet}$ play significant roles in the settlement and perpetuation of neuropathic pain, through several molecular interacting ways, as described above; nevertheless,

they can specifically contribute to the process through neuroexcitability enhancement of nociceptive neurons, activating CaMKII in excitatory glutamatergic neurons, potentiating synaptic transmission, increased calcium influx, or causing GABAergic interneurons inhibition, deriving in disinhibition (CHEN et al., 2010; MUSCOLI et al., 2003). Moreover, both species can cause TRP channels activation(JULIUS, 2013), critical for pain processing, preceding membranal proteins and lipids nitrosative modifications, and TLRs activation (iNOS) (GRACE et al., 2016), consequently, inducing nociceptive hypersensitivity. Once mitochondrial stress is a major contributor to neuropathic pain conditions, it has been seen that elevated levels of NOS and NOX derived species can cause mitochondrial homeostasis disturbance, and neuronal degeneration **(BENNETT, 2014)**. As an overall effect, both species contribute to central and peripheral sensitization following nerve injury.

2.3. Endogenous sources of reactive oxygen and nitrogen species (ROS/RNS)

Several sources of ROS and RNS have been identified in neurons during normal and neuropathic pain processing, giving its fundamental role in signaling pathways modulation, discussed later in this paper. Although, as previously stated, ROS and RNS are exogenously and endogenously formed, focus will be addressed towards the endogenous sources. Thus, at the cellular level, the majority of them comes from enzymatic activity **(Figure 4)** (BARTESAGHI; RADI, 2018).

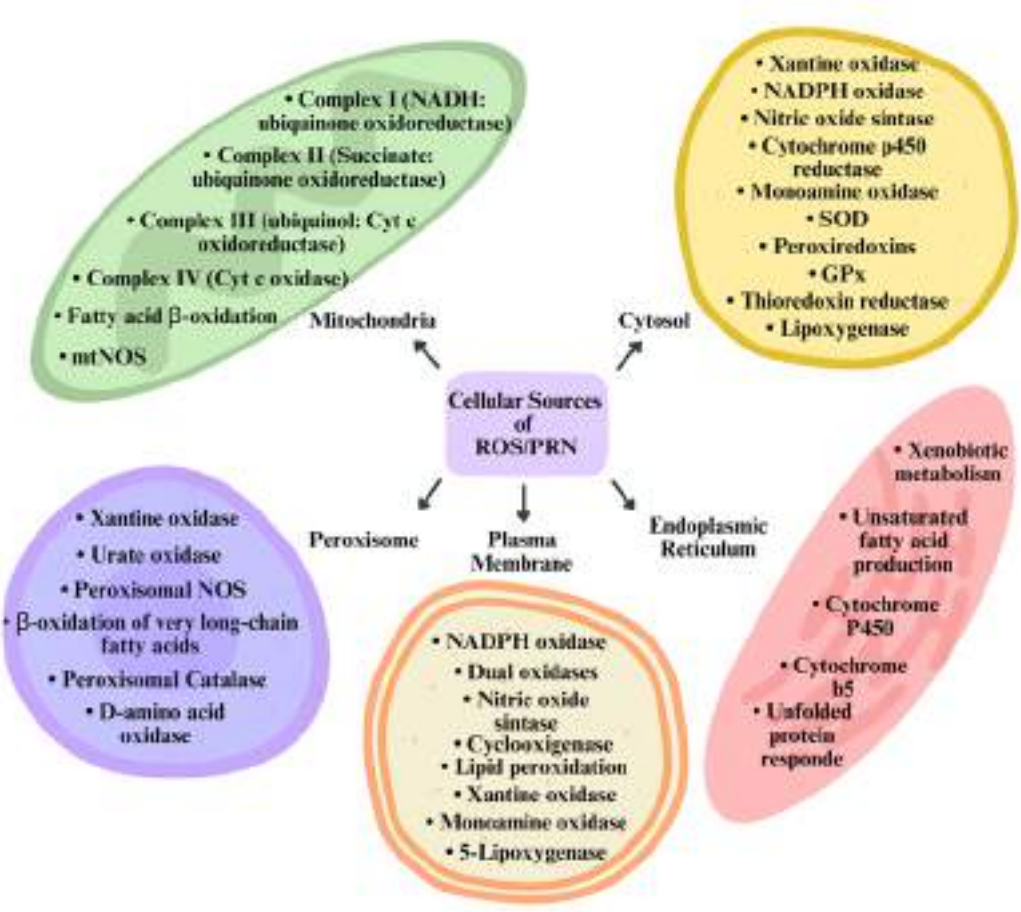


Figure 4 – Endogenous sources of reactive oxygen and nitrogen species. Figure illustrates the major cellular organelles and enzymatic sources involved in the endogenous production of reactive oxygen and nitrogen species (ROS/RNS) during physiological cell functioning.

2.3.1. Endoplasmic reticulum

The endoplasmic reticulum (ER) is formed as an intricate linked net with numerous tubules and sacs tightly interconnected and thought to occupy around 10% of the cellular space and has a pivotal role in lipids and proteins biosynthesis (ALBERTS et al., 2002). More specifically, it is involved in biosynthesis, folding, transport and posttranslational modifications of proteins, whether of the plasma membrane, Golgi apparatus or lysosomes, as well as calcium storage, and drugs detoxification, in certain cell types (DE SOUSA; OLIVEIRA; PINTO, 2011; DI MEO et al., 2016; HELENIUS; MARQUARDT; BRAAKMAN, 1992).

In the smooth ER, reactive species are produced by any of two systems in charge of xenobiotic metabolism and unsaturated fatty acid production. Regarding xenobiotic metabolism, through a two-phases process, an initial monooxygenase reaction (phase I) using a NADPH- cytochrome P450 reductase and a complex of cytochrome P450 react adding a polar group (OH^-) in a lipophilic substrate, using O_2 as a co-substrate, typically replacing a hydrogen atom, which introduces polarity into the molecule, and increases its water solubility. Following oxidation, the xenobiotic with added polarity undergoes a conjugation reaction (phase II) with endogenous molecules (glucuronic acid, sulfate, or glutathione), increasing further the hydrophilicity of the conjugate, facilitating its elimination(DI MEO et al., 2016).

In relation to the unsaturated fatty acids production, reactive species are produced by two ways of action of the cytochrome b5, which acts as an electron transfer. First, through a multienzyme – complex formed by cytochrome b5, NADH cytochrome b5 – reductase and a desaturase; here, cytochrome b5 transfers electrons from NADH cytochrome b5 – reductase to the desaturase, which inserts C–C double bonds in the fatty acids with the aid of an O_2 molecule, yielding as a result two molecules of H_2O (NAPIER; MICHAELSON; SAYANOVA, 2003). Secondly, through a direct electron transfer from the NADH cytochrome b5 – reductase to the catalytic site of a fused cytochrome b5/desaturase, adding the same C-C bond, previously mentioned. In both cases, cytochrome b5 acts positively over cytochrome P450 monooxygenase reaction, contributing to the availability of the second of two needed electrons for O_2 to activate cytochrome P450, accelerating the catalytic process and increasing the formation of H_2O_2 and $O^{\bullet-}$, as well as a direct input of $O^{\bullet-}$ via leakage of electrons to O_2 by NADH cytochrome b5 reductase (DI MEO et al., 2016; WIERCINSKA; LOU; SQUIRES, 2012).

Altogether, present in the ER, cytochrome P450 and cytochrome b5, can contribute to reactive species production through diverse mechanisms. Given that cytochrome P450 enzymes participates in the metabolism of xenobiotics and endogenous molecules, some of the reactions involved occurs with electrons transfer to O_2, that subsequently contributes to ROS production. Moreover, cytochrome P450 enzymes suffer redox-cycling processes; in such processes, electron transfer between heme-iron centers and additional molecules occurs, that can result in ROS generation, more precisely $O^{\bullet-}$ and H_2O_2, as derivatives (FORRESTER et al., 2018; VEITH; MOORTHY, 2018; ZANGAR; DAVYDOV; VERMA, 2004).

Into the bargain, it is believed that redox homeostasis in the ER can induce ER – associated stress, that triggers redox signaling mediators involved in ROS formation (JOMOVA

et al., 2023). As an example, unfolded or misfolded proteins that accumulate in the ER lumen can elicit such response, associated to chronic pain of neuropathic nature (BHANDARY et al., 2012) As the first of two possible manners, ROS can be formed during electron transfer from protein thiols to O_2 by the Endoplasmic Reticulum Oxidoreductin-1/Protein Disulfide Isomerase (ERO-1/PDI). Next in order, during the protein misfolding process due to the depletion of glutathione reductase (GSH), ROS are formed; when GSH is used, thiols are restored and can readily interact with ERO-1/PDI, so as to be re – oxidized. In this cyclic and repetitive process, disulfide bonds formation and scission generate ROS as derivatives (BHANDARY et al., 2012; HIGA; CHEVET, 2012; SANTOS et al., 2009).

Since ER stress in increasingly recognized as a relevant component in the development of neuropathic pain, ROS produced in the process play a significant role in it. Under high protein traffic in ER stress conditions, great quantities of misfolded proteins and ROS can be generated, triggering the unfolded protein response (UPR), which might lead to deviant cellular signaling, neurotoxicity, neuroinflammation, disruption of cellular functioning, and apoptosis, particularly relevant in neuropathic conditions, where ROS levels are boosted (KIM; LEE; SHEN, 2024; LEI et al., 2021).

ER stress and pain behavior were normalized in neuropathic animals undergoing nerve ligation, and BiP and *XBP1* expression in the spinal cord were reduced after treatment with tauroursodeoxycholic acid, an ER chaperone that aids ER in protein folding, indicating the participation of ER stress in the development of neuropathic pain (KAWANAKA; JIN; AOE, 2024).

2.3.2. Mitochondria

Among the most important functions of mitochondria can be named, production of energy in the form of ATP, either by the tricarboxylic acid cycle or oxidative phosphorylation, intracellular calcium concentration balance, and β-oxidation (DI MEO et al., 2016). Almost all of these cellular functions are associated with reactive species production; and mitochondria stands as one of the most important sources of reactive species with oxidant potentials. This amount of reactive species contributes to cellular damage and at the same time, account for relevant redox signaling pathways (MURPHY, 2009).

It is widely accepted that oxidative phosphorylation and ETC promotes mitochondrial ROS formation in higher concentrations than any other mitochondrial activity; even so, the relevance of the other sites should not be disregarded, solely based on the percentage of production, given that, there exist at least eleven different sites associated with substrate oxidation and oxidative phosphorylation electron leakage to O_2, that also produce $O^{\bullet-}$ and H_2O_2 (BRAND, 2016). These sites include, but are not necessarily limited to: 2-oxoglutarate dehydrogenase c (O_F site), pyruvate dehydrogenase (P_F site), branched-chain-2-oxoacids (B_F site), 2-oxoadipate (A_F site), flavin-containing site (I_F site), quinone-binding site (I_Q site), complex II site (II_F site), mitochondrial glycerol-3-phosphate dehydrogenase site (G_Q site), electron-transferrin flavoprotein:Q oxidoreductase system (E_F site), dihydroorotate dehydrogenase site (D_F site), outer Q-binding site of complex III (III_{QO} site), cytochrome b566 site (Cyt_{b566} site), and inner Q-binding site of complex III (III_{Qi} site) (BRAND, 2016; MESSNER; IMLAY, 2002; QUINLAN et al., 2013). In general terms, if electrons leak individually or in pairs, they will generate $O^{\bullet-}$ or H_2O_2, respectively. If they are transferred four-at-a-time, as expected, they will form H_2O by inflowing to oxidative phosphorylation (BRAND, 2016).

Mitochondrial ETC is responsible for ATP formation, through the process of oxidative phosphorylation. The oxidation of metabolites contained in energy sources undergo a transfer of electrons to electron carriers, such as nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD); the reduced form of these carriers, transfer the electrons to the respiratory chain, and finally to oxygen (JOMOVA et al., 2023; NAVARRO; BOVERIS, 2007). During this process, as electrons pass through the mitochondrial complexes, some may leak out and react with O_2, which then suffer either a reduction mediated by NADPH and xanthine oxidase, yielding $O^{\bullet-}$, or by ubiquinol, a reduced form of ubiquinone (VALKO et al., 2007). While ubiquinone itself does not react with $O^{\bullet-}$, it serves as an electron carrier from complexes I and II to complex III of the ETC, permitting ubiquinol's autoxidation. During this process, ubiquinol directly reacts with O_2 to form $O^{\bullet-}$. In this reaction, ubiquinol donates an electron to O_2 and a semiquinone radical intermediate is formed; the semiquinone radical furtherly reacts with O_2 to produce $O^{\bullet-}$, thus contributing to ROS formation (BRAND, 2016). Additionally, $O^{\bullet-}$ produced during univalent autooxidation of electron carriers can undergo a dismutation reaction by mitochondrial SOD, yielding H_2O_2, that can subsequently be turned into $OH^{\bullet}$ via Fenton reaction (MAILLOUX, 2015)

Yet, it is of remarkable notice that, the production of $O^{\bullet-}$ and H_2O_2 in the mitochondria will drastically depend on the protonmotive force, the NADH/NAD^+ and the $CoQH_2$/CoQ ratios, and the local concentration of molecular oxygen, which are unsteady and defiant to measure in vivo. Thus, $O^{\bullet-}$ and H_2O_2 levels will raise or lower depending on the redox state and the specific location of the electron donor; if they act upstream, causing oxidation of the site, they will lead to reduced electron leak; if they act downstream, causing reduction of the site, this will lead to increased electron leak. Altogether, makes troublesome to assume that any mitochondrial dysfunction will necessarily lead to increased $O^{\bullet-}$ and H_2O_2 levels, or to oxidative stress; instead, a wider frame must be taken into consideration (BRAND, 2016; BRAND; NICHOLLS, 2011).

ROS levels are found to be increased in neuropathic pain, more specifically in microglia, astrocytes and neurons of the dorsal horn in the spinal cord (KIM et al., 2010; SCHWARTZ et al., 2008). In the event of nerve injury, mitochondria dysfunction emerges as a consequence of excessive reactive species production, overpassed by the endogenous antioxidant systems, triggering mitochondrial fission and unpaired expression of mitochondrial transcription factor A (TFAM) and mitofusin 2 (MFN2) proteins, responsible for mitochondrial biogenesis and fusion, respectively; hence, contributing to oxidative stress and increased mitochondrial damage (LI et al., 2025). These events seem to be significantly regulated by the cellular energy sensor AMPK, which act upon oxidative stress conditions, triggering peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1α), that promotes mitochondrial genes expression related to biogenesis and mDNA replication and transcription, ultimately restoring mitochondrial function (ZHANG; LIANG, 2019). Furthermore, excessive ROS produced by mitochondria can trigger mitochondrial damage and exacerbate neuropathic pain through TRP channels activation, NMDA receptors upregulation, GABAergic gating mechanisms inhibition, inducing NLRP3 inflammasomes proinflammatory responses, and causing oxidative damage to neurons (KIM et al., 2004; KIM; CHUNG; CHUNG, 2008; SILVA SANTOS RIBEIRO; WILLEMEN; EIJKELKAMP, 2022).

2.3.3. Plasma membrane

Given its direct contact with the extracellular space, a high exposure to oxidizing agents and reactions are plausible to happen, leading to reactive species production (DI MEO et al., 2016). Several metabolic processes can be disturbed due to oxidatively damaged lipids of the

plasma membranes, that will precede transmembrane ion gradient alterations, interruption of secretory and signaling functions (SKULACHEV, 1996).

While undeniable weight relies on arachidonic acid (AA) derivatives, formed at the plasma membrane, such as prostaglandins, leukotrienes and thromboxane, through the action of membrane-bounded enzymes like lipoxygenase (MAHIPAL et al., 2007) and cyclooxygenase (HONG; JEON; KIM, 2008), several free radicals can be produced in the course, once arachidonic acid oxidation induces ROS generation and liberation (CHO; SEO; KIM, 2011). Correspondingly, AA and its metabolites spawned by lipoxygenase and cyclooxygenase promotes the generation of ROS by NADPH oxidase (NOX) (KIM; DINAUER, 2006).

The main source of reactive species generation is owed to the effect of NOX, which will indorse $O^{\bullet-}$ production. NOX are a group of enzymes bounded to the plasma membrane present in a myriad of cell types (VALKO et al., 2004). NOX are constituted by six subunits: one Rho-GTPase, either Rac1 or Rac2, and five phagocytic oxidases (phox): $gp91_{phox}$, $p22_{phox}$, $p40_{phox}$, $p47_{phox}$, $p67_{phox}$; being the first two restricted to the membrane, and the latest three, free in the cytoplasm (MASSAAD; KLANN, 2011). Upon stimulation, when proper spatial – temporary conditions allow the cytoplasmic subunit to migrate to the membrane subunits, assembly of all the components bound together, which conduces to allosteric alteration and subsequent activation. Once activated, NOX will catalyze the oxidation of cytoplasmic NADPH into NAD^+, releasing an electron to O_2, to produce $O^{\bullet-}$ onto the plasma membrane or in its outer side (LAMBETH, 2004; VIGNAIS, 2002).

Out of the NOX family, NOX1, NOX2, and NOX4 have been linked to the neuroinflammation process and the neuropathic pain development (KALLENBORN-GERHARDT; SCHRÖDER; SCHMIDTKO, 2022; ZHANG et al., 2024a, 2025a). NOX1 is expressed in DRG, spinal cord neurons and glial cells (SORCE; KRAUSE, 2009), and its activity inhibition by ML171 significantly reduced pain behavior through TRPA1 – mediated ROS production in a model of neuropathic pain induced by peripheral nerve injury in rats (DE LOGU et al., 2017). NOX2 is expressed in microglia from the spinal cord, while found in macrophages and neurons in DRG in neuropathic pain models induced by nerve injury; thus, its activity suppression was associated to decreased ROS production, microglia activation, and TNF-α and IL-1β, therefore, improving thermal and mechanical hypersensitivity (BERGER et al., 2011; KALLENBORN-GERHARDT et al., 2014); finally, NOX4 was detected in dorsal

horn neurons and interneurons of the spinal cord, as well as in the injured nerve and DRG in peripheral nerve injury and diabetic neuropathy (FU et al., 2021; GEIS et al., 2017) contributing to ROS production, peripheral myelin integrity impairment through myelin protein zero (MP) and peripheral myelin protein 22 (PMP22) degradation, associated to apoptosis, and consequently exacerbation of the manifestations of neuropathic pain, all of which was reduced when NOX4 activity was inhibited in knock-out mice (KALLENBORN-GERHARDT et al., 2012).

2.4. ROS/RNS – mediated cell signaling

As previously cited, RONS are endogenously or exogenously produced (VALKO et al., 2007), and in spite of having notorious deleterious or harmful effects over macromolecules, cellular pathways, cellular organelles, and to contribute to the establishment or development of pathological states when intrinsic antioxidant mechanisms fail to overpass the amount of RONS (FINKEL, 2011; JOMOVA et al., 2023), RONS also play pivotal roles in cell functioning, by eliciting or regulating changes in signaling cellular messages, enzymatic activity, response to growth factor, induction of inflammatory response (RIUS-PÉREZ et al., 2023), immune response, cellular adhesion, differentiation, proliferation, autophagy and apoptosis (TOUSOULIS et al., 2012), gene transcription (KASAI et al., 2020), proteins and membranes integrity (MARINHO et al., 2014; SIES; JONES, 2020), to name a few.

Given that the cellular activity is modified as a response to stimuli, mainly through activation of gene transcription, the two recognized ways by which ROS participate from such activation, are by (1) direct modification of transcription factors, that will be addressed to particular DNA promoters of target genes, or (2) via activation of mitogen – activated protein kinase (MAPK) pathways, like extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38, that will lead to phosphorylation and activation of other transcription factors, such as activator protein 1 (AP-1) and cAMP response element-binding protein (CREB) (SHI; DANSEN, 2020; ZHANG et al., 2016). The two mechanisms by which cell signaling happen, by means of ROS, can be explained as (1) modifications of target protein molecules, once reactive species oxidize main Cys residues, or by (2) changes in intracellular redox state by the redox – buffering capacity of the cells (DI MEO et al., 2016). The molecular alteration suffered from the former mechanism acts over enzymes, transporters, receptors, transcription factor regulatory sites, and allosteric and macromolecular sites (BAK et al., 2019); and can alter their localization, activity, and DNA – binding ability of the transcription factors, thus

repressing or enhancing the transcription of target genes (BEHRING et al., 2020; MARINHO et al., 2014).

Although some reactive species can cause irreversible structural modifications over molecules, with low specificity (ex.: $OH^{\bullet}$), turning them permanently damaged, other moderate oxidants, such as H_2O_2, will primarily target Cys residues of proteins, more precisely thiol groups (-SH) (DI MEO et al., 2016). Thiol groups from Cys residues can undergo several oxidative modifications, and the process typically occurs as follows: initially, ROS oxidize the -SH group of Cys to form sulfenic acid (-SOH); sulfenic acid (-SOH) can further react with a neighbor -SH group, either from the same protein or from another protein, yielding the formation of a disulfide bond (-S-S-). Under certain conditions, such as excessive reactive species, sulfenic acid (-SOH) can be further oxidized to sulfinic acid ($-SO_2H$) and then to sulfonic acid ($-SO_3H$). These two last higher oxidation states are usually irreversible. On the other hand, even though sulfenic acid formation has been seen has damaging, it has its own relevance as a redox-sensing signal for cellular functions, due to the reversible oxidation by ROS (ROOS; MESSENS, 2011; SEN, 2001).

Other targets of redox – signaling exist, such as iron – sulfur (Fe-S) cluster or Tyr residues, but the list could be vastly extensive for the purpose of this paper. Therefore, this section takes into consideration those redox – signaling targets deemed more sensitive to oxidative or nitrosative and/or implicated in regulation of protein function, associated to transcription factors control, well-characterized mechanisms, and that contribute to the regulation of oxidative stress, offering insights to how cells sense and respond to oxidative stress and neuropathic pain.

2.4.1. NRF2 – ARE pathway

Nuclear factor erythroid 2 – related factor 2 (NRF2) is a redox-sensitive leucine zipper (bZIP) transcription factor (CHEN et al., 2019), that portrays a significant role in neuropathic pain modulation through the inflammatory response and oxidative stress management by hyperalgesia and allodynia regulation, regulating the expression of HO-1 and IL-10 (CHEN et al., 2019; YARDIM et al., 2020). It is expressed in microglia in the spinal cord, DRG, and sciatic nerve (GUO et al., 2022). NRF2 binds to specific DNA sequences to control transcription from DNA to RNA (ZHOU et al., 2021). It is activated in response to oxidative and electrophilic stress (PARVEZ et al., 2018). Its major function is to initiate the expression of a wide range of cytoprotective genes once it has a role in maintaining redox homeostasis (SIES, 2017). In quiescent conditions, in the cytoplasm, NRF2 is bound to Kelch-like ECH – associated protein 1 (KEAP1) dimer, which acts as an adaptor protein that links NRF2 to Cullin 3 (Cul3) – based E3 ubiquitin ligase complex, that causes its ubiquitination, by attaching to lysine residues on NRF2, through a process involving ubiquitin-activating enzymes 1 (E1), ubiquitin-conjugating enzymes (E2), and E3 (SINGH et al., 2010); this tags NRF2 for subsequent degradation by the 26S proteosome into small peptides; this interaction maintains NRF2 cellular levels low, as expected in normal conditions **(Figure 5A.)** (FOURQUET et al., 2010).

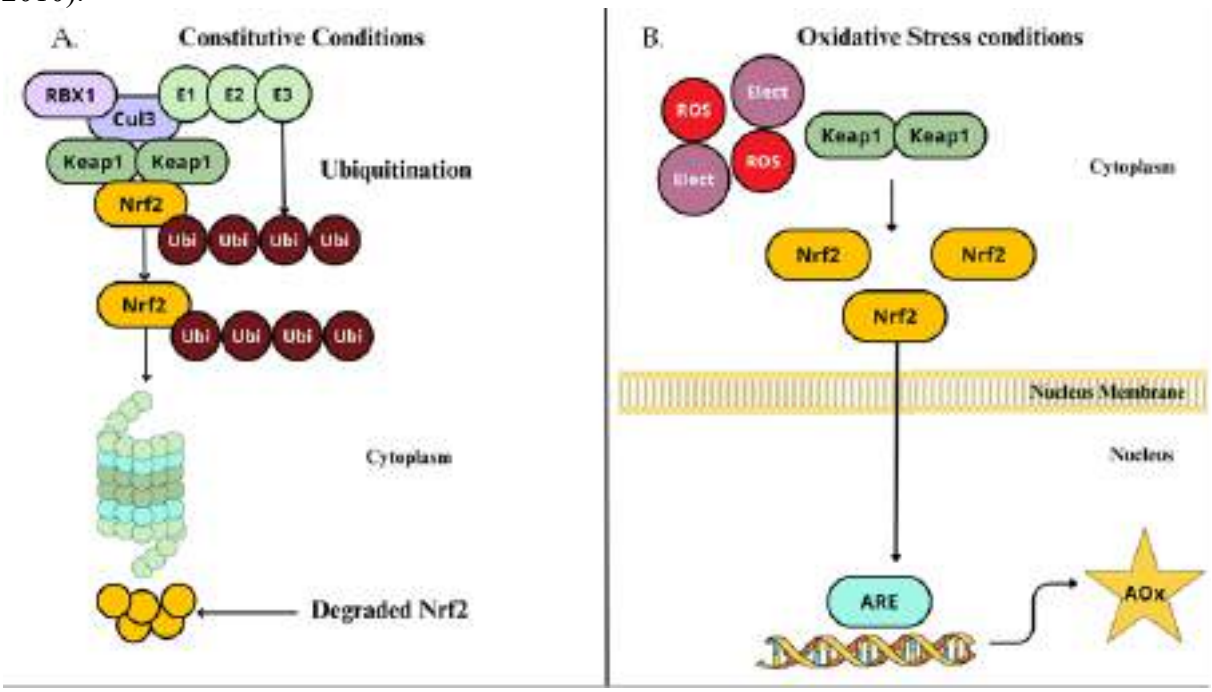


Figure 5 – NRF2 – ARE pathway. A. Constitutive conditions of normal Nrf2 degradation by ubiquitination process in the cytoplasm; B. Oxidative stress conditions of Nrf2 release by KEAP1 and translocation to the nucleus towards DNA regions responsible for the ARE encoding.

When an increase in oxidative stress occurs, NRF2 is released from KEAP1, accumulates in the cytoplasm, translocate to the nucleus, and heterodimerizes with specific

regions of DNA encoding antioxidant response elements (ARE), detoxification, and cytoprotective proteins, such as, heme-oxygenase 1 (HO-1) (LEE et al., 2013), CAT, SOD, and others **(Figure 5B.)** (VASCONCELOS et al., 2019; ZHOU et al., 2021).

As previously stated, KEAP1 is found to have a double function, as an oxidative and electrophilic stress sensor, and negatively regulator of NRF2, by degradation via ubiquitination (SINGH et al., 2010). Out of 25 cysteine residues of KEAP1, three are among the most critical for redox sensing, responsible for NRF2 dissociation (Cys^{151}) and degradation (Cys^{273} and Cys^{288}) (KOBAYASHI et al., 2006). KEAP1 is highly reactive and able to undergo modifications in response to alterations in the inner cellular redox state; these Cys can be oxidized or covalently modified, inducing the formation of disulfide bonds (MOTOHASHI; YAMAMOTO, 2004).

Thus, conformational variations reduce the affinity for NRF2; ultimately, preventing KEAP1 from enabling the ubiquitination and degradation of NRF2. This allows NRF2 to stabilize, and move forward with its transcriptional inducing capacity, which then triggers cellular protective antioxidant (YAMAMOTO; KENSLER; MOTOHASHI, 2018), anti-neurotoxic (KAIDERY et al., 2012), neuroprotection (LIU et al., 2020) and anti-inflammatory responses (ITOH et al., 2004).

NRF2 and HO-1 decreased levels were observed in neuropathic rats after vincristine administration, where oxidative stress , DNA damage, neuronal cell damage and inflammation, evidenced by increased 8OH-dG, diminished GFAP and NF-kB were present, and promptly reversed after quercetin administration, showing the role played by NRF2 pathway, and the protective effect of quercetin against neuropathic pain caused by vincristine (YARDIM et al., 2020)**.** To evaluate the role of NRF2 in neuropathic pain, NRF2 siRNA was administered to CCI-induced rats, which prominently decreased the expression of *NRF2* RNA and NRF2 and HO-1 protein and worsened the hyperalgesia behavior (withdrawal threshold and latency)(CHEN et al., 2019).

Also, in a study investigation the involvement of NRF2 expression in spared nerve injury in rats with our without an anhedonia phenotype, classified after hierarchical cluster analysis of sucrose preference test, showed that NRF2 expression was significantly decreased in medial prefrontal cortex, hippocampus and spinal cord of anhedonian rats, compared to non – anhedonian ones; and after sulforanphane, an activator of NRF2, was administered, it was

observed a reduced mechanical withdrawal threshold, but not sucrose preference by NRF2 levels normalization in those with the anhedonia phenotype (LI et al., 2018).

2.4.2. NF-κB signaling pathway

The nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) is a pleiotropic inducible transcription factor implicated in pro-survival regulatory inflammation and immune functions, cellular adhesion, differentiation, and proliferation, activated in a wide variety of neuroinflammation-associated stimuli and due to pro-apoptotic pathways, (GHOSH; MAY; KOPP, 1998; POPIOLEK-BARCZYK; MIKA, 2016). It is composed by homo- and heterodimers of five structural protein members, of which Rel-A(p65), Rel-B, and c-Rel contain C-terminal transactivation domains (TADs); and p50/p105 and p52/p100, that also serve inhibitory functions (MARINHO et al., 2014).

Conversely, in the nervous system, it is composed mainly by p50/Rel-A heterodimers (BAKALKIN; YAKOVLEVA; TERENIUS, 1993); but the most widely studied dimers are that of Rel-A and p50, also referred to as NF-κB_{can}, once they participate in the canonical activation pathway. On the other hand, Rel-B and p52 form dimer referred to as NF-κB_{non} for they participate in the non-canonical activation pathway, discussed later (MASSA et al., 2006). Additionally, NF-κB contains Rel-1-homology domains (RHD) that bears a nuclear localization signal, accountable for dimerization, detection, and binding to DNA, as well as communication with the NF-κB inhibitor (IκB) (SHIH; WANG; YANG, 2015).

As previously stated, NF-κB pathway is mainly activated by two distinct kinase-dependent manners, the canonical and the non-canonical. However, in the nervous system, the most studied and well known is the canonical pathway, that includes a series of cytokines and Toll-like receptors (TLR) (POPIOLEK-BARCZYK; MIKA, 2016). In normal conditions, NF-κB dimers form complexes with IκB, to retain them in the cytosol, by masking its nuclear localization signal (NSL), and also preventing NF-κB from binding to DNA by masking its DNA-binding sites (SHIH; WANG; YANG, 2015). There, H_2O_2, HOCl, $_1O^2$, and peroxynitrite act via oxidation or nitration of specific critical Cys residues on the IκB – kinase (IKK) complex, that ceases the joining of IκB to NF-κB; immediately after IκB is structurally modified, causes the NF-κB release **(Figure 6)** (GLOIRE; LEGRAND-POELS; PIETTE, 2006; SIES; JONES, 2020).

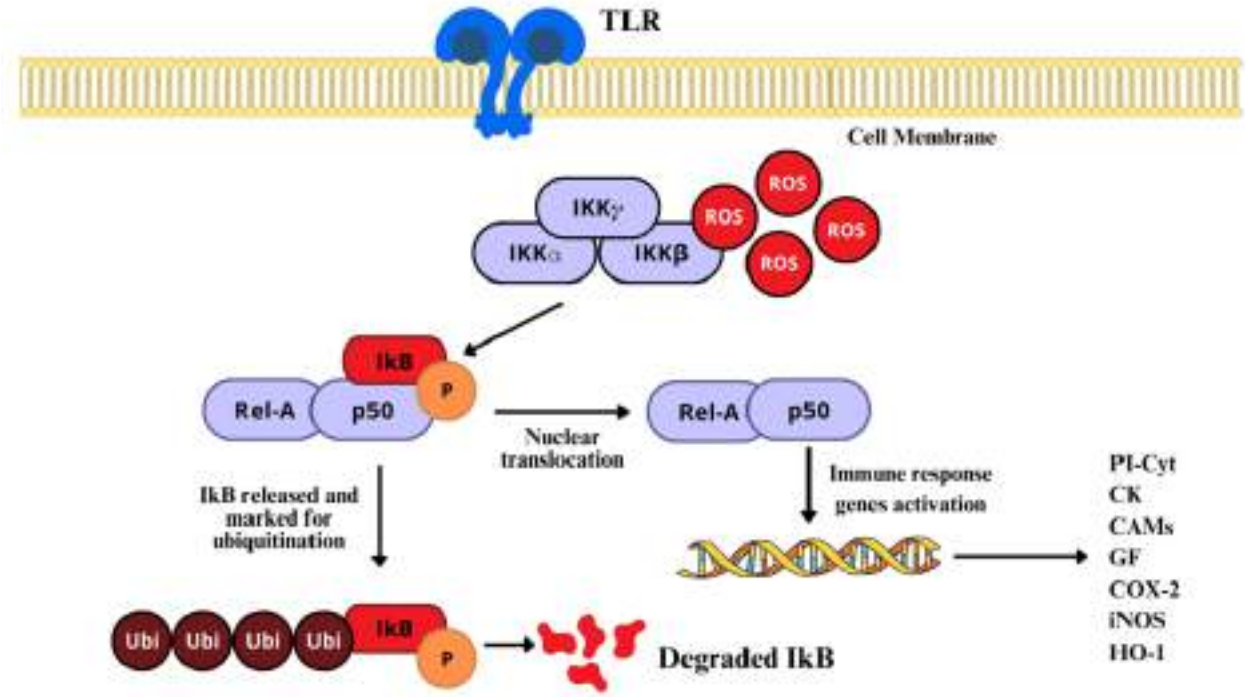


Figure 6 – Canonical pathway of NF-κB activation via TLR (Toll-Like Receptors) and IKK complex oxidation/nitration conduces to IκB – NF-κB complex separation and leads to NF-κB nuclear translocation. PI-Cyt: Pro-inflammatory Cytokines; CK: Chemokines; CAMs: Cell Adhesion Molecules; GF: Growth Factors; COX-2: Cyclooxygenase-2; iNOS: Inducible Nitric oOxide Synthase; HO-1: Heme-Oxygenase-1.

When phosphorylated at a specific regulatory aminoacidic residue by IKK complex, IκB is ubiquitinated and marked for 26S proteosome degradation, still in the cytosol (BRIGELIUS-FLOHÉ; FLOHÉ, 2011); NF-κB is freed for nuclear translocation, to activate the transcription of target genes (JOMOVA et al., 2023), where it binds to enhancer regions of specific genes, such as proinflammatory cytokines, chemokines, adhesion molecules, growing factors (GF), and promoters of COX-2, iNOS, heme-oxygenase (BRIGELIUS-FLOHÉ; FLOHÉ, 2011; DE OLIVEIRA-MARQUES et al., 2007) On the other side, H_2O_2 can regulate NF-κB by direct interaction with the DNA-binding regions Cys **(Figure 7)** (BASAK; HOFFMANN, 2008). In that sense, higher than normal levels of RONS in the nucleus can prevent NF-κB from interacting with DNA, reducing its transcriptional activity, although that same augment of H_2O_2 can induce peroxiredoxin 1 activity, that is a H_2O_2 scavenger.

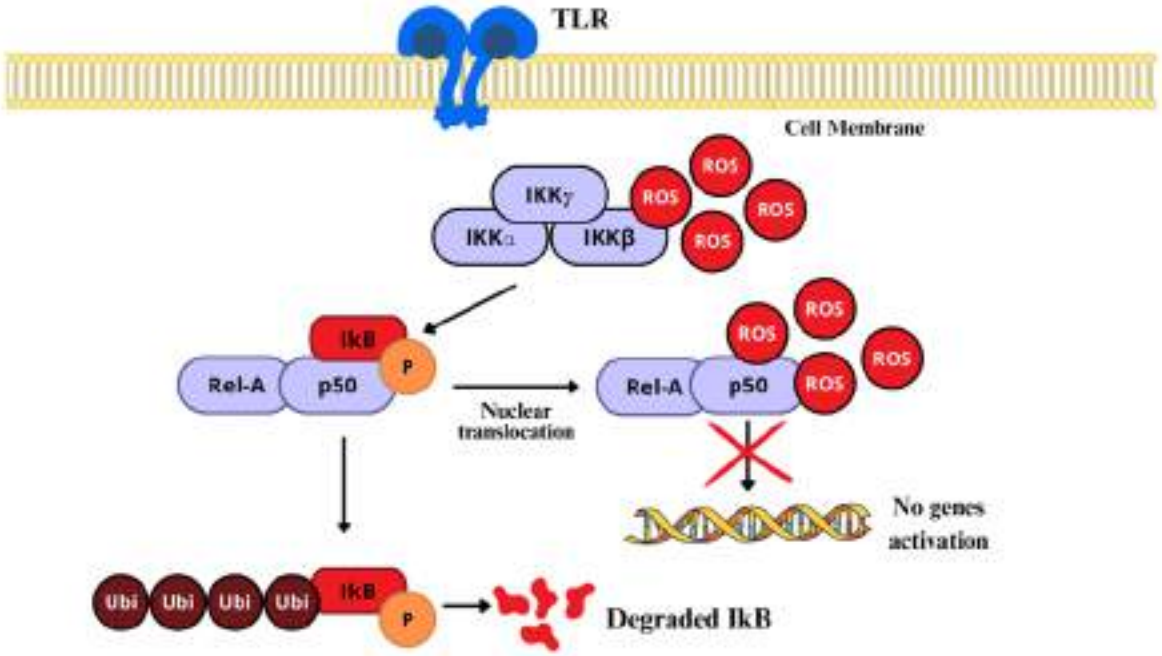


Figure 7 – NF-κB pathway inactivation via reactive oxygen and nitrogen species direct interaction with the DNA-binding regions Cys leads to inhibited genes activation in the nucleus under increased levels of ROS/RNS conditions.

A diverse array of genes with, sometimes differing functions respond to NF-κB$_{can}$; i.e.: pro-survival genes such as inhibitors of apoptosis (IAP), Bcl-2, Bcl-x, Bcl-w, Bfl-1, BIRCS, and MnSOD; yet, NF-κB$_{can}$ can also regulate proapoptotic genes like p53, TNF-α, Bax, c-myc, APO-1, and FasL (MASSA et al., 2006). Given the extremely specialized types and subtypes of neuronal cells, it is a major challenge to study NF-κB, due to traditional techniques limitations in their ability to mirror realistic and accurate locations and activities of proteins that are regulated by specific dimer combinations and several post-translational modifications.

NF-kB is widely implicated in spinal microglial activation to M1 phenotype in the establishment of neuropathic pain, as seen in the examination of growth and differentiation factor 11 (GDF11), an inhibitor of macrophage activation, impact on microglia polarization, thermal and mechanical hyperalgesia in sciatic nerve injury mice, showing a marked reduction of pain behaviors, enhancing the swift from M1 to M2 phenotype via TGF-βR1/SMAD2/NF-κB pathway modulation (CHEN et al., 2018; LIU; ZHANG, 2025). Another study aimed to investigate the participation of Pellino 1 (Peli1), a component of the E3 ubiquitin ligase, in the activation of microglia in neuropathic pain induced by sciatic nerve injury. It was found higher levels of Peli1 and NF-κB expression, as well as pain behavior after SNI – induced neuropathic pain. Also, administration of intrathecal Peli1 shRNA prior to SNI and siRNA after the

establishment of SNI, lead to a great lower expression of Peli1 and NF-κB in the spinal dorsal horn of mice, alleviating thermal and mechanical threshold through NF-κB pathway modulation, once Peli1 is needed for ubiquitination of proteins regulating NF-κB activation (WANG et al., 2020).

2.4.3. MAPK/AP-1 signaling pathway

The activator protein 1 (AP-1) is a heterogenous family constituted by the basic – region leucine zipper (bZIP) transcription factor, accountable for the regulation of cellular responses to numerous extracellular stimuli, including ROS, (AMOUTZIAS et al., 2007). This vast group is represented by the members of the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra-1, Fra-2), Maf (c-Maf, MafA, MafB, MafG/F/K, Nrl) and ATF (ATF2, ATF3, B-ATF, JDP1, JDP2) proteins subfamilies (HONG et al., 2024; KARIN; LIU; ZANDI, 1997). They harbor a C-terminal leucine zipper domain that allows them to form hetero- and homodimers, which subsequently bind to specific DNA sequences known as 12-O-tetradecanoylphorbol-13-acetate (TPA) – responsive elements (TRE) or cAMP response elements (CRE) in the promoter regions of target genes, where they regulate gene expression, mediated by basic N-acid-rich domains, parallelly to the leucine zipper (KARIN; LIU; ZANDI, 1997). The specific arrangement of the dimers composing the AP-1 complex establishes the interaction patterns, molecular affinities, and functional specificity that exhibits their tasks in the cell (BHOSALE et al., 2022; SHAULIAN; KARIN, 2002).

AP-1 transcription factor can be redox activated by ROS, ROS – dependent factors, cytokines, GF, neurotransmitters, UV-light, and ionizing radiation (WISDOM, 1999). The increased exposure to H_2O_2, OH^-, and $O^{\bullet -}$ can directly determine the redox state of several conserved cysteine residues included in the AP-1 pathway (GIUS et al., 1999; XANTHOUDAKIS' et al., 1992). Also, AP-1 can be indirectly regulated by ROS signaling pathways, like NF-κB and Interferon-γ. The AP-1 transcription factor family are targets of the ROS – sensitive MAPKs cascades for phosphorylation, this conduces to enhanced transcriptional activation (SHAULIAN; KARIN, 2001; TANOS et al., 2005). Accurately, ROS activate ERK1/2, JNK and p38 MAPKs **(Figure 8)**, which are crucial mediator for signal transduction pathways conducing to AP-1 activation, through phosphorylation of c-Jun on serine and threonine residues, enhancing its stabilization and c-Fos transcriptional activity over genes involved in neurons, microglia, and astrocytes activation, in a sequential manner (ZHUANG et al., 2005), particularly relevant in dorsal horn neurons of the spinal cord and

DRG, and playing distinct roles in different cells across the development of neuropathic pain (JIN et al., 2003). The accurate redox control of AP-1 expression and activation is essential for homeostasis, and its alterations has been implicated in altered neurons states, for it is a key molecular switch that controls the expression of downstream proinflammatory factors (WEI et al., 2019). Nevertheless, the regulation of AP-1 activity is complex once, briefly, occurs through and depends on changes in Jun and Fos gene transcription and mRNA expression; effects in Jun and Fos protein turnover; post-translational modifications of Jun and Fos proteins that modulates its transactivation capacity; and finally, interaction with other transcription factors that can positively or negatively affect their activity (CHINENOV; KERPPOLA, 2001).

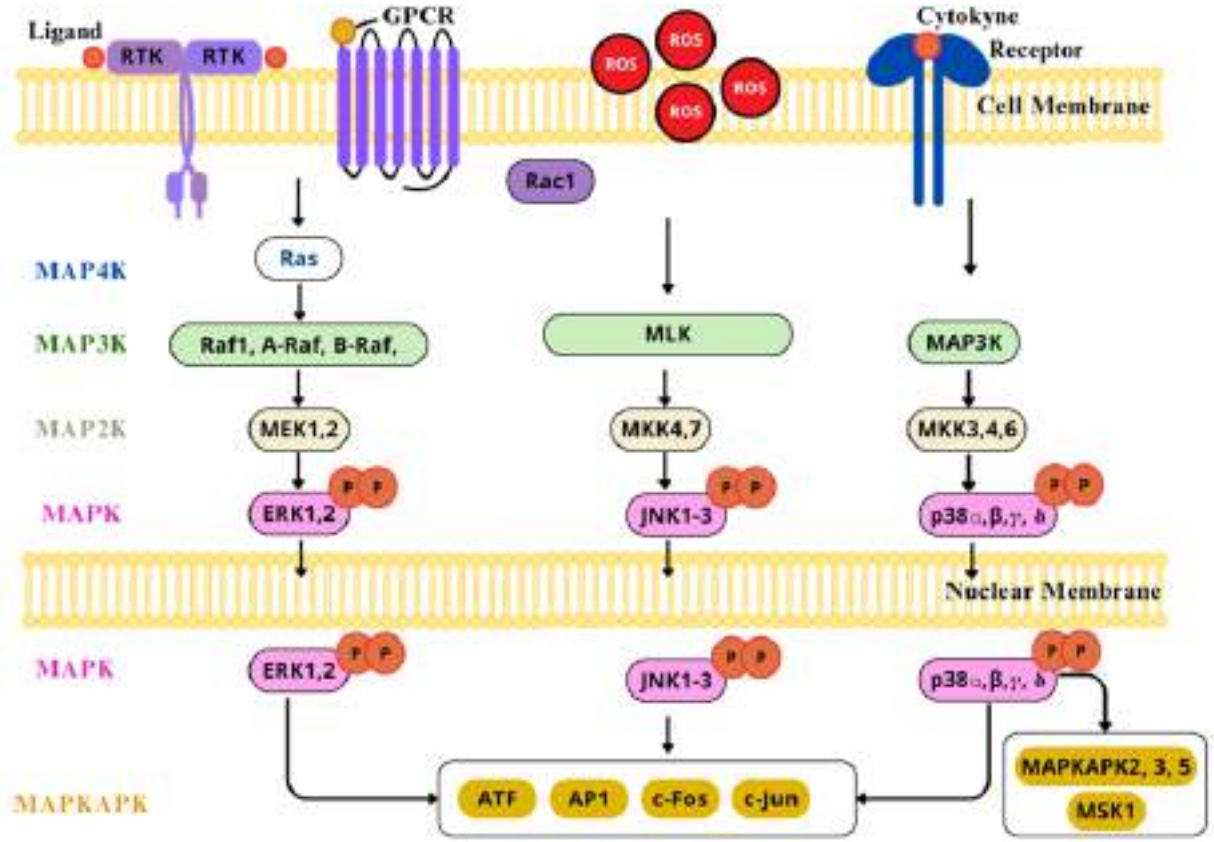


Figure 8 – MAPK/AP-1 signaling pathway. MAPKs are regulated by a cascade of phosphorylation and dephosphorylation of threonine and serine residues, triggered by tyrosine kinase receptors (RTK) activation, protein tyrosine kinase (TK), cytokine receptors (CR), heterotrimeric G protein – coupled receptors (GPCR), growth factors (GF), or ROS/RNS. ATF: Activating Transcription Factor; AP-1: Activator Protein – 1; MLK: Mixed-Lineage Kinase; MEK: MAPK/ERK Kinase; MKK: Mitogen-Activated Protein Kinase; ERK: Extracellular Signal – Regulated Kinase ; JNK: c Jun N-terminal Kinase.

As previously stated, MAPK cascades are intracellular critical signal transduction pathways that transmit extracellular stimuli to the cytoplasm and nucleus, influencing a wide variety of cellular activities as growth, proliferation, migration, differentiation, inflammation, stress, survival and apoptosis (SHAUL; SEGER, 2007). Among those stimuli, oxidative shift

of the intracellular thiol/disulfide redox state, or ROS are accountable for significant activation of the MAPK cascade elements, as seen in neurons (SAMANTA et al., 1998; SATOH et al., 2000). They consist in a series of protein kinases that activate each other in specific sequences. MAPKs are regulated by phosphorylation and dephosphorylation of threonine and serine residues, and are triggered by tyrosine kinase receptors activation, protein tyrosine kinases, cytokine receptors, heterotrimeric G protein – coupled receptors, and GF (DRÖGE, 2002). The major MAPK cascades include ERK1/2 (extracellular signal – regulated kinase 1 and 2), JNK (c Jun N-terminal kinase), p38 pathway, and ERK5, each of which has specific upstream activators, downstream targets, and biological actions (KIM; CHOI, 2015).

Each cascade is composed by three central kinases (MAP3K, MAP2K, and MAPK); an additional upstream MAP4K and downstream MAPKAPK components are included. Respectively, each cascade has its own signal transmitted by successive phosphorylation and activation of sequential kinases, that at a given point lead to the phosphorylation of the target regulatory proteins by the MAPK and MAPKAPK constituents (PLOTNIKOV et al., 2011). With the aim of executing their functions, MAPKS and MAPKAPKs phosphorylate and regulate their substrates, so as to induce and regulate the de-novo gene expression of other transcription factors and suppressors, and chromatin remodeling proteins, at the nucleus level, after being physically transported across the nuclear membrane (WHITMARSH, 2007).

The ERK1/2 cascade is activated through various membrane receptors, but mostly via GTPase Ras, which receives extracellular signals at the plasma membrane, that in turn conscripts the Raf-1 and B-Raf (MAP3K) tier of the cascade to the plasma membrane, probably through homo- or hetero-dimerization and phosphorylation (NIAULT; BACCARINI, 2010). Therefore, the kinases activating the Raf components are considered MAP4Ks. Afterward, the signal is transmitted to the MEK1/2 (MAP2K), through phosphorylation of serine residues in their activation loop. The activated MEK1/2 transmit the signal to ERK1/2 (MAPK) also by phosphorylation of the threonine and tyrosine regulatory residues in the Thr-Glu-Tyr domain, in the activation loop (KIM; CHOI, 2010). Ultimately, the signals pass on to the MAPKAPK elements, RSKs, MNKs and MSKs, and other substrates located in the cytoplasm or the nucleus. Both ERK1/2 and MAPKAPK can phosphorylate substrates involved in cellular proliferation, differentiation, neuroplasticity, stress response, pro-survival, and pro-apoptotic processes (YOON; SEGER, 2006). As a matter of fact, ERK is phosphorylated in dorsal horn neurons of the spinal cord, in DRG neurons and peripheral nerve terminals after nerve injury, contributing

to peripheral and central sensitization (CHENG et al., 2003; ZHUANG et al., 2005). ERK activation occurs in a sequential manner, starting in the dorsal horn neurons after SNL within 10 minutes through transcriptional modulation of NK-1 and prodynorphin (JI et al., 2002; WANG et al., 2004), followed by microglia for some days, having its most robust expression in 3 days; and finally, in astrocytes for weeks, supporting the role of ERK in astrocytes in the maintenance of neuropathic pain, whose inhibition reduced behavior associated to neuropathic pain (ZHUANG et al., 2005). Isoliquiritigenin reduced mechanical and thermal hyperalgesia sued by a CCI in mice, inhibiting the activation of spinal microglia and neuroinflammation through the ERK pathway inhibition (WANG et al., 2024).

The JNK cascade is usually activated by stress signals such as, UV radiation and cytokines (WESTON; DAVIS, 2007). It includes MLK (MAP3K), MKK4/7 (MAP2K), and JNK (MAPK). Once activated by stress-dependent and -independent stimuli and receptors including mitogens, they transmit their signals to GTPases such as Rac1, that activates the MAP3K level kinases directly or via MAP4Ks (JOHNSON; NAKAMURA, 2007). The MAP3K tier transmit the signals through phosphorylation of the threonine and serine residues in the activation loop, that consequently, activates the MKK4/7; finally, these kinases activate any of the components of the MAPK level, by direct phosphorylation of tyrosine and threonine residues in the activation loop. As expected, the JNKs and their respective MAPKAPKs, phosphorylate substrates in the cytoplasm and nucleus, that modulate the transcription of genes mediating apoptosis, immunological responses and neuronal activity (HAEUSGEN et al., 2009). JNK-1 is activated in early (three days) and late (21 days) in GFAP- expressing astrocytes in neuropathic pain caused by SNL, and transiently (12 hours) in DRG neurons after SNL, specifically in small C-fibers; therefore, supporting the idea that spinal astrocytes contribute to the continuance of neuropathic pain (JIN et al., 2003). The neuroprotective effect of Orexin B was evaluated in a model of neuropathic pain induced by CCI in rats. Orexin was found to be effective in improving mechanical and thermal hyperalgesia, preventing microglia activation reducing Iba1 levels and preventing JNK/NF-κB signaling pathway activation (ZHU et al., 2024).

As for the p38 cascade, it is activated by stress signals and environmental stressors, but it also responds to normal cellular processes, like inflammatory cytokines (ZARUBIN; HAN, 2005). It involves MKK3/4/6 (MAPKK), and p38 (MAPK) (PLOTNIKOV et al., 2011). After activation by stress conditions or receptors, the signals are transmitted via GTPases, MAP4K

and MAP3K, in a similar manner to JNK cascade. The MAP3K level of the cascade phosphorylate and activates MKK3/6, and under certain conditions, MKK4 (MAP2K) components of p38 cascade (WHITMARSH; DAVIS, 2007). At this point, the p38 isoforms and functional unconventional spliced forms at the MAPK level are activated via phosphorylation of the threonine and tyrosine residues in the regulatory domain in their activation loop. Lastly, the signals are transmitted by the p38, or MAPKAPKs to target motifs, accountable for regulatory processes (PLOTNIKOV et al., 2011). p38 MAPKs are particularly expressed in the superficial laminae of dorsal horn neurons in the spinal cord, whose increase correlates with lower mechanical withdrawal and thermal latency thresholds in SNL induced neuropathic pain (JIN et al., 2003). Increasing evidence points the role of p38 MAPK in the activation of spinal microglia after nerve injury and its contribution to neuropathic pain, via downstream mechanisms of proinflammatory mediators, after nuclear translocation, where influences genes expression of TNF-α, IL-1β and IL-6, critical mediators of neuroinflammation and pain, that also exacerbate other pain signaling pathways (JI; SUTER, 2007). p38 MAPK involvement in the pathophysiology of neuropathic pain mediating microglial activation was evaluated in SNL rat models treated with electroacupuncture to assess the analgesic effect of such treatment; it was found a significant decrease in p38 MAPK activation and enhanced mechanical withdrawal threshold, both in the ipsilateral and contralateral dorsal horn of the spinal cord, not only remarking the beneficial effect of electroacupuncture, but the potential of targeting p38 MAPK pathway to treat neuropathic pain (LEE; YU; WANG, 2016).

2.4.4. Phosphoinositide 3-kinase (PI3K)/Akt pathway

The PI3K pathway constitute a conserved family of kinase present in the inner side of the plasma membrane, capable of acting specifically on the D-3 position of the inositol ring of inositol phospholipids (VANHAESEBROECK; WHITEHEAD; PIÑEIRO, 2016). Such phosphorylation produces phosphatidylinositol-3,4,5-triphosphate (PIP3), phosphatidylinositol-3,4-biphosphate (PIP2), and phosphatidylinositol-3-phosphate (PIP) (HAWKINS; STEPHENS, 2016). The PI3K enzymes, when activated, transmit intracellular signaling cascades concerning signal transduction, protein synthesis, vesicular traffic, cytoskeletal reorganization, cell growth, proliferation, survival, metabolism, apoptosis, autophagy, (DEANE; FRUMAN, 2004; VANHAESEBROECK; WHITEHEAD; PIÑEIRO, 2016), drug resistance in response to GF, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), nerve growth factor, (NGF), vascular endothelial growth factor

(VEGF), and brain-derived neurotrophic factor (BDNF) (MANNING; TOKER, 2017; ZHANG et al., 2016). PI3K has also been found to be essential in the initiation and maintenance of neuropathic pain (CHEN et al., 2017).

Although in mammalians cells, the PI3Ks exists in three classes (I, II, and III), and each of these have different structural characteristics, functional homologies and substrate specificity, the most prevalent and significant in the nervous systems is the class I PI3Ks, where they play a crucial role in neuronal development, synaptic plasticity, and neuroprotection (CIANCIULLI et al., 2020). Class I PI3K are further divided into class IA and class IB subgroups, (DEANE; FRUMAN, 2004; RYAN et al., 2024; SAPONARO et al., 2012). The heterodimeric class IA PI3Ks are composed of a regulatory subunit p85, including five polypeptides p85α, p85β, p55α, p50α, p55γ; and a catalytic subunit p110 (p110α, p110β, or p110δ), which are activated by receptor tyrosine kinase (RTK) and G-protein-coupled receptors (GPCRs), small G protein Ras, and cytokines (ZHU et al., 2014). The p110α and p110β are ubiquitously expressed in all cell types, while p110δ expression is more restricted to leukocytes (WHITEHEAD et al., 2012). The catalytic p110 subunit contain a C-2 region for membrane anchoring; a Ras binding region (RBD); an adaptor binding region (ABD), which holds a N-terminal region for interaction with the regulatory p85 subunit; a helical region, and a catalytic region. The p85 regulatory subunits have in common a p110 binding region (inter-Src-homology-2 region, iSH2); and two SH2 regions for p110 ABD binding, constitutively (AMZEL et al., 2008; VANHAESEBROECK; WHITEHEAD; PIÑEIRO, 2016). On the other hand, class IB PI3Ks enzymes are formed by p110γ polypeptide, associated to an adaptor subunit p101 or p84 and p87, to form heterodimers, p101/p110γ or p84/p110γ, which are primarily activated by GPCRs, due to the interaction of its regulatory subunit with the subunit of trimeric G protein (KATSO et al., 2001); this isoform is present in the nervous system.

Either of these PI3Ks catalyzes the phosphorylation of PIP2 to produce PIP3 (FRITSCH; DOWNWARD, 2013; HAWKINS; STEPHENS, 2015; VANHAESEBROECK et al., 2010). Typically, its activation starts with the union of a ligand to RTKs or GPCRs, through its regulatory p85 subunit. In non-activated states, the p85 subunit remains bonded to catalytic subunit p110 ABD region, through its iSH2 region, which stabilize p110 catalytic subunit. Once properly activated through cytokines, GF, insulin and LPS, the SH2 regions bind to its respective receptors or adapter proteins, leading to allosteric activation of the p110 catalytic subunit of the PI3K (CIANCIULLI et al., 2020; HEMMINGS; RESTUCCIA, 2012). When

activated, PI3K transforms the PIP2 to PIP3, that serves as membrane embedded second messenger, inducing the repositioning and activity of pleckstrin-homology (PH) region containing proteins, in the inner side of the plasma membrane (MANNING; TOKER, 2017). Among these proteins, can be included Akt (protein serine/threonine kinase), also knowns as PKB, and phosphoinositide dependent kinase 1 (PDK1) (HAWKINS; STEPHENS, 2015). Akt activation permits the transportation on protein kinase to the cytoplasm and nucleus, where it downstream modulates pro-apoptotic proteins, such as Bcl-2, caspase 9, fork head transcription factors (FOXOs), NF-κB, mTOR, and glycogen synthase kinase 3 (GSK3) **(Figure 9)** (MANNING; TOKER, 2017; RAUF et al., 2023; ZHU et al., 2014).

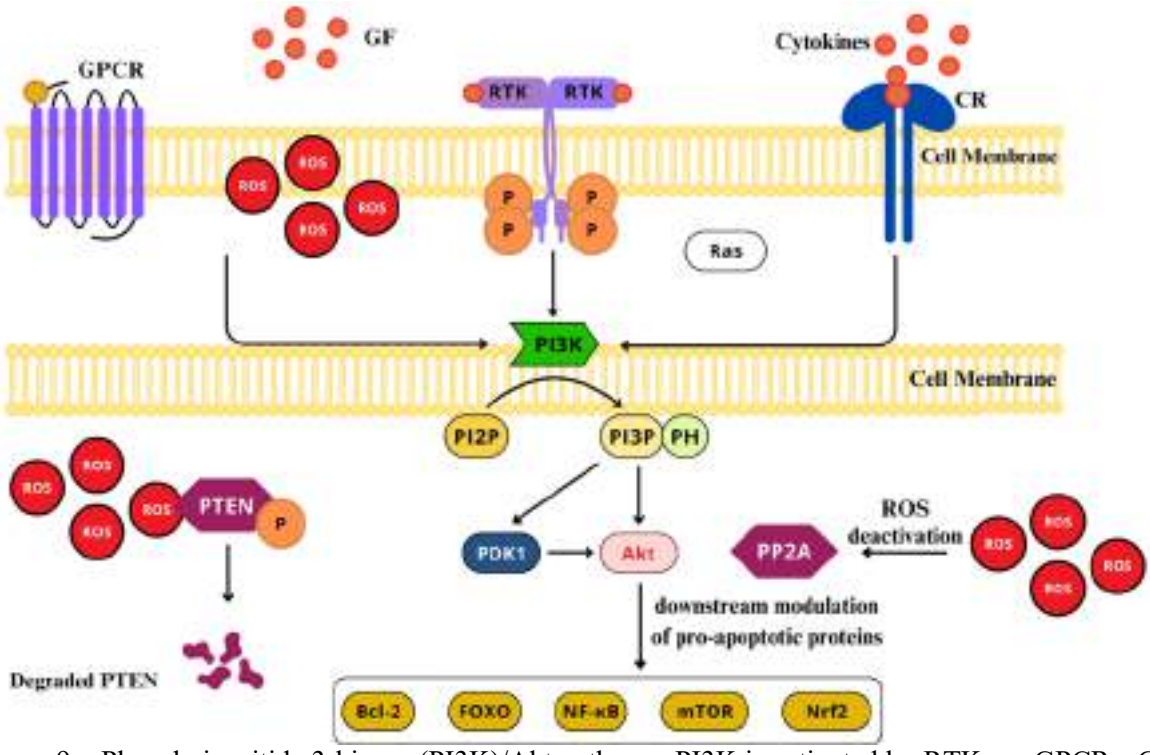


Figure 9 – Phosphoinositide 3-kinase (PI3K)/Akt pathway. PI3K is activated by RTKs or GPCRs, GF, CR, and ROS. Once activated, PI3K transforms the PIP2 to PIP3. This serves as a second messenger for Akt (protein serine/threonine kinase) and phosphoinositide dependent kinase 1 (PDK1) activation. Akt activation allows downstream modulation of pro-apoptotic proteins such as Bcl-2, caspase 9, fork head transcription factors (FOXOs), NF-κB, and mTOR. ROS can inhibit phosphatase and tensin homolog (PTEN), which negatively regulates PIP3 synthesis and inhibits Akt activation, leading to further activation of PI3K/Akt signaling pathway. Phosphatase 2A (PP2A), a Akt/PKB inhibitor, can be deactivated by ROS.

ROS and RNS interact with specific amino acids residues on tyrosine phosphatases, protein tyrosine kinase, and protein kinase C, that can trigger kinase cascades, MAPKs, and PI3Ks (KMA; BARUAH, 2022). ROS can directly activate PI3K, increasing its downstream signaling pathway (TROUTMAN; BAZAN; PASARE, 2012). At the same time, H_2O_2 can

oxidize and inhibit phosphatase and tensin homolog (PTEN), which negatively regulates PIP3 synthesis and inhibits Akt activation, hence, conducing to further activation of PI3K/Akt signaling pathway (NAKANISHI et al., 2014). Additionally, ROS enhance phosphorylation of PTEN through casein kinase II, allowing PTEN to undergo proteolytic degradation (LESLIE; DOWNES, 2002). Moreover, phosphatase 2A (PP2A), a Akt/PKB inhibitor, can be deactivated by ROS (INNOCENTI et al., 2003; LESLIE; DOWNES, 2002). Nevertheless, low ROS levels cause oxidation of Akt/PKB -S-S- bridges, promoting PP2A association with the Akt/PKB complex, promoting short-term activation of the Akt/PKB pathway (LEE et al., 2002; MURATA et al., 2003).

Dysregulation of this pathway has major implications in neuropathic pain (CHEN et al., 2017). PI3K/Akt inhibition or alteration conduce to elevated states of ROS production, membrane depolarization, mitochondrial membrane stability impairment, diminishment of oxidative phosphorylation and ATP production (ARCARO et al., 2002; SEITZ et al., 2013). The class I PI3K catalytic subunit p110α has a role in controlling ROS levels at a physiologic rate through the NRF2/ARE dependent pathway (WANG et al., 2008), which otherwise, not controlled, could lead to the activation of the autophagy machinery (KMA; BARUAH, 2022). Dysregulation of the PI3K/Akt pathway has a relevant role in the establishment of chronic pain conditions derived from traumatic lesions (CHEN et al., 2017; MANNING; TOKER, 2017; XU et al., 2014, 2007). PI3K has also been linked to TRK systems NGF/TrkA and BDNF/TrkB activation, displaying central sensitization and hyperalgesia mediation induced by G-CSF (CARVALHO et al., 2011), and mediating pain induced by plantar incision in mice (XU et al., 2014). Additionally, PI3K mediates peripheral and central sensitization and hyperalgesia caused by intraplantar injection of EphrinB1-Fc, triggering the EphrinBs (ligand) and EphBs (receptor) pathway activation (GUAN et al., 2010), affecting neuronal excitability, leading to increased firing of pain signals, contributing to pain hypersensitivity (SONG et al., 2008). PI3K was proved to be crucial for the DRG and dorsal horn neurons upregulation of the kinesis family member 1a (Kif1a), following a positive correlation between PI3K/Akt/CREB higher levels and Kif1a protein and mRNA expression in a chronic pain model induced by chronic constriction injury; the process starts with CREB phosphorylation by PI3K, that recruits the DNA demethylation TET1 towards the *Kif1a* promoter region, inducing upregulation its expression, which then leads to pain sensation, such insight offers a novel target for managing neuropathic pain, once inhibition of PI3K lead to decreased Kif1a expression, and alleviating pain associated behaviors (JIANG et al., 2025).

2.4.5. Calcium signaling

Divalent calcium ions (Ca^{2+}) are widely implicated in a plethora of pro-survival and pro-apoptotic cell processes, considering that it is one of the most relevant second messengers for intracellular signaling in most cells, but particularly in neurons and astrocytes, where serves intricate and integrated functions like, dendritic response to neurotransmitters, gene expression through nucleus signaling, and neurotransmitter release from presynaptic terminals (GLEICHMANN; MATTSON, 2011). Ca^{2+} aids to communicate depolarization status and synaptic activity to neurons (BERRIDGE; LIPP; BOOTMAN, 2000). The biochemically elegant mechanism through which it exerts signaling properties relies on the calcium concentration gradient relation between the extracellular space and the cytosolic space, and the later with intracellular compartments, such as endoplasmic reticulum and mitochondria (BERRIDGE; LIPP; BOOTMAN, 2000). In this context, given that Ca^{2+} is a main element to maintain and control neuronal excitability, it is not absurd to consider the high energetic demand that this implies for neurons, once all the Ca^{2+} that enters to the cytosolic space, must be removed from within, through either plasma membrane calcium ATPase (PMCA), sodium-calcium exchanger (NCX), mitochondrial calcium uniporter (MCU), mitochondrial efflux systems, endoplasmic reticulum calcium ATPase (SERCA), calcium release channels, and cation-dependent calcium pumps **(Figure 10)** (BRINI et al., 2014).

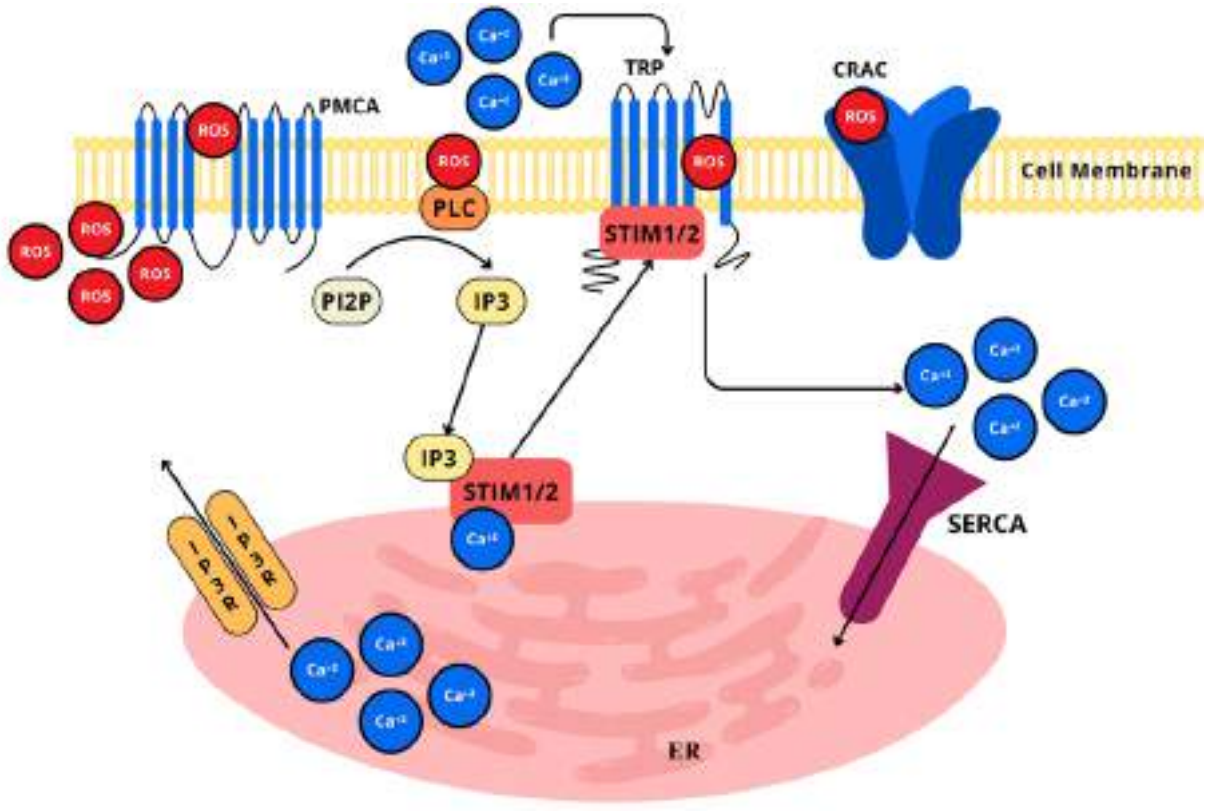


Figure 10 – Calcium signaling induced by reactive species. ROS can stimulate calcium signaling in cells of the nervous system, initiating lipid peroxidation and activation of phospholipase C (PLC) and inositol 1,4,5 – triphosphate (IP3) receptor (IP3R) – mediated calcium signaling. Also, ROS/RNS can directly oxidize or nitrosylate cysteine residues in Ca^{2+} channels and can influence Ca^{2+} channels gene expression, affecting their quantity and proportion in the cellular membrane, open-time probability, and trafficking. PMCA: Plasma Membrane Calcium ATPase; STIM1/2: Stromal Interaction Molecule 1/2; CRAC: Calcium Release-Activated Calcium channel; SERCA: Endoplasmic Reticulum Calcium ATPase.

The coordinated actions of the aforementioned mechanisms of Ca^{2+} transport and exchange ensure that intracellular calcium levels are precisely regulated, avoiding toxic levels, this allows proper neuronal function and signaling (BAEV et al., 2022). As previously described, mitochondria are responsible for ATP production, and the ETC functioning; yet another significant task it performs is related to temporary calcium buffer, when cytosolic levels of Ca^{2+} are above normal concentrations, therefore protecting neurons against high Ca^{2+} excitotoxicity (ABETI; ABRAMOV, 2015).

In the mitochondria, $Ca^{2+}$is transported through the MCU, against the concentration gradient, by means of electrochemical gradient – mitochondrial membrane potential; once in the mitochondria, Ca^{2+} forms inactive precipitates bounded to inorganic phosphates (BAUGHMAN et al., 2011). When Ca^{2+} is not bounded, it is transported back to the cytosol by the NCX. Nevertheless, when mitochondrial levels of Ca^{2+} are excessively high, this will force its way out through the mitochondrial permeability transition pores (mPTP); this marks the initial steps towards the release of proapoptotic proteins and the beginning of cell death through necrosis or apoptosis (ANGELOVA, 2021; OTT et al., 2007). Cytosolic Ca^{2+} levels increase mediated by ROS induces the AP-1 proteins c-Fos and c-Jun (DRÖGE, 2002; JIMÉNEZ et al., 2004), as well as PKC-α activation through oxidative stress (COSENTINO-GOMES; ROCCO-MACHADO; MEYER-FERNANDES, 2012).

As formerly stated, mitochondria is a paramount producer of reactive species, through the ETC as by-products, mainly $O^{\bullet-}$ and H_2O_2; as well as through the TCA cycle, α-ketoglutarate dehydrogenase or pyruvate dehydrogenase complexes, which mainly produces $O^{\bullet-}$ and H_2O_2; or through enzymes not related to the ATP production, yet residents of the mitochondrial membrane, such as monoamine oxidase (MAO), and cytochrome b5 reductase (Cb5R), and glycerol–3–phosphate dehydrogenase (ANGELOVA; ABRAMOV, 2016). Altogether, mitochondria can be considered a producer and a target itself of ROS actions, due to their intricate interaction, mutual regulation and complementary functions, either in physiological or pathological conditions (KANIA et al., 2017).

ROS can stimulate calcium signaling in cells of the nervous system, as seen in cases where astrocytic MAO produced H_2O_2, initiating lipid peroxidation and activation of phospholipase C and inositol 1,4,5 – triphosphate (IP3) receptor (IP3R) – mediated calcium signaling (NOVIKOVA et al., 2020). Astrocytes in hypoxia conditions have shown to trigger Ca^{2+}signaling as a response to ROS; also, RONS can directly oxidize or nitrosylate cysteine residues in Ca^{2+} channels, specifically in the pore – forming α-1-subunit (GÖRLACH et al., 2015), altering its conformational structure and function, modifying neurotransmitter release, synaptic plasticity, essential for learning, memory and chronic pain (TODOROVIC; JEVTOVIC-TODOROVIC, 2014). Additionally, RONS can influence Ca^{2+} channels gene expression, affecting their quantity and proportion in the cellular membrane, open-time probability, and trafficking (BOGESKI et al., 2010). Two major types of channel proteins seem to be involved in receptor-induced Ca^{2+} signals, the store-operated Ca^{2+} channels (SOC) and transient – receptor potential channels (TRP). Both are of major importance for cell functions, thus been accounted for the entrance and release of Ca^{2+}through plasma membrane channels, and from its sites of storage, respectively. TRP, which are known to transport mainly Ca^{2+} ions, have six non-voltage sensitive transmembrane channels, divided based on their activation mechanism and N-termini or C-termini regulatory domain, the canonical (TRC1 – TRC7), melastatin -related TRP (TRPM1 – TRPM8), and vanilloid – receptor related TRP (TRPV1 – TRPV4) (GÖRLACH et al., 2015).

All of the TRP family members are redox sensitive and participate of redox regulation and oxidative stress, either through activation by H_2O_2 mediated by ADP-ribose or cyclic ADP-ribose reacting in the binding cleft of the C-terminal (PERRAUD et al., 2005), by other oxidizing agents that sensitize TRP to pH alterations (SUSANKOVA et al., 2006), or by modulation of SOC, which is regulated by translocation of the ER Ca^{2+} sensors stromal interaction molecule 1/2 (STIM1/2) to the plasma membrane, where they activate Orai channels to ignite calcium entry and storage (BOGESKI; KILCH; NIEMEYER, 2012). The TRP channels are of paramount relevance in the processing and discrimination of heat, cold, pain and stress components and in the establishment of neuropathic pain derived from chemotherapy treatment. Paclitaxel can cause severe peripheral neuropathic pain, demonstrated by mechanical withdrawal threshold decrease in rats, which was inhibited after intrathecal and intraperitoneal administration of TRPV1 antagonist, capsazepine; TRPM8 protein expression was also reduced in DRG neurons. Topical application of menthol increased TRPM8 activity and reduced TRPV1 activity. In that sense, TRPV1 upregulation and TRPM8 inhibited activity are related

to neuropathic pain induced by paclitaxel (LI et al., 2024). Following nerve injury or neuron dysfunction, TRP channel expression is altered affecting neuronal excitability, neuroplasticity and neuroinflammation, conducing to neuropathic pain, as evidenced by TRPC1, TRPC4-6 increased expression associated to mechanical hypersensitivity in SNI (ALESSANDRI-HABER et al., 2009; CHU et al., 2020; XU; WANG, 2024).

In this sense, both mitochondrial ROS production and Ca^{2+} have physiologic roles in normal cell functioning. Nevertheless, dysfunction in both equilibriums also play head roles in the pathogenesis chronic pain states, and cell death, once neurons are vulnerable to greater redox and energetic disturbs (GODOY et al., 2021).

2.4.6. Unfolded protein response (UPR) pathway

As formerly mentioned, when ER undergoes stressing conditions, such as alterations in redox homeostasis, hypoxia, altered Ca^{2+}regulations, sustained hyperglycemia, and accumulation of unfolded/misfolded proteins in the ER lumen (GONG et al., 2017; MALHOTRA; KAUFMAN, 2007), a series of adaptative/protective or maladaptive responses initiates, named unfolded-protein response (UPR), which directly or indirectly affects the ER itself, or other organelles (SANTOS et al., 2009). The primarily objective of this elicited response is to reestablish normal ER function and homeostasis; nevertheless, when stress conditions are indeed critical and chronic, cell is driven towards dysfunctionality, oxidative stress, ER stress, and ultimately, cell death (CHAUDHARI et al., 2014). ER stress is widely implicated in neuronal injury (NIELLA et al., 2024; SOKKA et al., 2007). SNL induces increased expression of ER stress markers such as *sXBP1*, BiP, as well as inducing the activation of the ATF6 and PERK/eiF2 pathway in dorsal horn neurons of the spinal cord (ENJI ZHANG, 2015). Use of chaperones such as tauroursodeoxycholic acid has been effective in reversing ER stress, elicited by the UPR in neuropathic conditions like, CCI, diabetic peripheral neuropathy and that induced by formalin (NIELLA et al., 2024; PATEL et al., 2023; ZHOU et al., 2017). The influence of the UPR towards neuropathic pain involves central and peripheral sensitization, either by increased ER stress markers, or impairing calcium signaling, for the trigger of nociceptive pathways, leading to hyperalgesia .

The initiation and development of the UPR involves three proximal ER stress – sensor transmembrane kinases/transcription factor, namely inositol – requiring kinase 1 (IRE-1α), activating transcription factor 6 (ATF-6), and double stranded RNA-activated protein kinase

(PKR)-like endoplasmic reticulum kinase (PERK), each of which has its own ER branch, but also work complementarily, to restore normal ER functions, which are, under non-stressed conditions, attached to the immunoglobulin heavy chain binding protein (GRP78/BIP), through their amino terminals, at their ER luminal ends **(Figure 11)** (READ; SCHRÖDER, 2021; SANTOS et al., 2009). ER embodies a highly oxidizing-folding environment to enable disulfide bond formation as one of the fundamental steps in normal protein structural formation, thus, it is attributed to it the contribution of 25% of ROS produced by cells (MALHOTRA; KAUFMAN, 2007).

This oxidative process of intramolecular disulfide bonds formation, known as oxidative protein folding (OPF) is one of the most usual posttranslational protein modifications (BORGES; LAKE, 2014). The catalyzation of the disulfide bonds formation is primarily carried out by the thioredoxin protein disulfide isomerase (PDI), through thiol-disulfide oxidation. PDI are formed by four thioredoxin (Trx) domains, namely a, a', b, and b', and a KDEL ER retention group of c-domain (KOZLOV et al., 2010). The redox state of the catalytic a-domains in the PDI enzyme stablishes the oxidase or isomerase activity, depending on the CGHC (cysteine-glycine-histidine-cysteine) active sites motifs; thus, PDI in its reduced state, due to the active site cysteines within the CGHC motif present as thiols (-SH), allows PDI to disrupt incorrect disulfide bonds, acting as disulfide reductase, as well as rearrange incorrect disulfide bonds into correct ones, acting as isomerase; while in the oxidized form, the active site cysteines within the CGHC are present as disulfide (-S-S-) bonds, allowing PDI to oxidize substrates, to form new disulfide bonds between cystine residues, working as an electron acceptor (ZHANG et al., 2019).

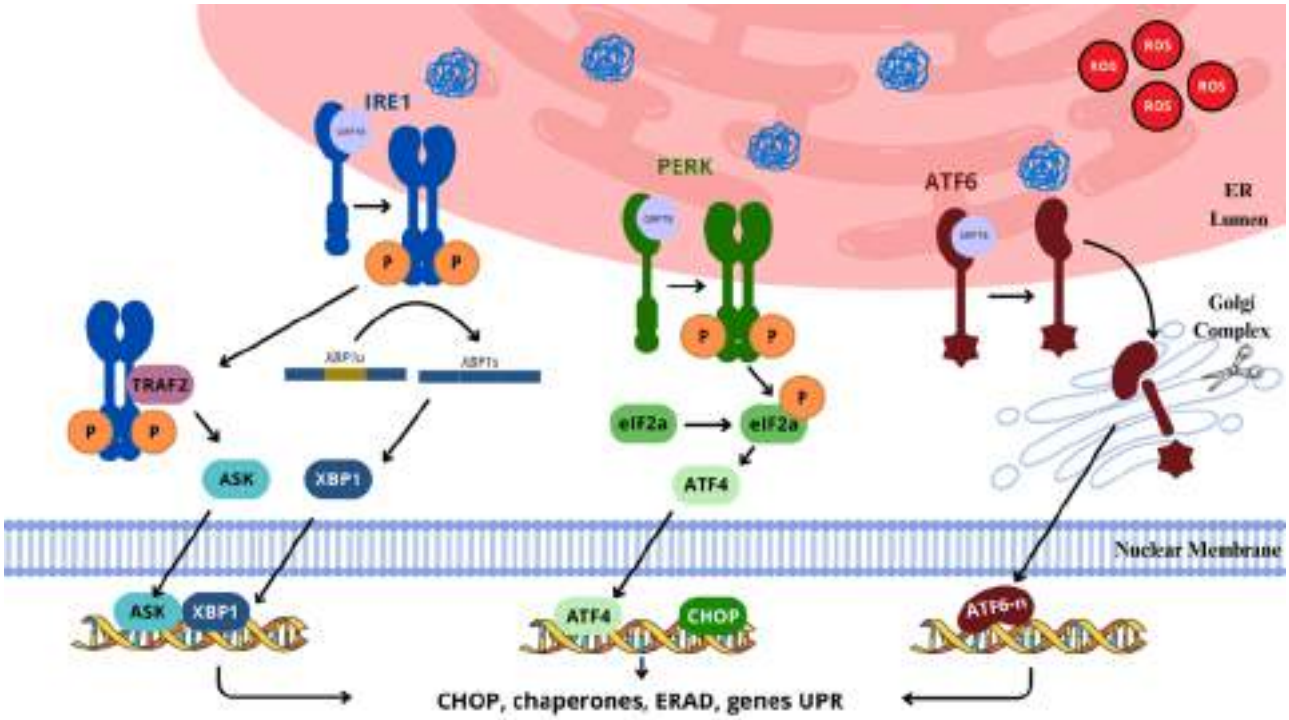


Figure 11 – Unfolded protein response (UPR) pathway. Under non-stressed conditions, GRP78 attaches to unfolded protein response (UPS) sensors, IRE-1α, ATF-6, and PERK. In the presence of unfolded/misfolded proteins, GRP78 is detached from the UPR sensors; once activated induce complex pro-survival autophagy, antioxidant ERAD pathway, and ER regeneration, or pro-apoptotic and ferroptosis mechanisms. Also, ROS produced serves as signal for activation or modification of the cellular stress response, once ROS-oxidated cysteine residues in the UPR sensors can mediate UPR signals. IRE-1α: Inositol – Requiring Kinase 1; ATF-6: Activating Transcription Factor 6; PERK: RNA-activated protein kinase (PKR)-like Endoplasmic Reticulum Kinase; eiF2a: Eukaryotic Initiation Factor 2 Alpha; XBP1: X-box Binding Protein 1; **CHOP:** C/EBP Homologous Protein; ASK1: Apoptosis Signal-regulating Kinase 1; TRAF2: Tumor Necrosis Factor Receptor-Associated Factor 2.

On the other hand, b' domains are responsible for the determination of misfolded or unfolded protein, through identification of hydrophobic exposed terminals of the proteins (KLAPPA et al., 1998). H_2O_2, besides being the main source for oxidation, produced by O_2, is also used by peroxidases to shape disulfide bonds to re-oxidize PDI. The cyclic reoxidation of PDI in the active sites help maintain the redox balance within the ER, which is more oxidative than the cytosolic environment. Yet, under highly oxidizing conditions, CGHC motif can be hyperoxidized to its cysteine sulfinic acid or sulfonic acid form, turning it unactive, and unable to catalyze further reactions, leading to accumulation of unfolded or misfolded proteins (ZHANG et al., 2019).

Successively, the ER membraned associated oxidoreductin (ERO-1), that possess two cysteine pairs on a flexible loop and on the CXXC motif, transfers sulfhydryl electrons from the reduced PDI to O_2, yielding H_2O_2, that adds to maintaining the oxidizing environment (ARAKI et al., 2013; CHAUDHARI et al., 2014). In this sense, the ER redox state is tightly

related to ER homeostasis, that depends on ROS production during disulfide bonds formation in protein folding, to participate in the pro-oxidizing environment in the ER (BHATTARAI et al., 2021). Additionally, quiescin sulfhydryl oxidase (QSOX), a FAD domain and Trx domain containing enzyme, catalyzes the formation of disulfide bonds, via free thiol groups oxidation on cysteine residues of substrate proteins, transferring electrons from the cysteines of substrates to the cysteines in the Trx domain of QSOX, which are then shifted to its FAD domain. The reduced $FADH_2$ domain acts as an electron acceptor, ready to be re-oxidized, conveying the electrons to O_2, yielding H_2O_2, allowing QSOX to reenter a new cycle of disulfide bonds formation (SEVIER, 2012).

Further increase in ROS formation is triggered by Nox4 activity after UPR (ZEESHAN et al., 2016). Additionally, another source of ER-stress is attributed to ROS – dependent Ca^{2+} depletion from the ER, that restrains the ability of Ca^{2+} to activate ER chaperones, such as calreticulin and calnexin. This can be explained due to the fact that IP3R, the ryanodine receptor (RyR), and Ca^{2+} pumps are redox regulated. For example, SERCA activity is inhibited when Cys674 residue undergoes sulfoxidation, and solely activated after the same cysteine residue undergoes glutathionylation by NO; IP3Rs sensitivity to IP3 and RyR activity are enhanced by ROS, leading to augmented Ca^{2+} release from the ER (ELETTO et al., 2014).

However, excessive RONS can conduce to dysregulation of these receptors, contributing to ER and cellular stress and death through disrupted calcium homeostasis. As another source of ER oxidizing environment, when misfolded proteins appear, disulfide bonds are corrected by reduction through GSH, resulting in higher oxidized glutathione (GSSH) rates; this also participates in redox ER homeostasis (SANTOS et al., 2009).

In this process, paradoxically, the UPR induces increased ROS levels, while at the same time, ROS-mediated ER stress is expected to be restored by UPR. in this context, ROS ER levels increase due to higher ERO-1-PDI activity , but ROS produced also serves as signal for activation or modification of the cellular stress response, once ROS-oxidated cysteine residues in the UPR sensors, IRE-1α, ATF-6, and PERK can mediate UPR signals, being activated to induce complex pro-survival autophagy, antioxidant ERAD pathway, and ER regeneration, or pro-apoptotic and ferroptosis mechanisms (KIM; LEE; SHEN, 2024). Similar to ROS, RNS induce nitrosative stress, being linked to UPR – mediated cytotoxicity via cysteine residues inactivation in the active sites of PDI (UEHARA et al., 2006).

3. ANTIOXIDANT ENZYMES

The nervous system exhibits a high oxidative metabolic activity rate with a consequent preeminent amount of reactive species production via enzymatic and non-enzymatic reactions as by-products of normal neuronal functioning (MASSAAD; KLANN, 2011), being particularly elevated during neurons and glia activation by glutamate and ATP, and more specifically in neurons of the midbrain and cerebellum, mainly attributed to glial cells (VINOKUROV et al., 2021). Moreover, the relatively low levels of antioxidants (LEE; CHA; LEE, 2020), the high membrane surface to cytoplasm rate, and the high lipid content makes neurons particularly vulnerable targets to oxidative damage over macromolecules and lipid peroxidation (NIEDZIELSKA et al., 2016). Although, as above mentioned, redox signaling works as an intrinsic sensor for oxidative stress, serving several physiological functions, when the cellular antioxidant systems fail to prevent overproduction of such reactive species, detrimental pathways can be activated, inducing cellular and tissue damage.

The cellular antioxidant systems, that is, those compounds present at lower concentration in relation to oxidizable substrate that are able to hinder or preclude the oxidation of the substrate (HALLIWELL, 1990) are composed by enzymatic and non-enzymatic molecules and are chemically structured for and capable of reducing other compounds with oxidizing potential of different chemical natures (KADIISKA; MASON, 2002). The first line of endogenous defense is constituted by an enzymatic antioxidant system that include SOD, CAT, GPx, whereas the second line of defense is formed by non–enzymatic antioxidants, such as antioxidant enzyme cofactors, reactive species scavengers, oxidative enzyme inhibitors, and Cu, Fe, Zn, and Mn transition metals, by participating in reactions that neutralize ROS directly or catalyzing the formation of less reactive species **(Figure 12)** (LEE; CHA; LEE, 2020; PISOSCHI; POP, 2015). Further processes of restoration of damage by a repair system can be activated, eliminating oxidized nucleic acids by proteolytic enzymes, and lipids, either by phospholipases or peroxidases (CADET et al., 2017; LOBO et al., 2010).

Most of the antioxidants develops its actions in a series of steps similar to those occurring during lipid peroxidation, that is, initiation, propagation, and chain termination (AYALA; MUÑOZ; ARGÜELLES, 2014). For instance, antioxidants inhibit lipid peroxidation removing O_2, reducing local concentration of O_2, removing oxidative metal ions, neutralizing hostile reactive species, scavenging chain initiating radicals, stopping the progress of chain-reactions, extinguishing $^1O_2$ (PISOSCHI; POP, 2015). Antioxidants act controlling excessive

reactive species formation, neutralizing by direct scavenging through donation of an electron to the reactive species/free radicals, inducing the formation of less reactive or stable products, preventing further oxidation, regenerating other antioxidant to its active form, chelating metal ions, avoiding them to enter catalytic reactions that yields free radicals, like Fenton reaction, and enhancing other endogenous antioxidant systems (LOBO et al., 2010).

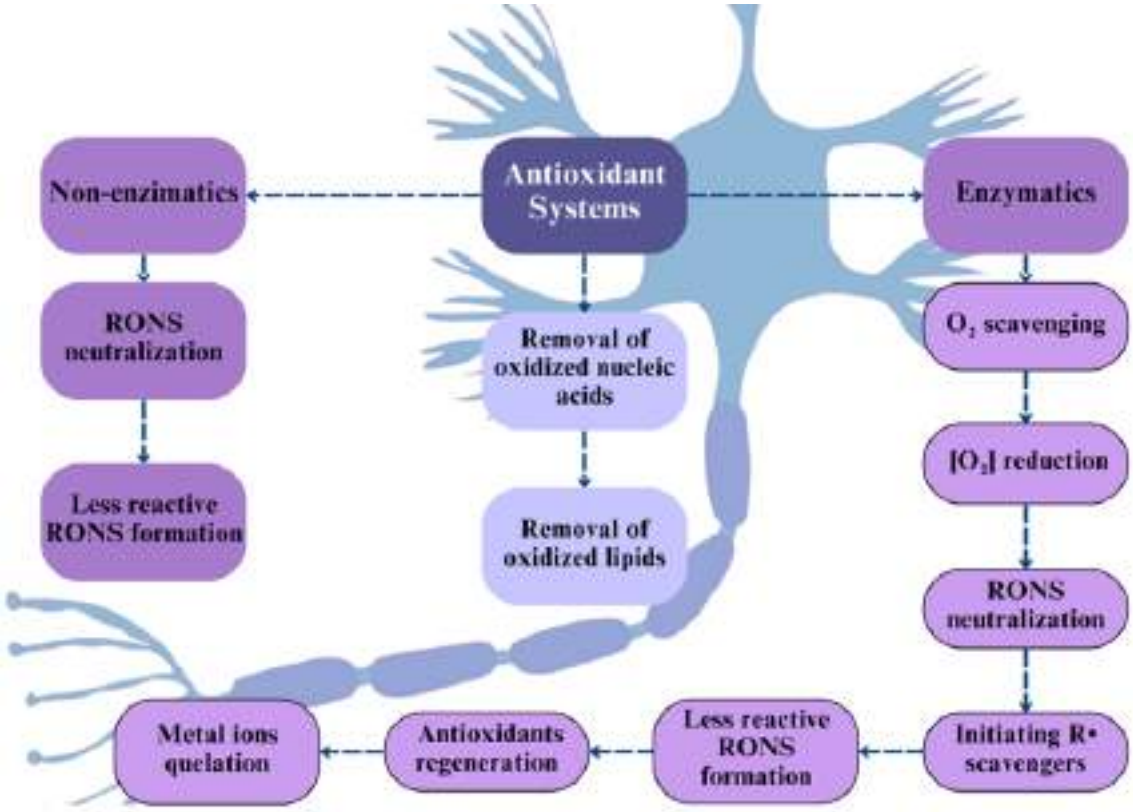


Figure 12 – Enzymatic and non-enzymatic antioxidant endogenous systems mechanisms of action.

For the purpose of this review, focus will be addressed to those able to catalytically deplete reactive species, without being consumed in the reaction, that is, preventative enzymatic antioxidants, as opposite to non-enzymatic chain-breakers, singlet oxygen quenchers, and metal chelator antioxidants, that are consumed in the reaction, once these are the most widely studied and considered the most important among the endogenous antioxidant systems, for its compelling contribution to the normal redox state, and for its role in promoting the synthesis or regeneration of further antioxidant or pro – survival mechanisms (JOMOVA et al., 2023, 2024). In addition to SOD, CAT, and GPx which are considered to be the first front line of endogenous antioxidant defense system, it will be also included the Trx enzyme and the thioredoxin reductase (TrxR), for its enzymatic nature, as opposite to that considered by other authors (IGHODARO; AKINLOYE, 2018).

3.1. Superoxide dismutase

Superoxide dismutase family (SODs) are transition metal – containing enzymes present in all living organisms existing in the presence of oxygen. SODs are considered one of the most powerful antioxidant enzymes against biological oxidants and possess the ability to transforms two $O^{\bullet-}$ molecules into H_2O_2 and O_2 in a pH-independent medium (JOMOVA et al., 2024). In mammals, three forms of SOD are acknowledged, the Cu, Zn – containing SOD-1, is a homodimer constituted by eight beta strands, with one Cu atom serving for catalytic function, and one Zn atom relevant for structural stability, in each of the two subunits, respectively; it is ubiquitously expressed, and found predominantly in the cytoplasm, but also expressed in the mitochondrial intermembrane space and the nucleus (DAVIS; PENNYPACKER, 2017; WANG et al., 2018b); SOD-2 is a homotetrameric enzyme that contains Mn in its active site. It is found in the mitochondrial matrix where the pH is considerably higher (pH approx. 7.8), compared to that of the intermembrane space (approx. 7.0 – 7.4); it is expressed at different amounts depending on the type of cell, being significantly higher in those cell that encompass elevated proportions of mitochondria (JOMOVA et al., 2024).

They perform its dismutation power over the $O^{\bullet-}$ molecules produced in the ETC, providing electrons from the metal core directly to the negatively charged $O^{\bullet-}$ molecules and a protonation to yield H_2O_2 (DAVIS; PENNYPACKER, 2017); and finally, the Cu and Zn containing enzyme SOD-3, produced intracellularly and found predominantly in the extracellular space (ADACHI; WANG, 1998), is homotetrameric in humans and mice, but dimeric in rats **(Figure 13)** (ZELKO; MARIANI; FOLZ, 2002). Opposed to SOD-1 and SOD-2 that are ubiquitously expressed, SOD-3 is diversely expressed across different types of cells, found to be highly expressed in heart, lungs, and pancreas, but low in the brain (ZELKO; MARIANI; FOLZ, 2002). Additionally, SOD-3 is generally less responsive to direct induction by $O^{\bullet-}$ molecules or other oxidants, due to its presence and role in the extracellular matrix, where exposure to regulatory signals others than those encountered in the intracellular space are found. SOD3 is regulated in conjunction with signaling molecules and cytokines, such as interleukin-1 (IL-1β), and TNF-α, as part of a coordinated response to inflammation, contributing to modulate oxidative stress in the extracellular environment (AGRAHARI et al., 2021).

The dismutation reaction catalyzed by SOD is considered to be highly efficient given that it happens at a diffusion – limited rate of $\sim 2 \times 10^9\ M^{-1} \cdot s^{-1}$, that is $\sim 10^4$ the rate constant during spontaneous dismutation (MCCORDS; FRIDOVICH, 1969). In the process, an electron reduction and protonation of $O^{\bullet -}$ radical results in the formation of H_2O_2, by means of an initial step of $O^{\bullet -}$ radical binding to the core Cu(II) ion, resulting in the oxidative transfer of an electron to $O^{\bullet -}$ and production of reduced Cu(I); then, a second $O^{\bullet -}$ radical is bounded to a partly protonated Arg143 in the anion-binding site of the SOD that oxidize the $O^{\bullet -}$ radical. Moreover, His63 donates a proton and an electron from reduced Cu(I) to $O^{\bullet -}$ radical, producing H_2O_2, and the oxidation of Cu(I) to Cu(II), to pursuit further dismutation (QUIST et al., 2017).

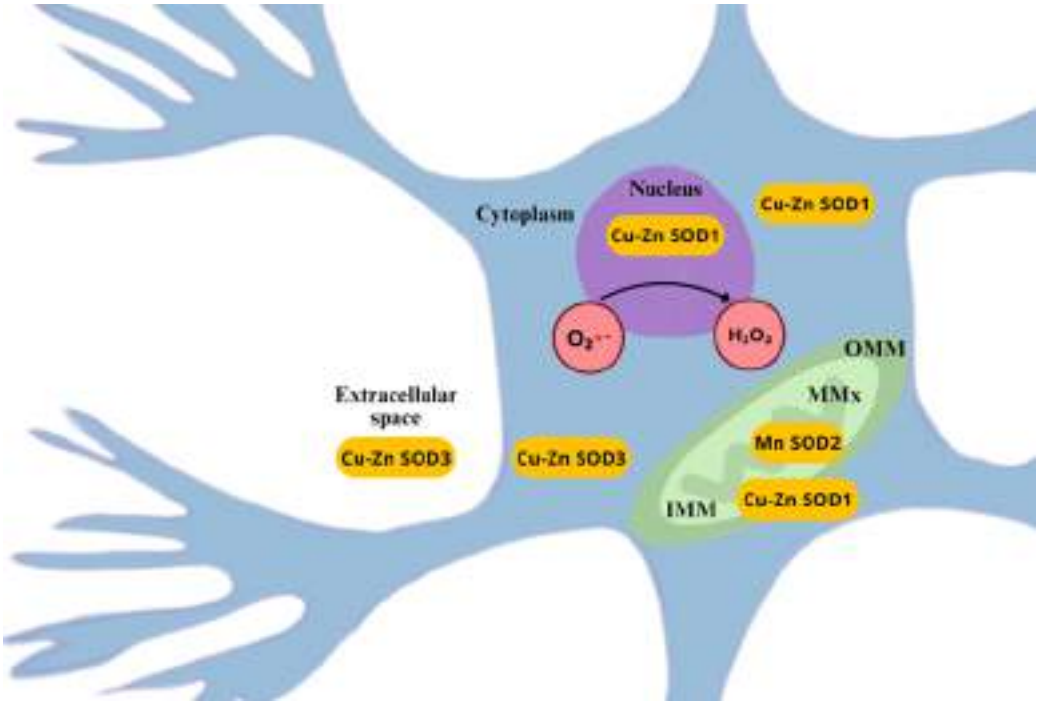


Figure 13 – Superoxide Dismutase (SOD) sites of expression in neuronal cell. IMM: internal Mitochondiral Membrane; OMM: outer Mitochondrial Membrane; MMx: Mitochondrial Matrix.

Changes in SOD expression or function are linked to chronic pain. Maropitant treatment increases SOD expression, reducing pain threshold in peripheral nerve injury, often marked by neuronal loss (DAVIS; PENNYPACKER, 2017; NIELLA et al., 2024). Enhanced expression of SOD-1 was observed to reduce lipopolysaccharide-mediated superoxide production, suppress cyclooxygenase (COX)-2 and iNOS expression, and confer protection against microglia-mediated inflammatory responses (FAN et al., 2023). Compromised SOD-2 function is a common characteristic of pathogenesis associated to oxidative stress in brain injury (HALL et al., 2019). Moreover, an increase in SOD-2 expression avoided neuronal death from oxidative stress (MURAKAMI et al., 2011).

3.2. Catalase

Catalase (CAT) is a tetrameric heme-containing enzyme, formed by four tetrahedrally arranged subunits of 60kDa each, containing an active heme group and NADPH bound to each subunit (KIRKMAN; GAETANI, 1984). It is part of the front-line defense antioxidant system, commonly found in mammalian cells as part of the peroxisome (LEE; CHA; LEE, 2020). CAT is produced due to oxygen exposure and is characterized by the ability to catabolize the rupture of two H_2O_2 molecules to form two molecules of H_2O and one of O_2, through a two-step reaction, using Fe as a cofactor **(Figure 14)** (AMIR ASLANI; GHOBADI, 2016). It is expressed ubiquitously in central nervous systems cells, including neurons and glia, where works protecting cells by detoxification of H_2O_2, capacitating the body to tolerate and adapt to oxidative stress, as an adaptative response (USUI et al., 2009). In addition to its protective effects against ROS, CAT offers influence over several pathways and metabolic processes such as growth, proliferation and apoptosis (UTTARA et al., 2009) and influence over redox sensitive signaling molecules, like protein kinases and transcription factors, through modulation of H_2O_2 levels, impacting cellular responses to environmental or endogenous stressor (BAKER et al., 2023).

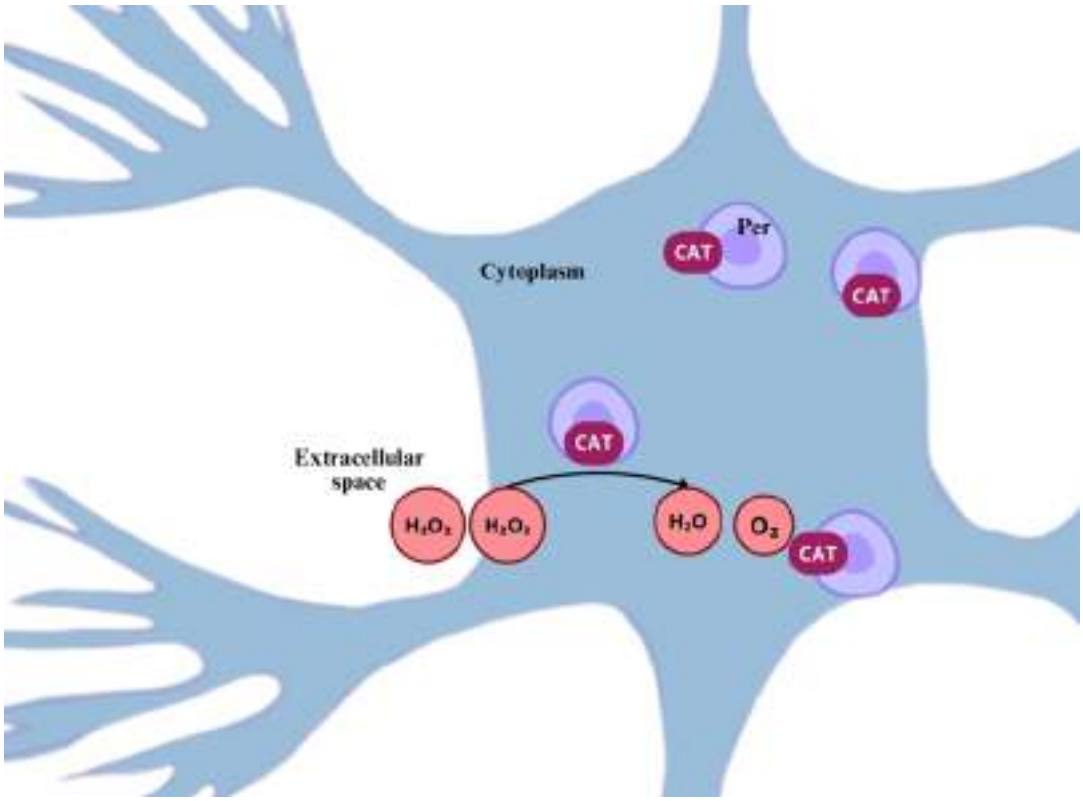


Figure 14 – Catalase (CAT) sites of expression in neuronal cell. CAT is characterized by the ability to catabolize the rupture of two H_2O_2 molecules to form two molecules of H_2O and one of O_2. Per: peroxisome.

The reaction is described as a two-step reaction, as follows: initially, a compound I, the covalent oxyferryl species Fe(IV) possessing a porphyrin π cation radical is formed from Fe(III), via reduction of one H_2O_2 molecule; following, the Fe(IV) compound is decomposed through redox reactions via two-electron transfer from the second H_2O_2 molecule, which works as an electron donor to the reaction, therefore, producing O_2 and H_2O, and restoring the compound I to its resting state Fe(III) (NANDI et al., 2019) after so, the final products, O_2 and H_2O, are released from the catalase, and the later returns back to its original state, to pursuit further reactions, binding and decomposing another H_2O_2 molecule. CAT is maintained chemically and structurally available after repeated cycles of chemical reductions of H_2O_2, contributing to steady O_2 concentrations states, and redox balance (NANDI et al., 2019).

Also, it is considered one of the most powerful antioxidant molecules in biological systems, once it has the highest turnover of all enzymes, degrading millions of H_2O_2 per second (JOMOVA et al., 2024). Inhibition of CAT activity induced increased cytotoxicity and higher ROS rates, denoting a pivotal role in oxidative balance (LEE; CHA; LEE, 2020).

Therefore, CAT dysfunction or deficiency is thought to cause impaired glucose metabolism and lipid accumulation (DUTTA et al., 2021), as well to contribute to oxidative stress and neuronal damage in paclitaxel induced neuropathic pain, where it is decreased together with NRF2, SOD2, HO-1, and can be reversed by cannabidiol (CBD) and tetrahydrocannabivarin (THCV) by upregulation of catalase, but also other protective proteins such as p-AMPK (KUMAR KALVALA et al., 2022) contributing to pain relief and neuronal protection.

CAT is increasingly recognized as a therapeutic target for diseases , founded on CAT-based therapeutic strategies providing neuroprotective effects through integrity and function neuronal preservation, for treating or averting neuroinflammatory events, using viral-cultured CAT or gene-therapy, delivering CAT genes and enhancing endogenous CAT gene expression. Moreover, innovative lines addressed to enhance CAT-based therapy comprise protein engineering, with improved stability, activity and specificity remains promissory, expanding therapeutic potential and strategies (LUANGWATTANANUN et al., 2016).

3.3. Glutathione peroxidase

The family of antioxidant enzymes glutathione peroxidase (GPx) consist of a group of, to date identified, eight isoforms (GPx1 – GPx8) present in all living organisms. Among them, GPx1 is the most abundantly found in almost all tissues and is highly expressed in the cytoplasm and mitochondria; GPx2 is found mostly in the gastrointestinal tract and human liver; GPx3 is found in the plasma and in the extracellular space; GPx4 is expressed ubiquitously, but specially in brain, testis, and sperm cells where cell membranes need to be protected from lipid peroxidation; GPx5 is specific from the epididymis; GPx6 is expressed in the olfactory epithelium and embryonic tissues; and GPx7-8 are expressed predominantly in the ER, where they play a role in protection from oxidative stress, and maintaining the oxidative balance for oxidative protein folding and the secretory pathway **(Figure 15)** (PEI et al., 2023). Chemically, GPx are oligomeric enzymes, formed by subunits arranged with either a selenocysteine or cysteine amino acid redox-sensitive active site, both playing main functions in the catalytic reduction of hydroperoxides and H_2O_2, which are transformed to alcohols (ROHs) or H_2O, respectively (HANDY; LOSCALZO, 2022).

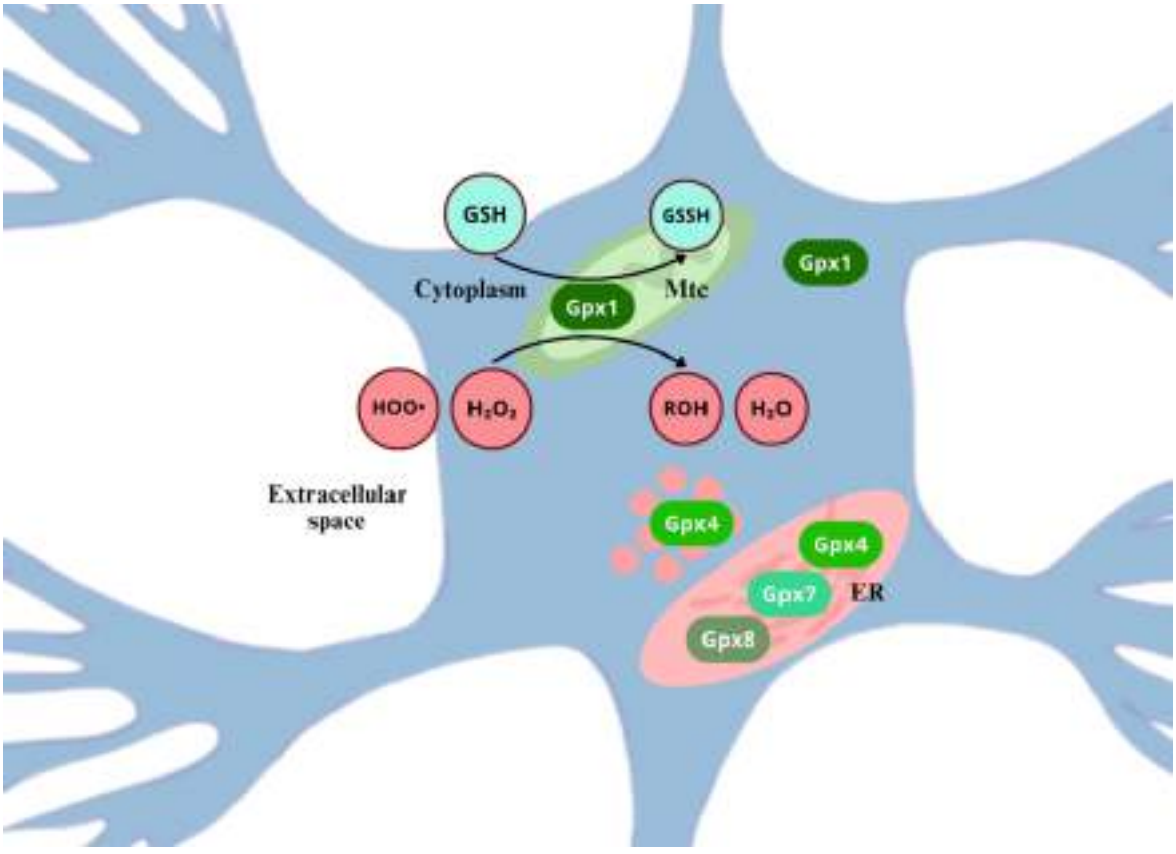


Figure 15 – Glutathione peroxidase (Gpx) sites of expression in neuronal cell. Gpx drives the enzymatic reduction of H_2O_2 and hydroperoxides via oxidation of reduced glutathione (GSH) to its oxidized form (GSSH) in a ping-pong mechanism involving oxidation of the Gpx followed by a two-step reduction.

The GPx family has a vast number of physiological functions, although the most important role played is related to enzymatic reduction of H_2O_2 and hydroperoxides via oxidation of reduced glutathione (GSH) to its oxidized form (GSSH) in a ping-pong mechanism involving oxidation of the GPx followed by a two-step reduction (WEAVER; SKOUTA, 2022). As a first phase, the catalytic site of GPx consisting of a selenocysteine or a cysteine amino acid is oxidized to selenenic acid or sulfenic acid, respectively, by hydroperoxides or H_2O_2 which successively are transformed to its corresponding reduced forms, alcohols (ROHs) or H_2O (BRIGELIUS-FLOHÉ; FLOHÉ, 2020); thereafter, the second phase involves the return of GPx to its initial reduced state, through a series of reduction reactions by the GSH and NADPH as a cofactor. Here, the two-step reduction process starts with selenenic acid and sulfenic acid return to its oxidized states, selenocysteine and cysteine, with the help of two GSH molecules, that becomes oxidized (GSSG) in the process (PEI et al., 2023). The return of the oxidized GSSG form to the reduced GSH form involves the enzyme glutathione reductase (GR); for such, NADPH binds to the active site of the GR that has a FAD cofactor; here, the NADPH donates electrons to FAD, reducing it to $FADH_2$, that in turn transfers these electrons to the disulfide bond in GSSG, disrupting the bond and yielding two molecules of GSH. As FAD is reduced, NADPH is oxidized to NADP+, that is released from the enzyme, finishing the cycle (PEI et al., 2023).

Intracellular depletion of GSH and decreased GPx4 activity caused by ferroptosis, an iron – dependent cell death pathway seen in pathological processes, results in the accumulation of lipid peroxides, Fe(II) – catalyzed oxidation of lipids and increased production of reactive species (LI et al., 2020). In a spinal cord injury model in rats, GPx was upregulated following treatment with L-carnitine and atorvastatin, as opposed to non-treated animals, improving neuronal function (HAZZAA et al., 2021). GPx is also upregulated in SNL – induced neuropathic pain (DRUMMOND et al., 2024)**.** GPx4 was downregulated in a model of spared nerve injury in rats; after methyl ferulic acid administration, GPx4 levels were increased, accompanied by significant NOX4, ferroptosis – related protein ACSL4 decreased, and consequent mechanical withdrawal threshold and thermal withdrawal latency improvement (LIU et al., 2023). Another study assessing whether sirtuin 2 (SIRT2) attains a neuroprotective effect in spared nerve injury – induced neuropathic pain, found enhanced levels of GPx4 and ferroportin 1 (FPN1) after SIRT2 recombinant adenovirus intrathecal administration, reducing intracellular iron accumulation and oxidative stress, hence, attenuating mechanical hypersensitivity (ZHANG et al., 2022c). It has also been demonstrated that GPx4 upregulation

can reverse and inhibit ferroptosis – induced stress, blocking neurons and glia activation from the dorsal horn of spinal cord in neuropathic pain induced by chronic constriction injury in rats (GUO et al., 2021; WANG et al., 2021). Altogether, GPx is a relevant target for therapeutic approach towards neuropathic pain (FAN et al., 2025).

3.4. Thioredoxin

The thioredoxin (Trx) systems is formed by two oxidoreductase enzymes, Trx and TrxR, and NADPH as an electron donor. Trx are highly conserved across living cells, including mammals. The Trx system has greatly important functions protecting cells against oxidative stress and damage, being particularly important in the CNS cells, where assists correct protein synthesis and modulation of apoptosis (BJØRKLUND et al., 2022). Trx serves as a redox homeostasis regulator through modulation of signaling pathways, induction of transcription factors and pro-survival mechanisms (BURKE-GAFFNEY; CALLISTER; NAKAMURA, 2005). Trx is depicted with a Cys-Gly-Pro-Cys active site vital for protein disulfide reductase of enzymes like peroxidredoxins (Prxs), ribonucleotide reductase (RNRs), and methionine sulfoxide reductase (MSRs), once it serves as an oxidoreductase and an electron donor (ARNÉR; HOLMGREN, 2000). Trx has the ability to catalyze the reductive conversion of disulfide (S-S) bonds into dithiol (-SH) bonds on substrates, due to its reductase power, acting through disulfide-dithiol exchange (LEE; CHA; LEE, 2020). The whole process happens as follows: Trx binds to the protein substrate that contains a disulfide bond (S-S), then, one thiol (-SH) group of the N-terminal cysteine in the active site (Cys-Gly-Pro-Cys) of the Trx performs a nucleophilic attack to the disulfide (S-S) bond of the substrate by electron donation, yielding an intermolecular mixed S-S between the Trx and the substrate; after, the second thiol (-SH) group in the C-terminal cysteine in the active site (Cys-Gly-Pro-Cys) of the Trx attacks the intermolecular mixed S-S bond, releasing the reduced substrate and conducing to the creation of a disulfide (S-S) bond in the Trx itself; finally, the disulfide (S-S) bond in the oxidized Trx is reduced back to its original free thiol (-SH) groups state, through the enzyme TrxR, using NADPH as an electron donor, ready to engage in the next catalytic cycle **(Figure 16)** (HASAN et al., 2022).

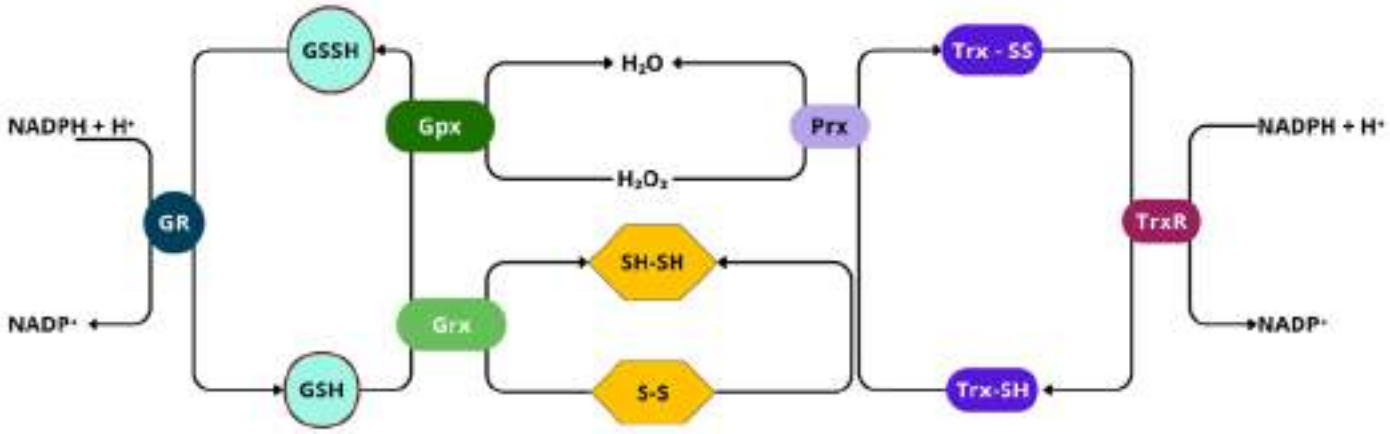


Figure 16 – The Thioredoxin System in neuronal antioxidant defense. Trx has the ability to catalyze the reductive conversion of disulfide (S-S) bonds into dithiol (-SH) bonds on substrates, due to its reductase power, acting through disulfide-dithiol exchange. The disulfide (S-S) bond in the oxidized Trx is reduced back to its original free thiol (-SH) groups state, through the enzyme TrxR, using NADPH as an electron donor, ready to engage in the next catalytic cycle. GPx catalyzes the reduction of hydrogen peroxide (H_2O_2) to water. To do this, GPx utilizes Glutathione (GSH) as an electron donor, here, two molecules of GSH are oxidized to form a disulfide bond (S-S), resulting in GSSG (oxidized glutathione). Now, the oxidized glutathione (GSSG) is recycled back to its reduced form (GSH) to maintain its antioxidant capacity. GSSH: Oxidized Glutathione; GSH: Glutathione; GPx: Glutathione Peroxidase; Grx: Glutathione Reductase; GR: Glutathione Reductase; Trx: Thioredoxine; TrxR: Thioredoxine Reductase; SH: dithiol bonds; SS: disulfide bonds.

There are two primaries strict Trx proteins expressed in mammals, Trx1 and Trx2. Trx1 is the most widely studied and present mainly in the cytosol but can be found in the nucleus upon oxidative/nitrosative stress (MONTEIRO; OGATA; STERN, 2017), or released through the cellular membrane (SILVA-ADAYA; GONSEBATT; GUEVARA, 2014); Trx1 is coupled to Prx1 and Prx2, and MSR, being critical for cellular growth and apoptosis, and is expressed in neuronal cells **(Figure 17)**. Thus, neurons with dysfunctional mitochondria induced by complex IV inhibition show H_2O_2 increased vulnerability due to low Trx expression (REN et al., 2017). Trx2 isoform is localized in the mitochondria and is exceptionally relevant for maintaining mitochondrial redox balance, as well as protection of mitochondrial DNA and proteins from oxidative damage (VILHARDT et al., 2017). Similarly to Trx1, Trx2 is expressed in neurons, and shows lower expression levels after complex IV inhibition, turning the cells more susceptible to H_2O_2 attack (REN et al., 2017). Both, Trx1 and Trx2 are primarily expressed in sensitive neurons in DRG and in the spinal cord and sciatic nerve and are particularly altered in nociceptive signaling sites after sciatic nerve injury, participating in the

regulation of oxidative stress and redox homeostasis, relevant in the pathophysiology of neuropathic pain (VALEK et al., 2015).

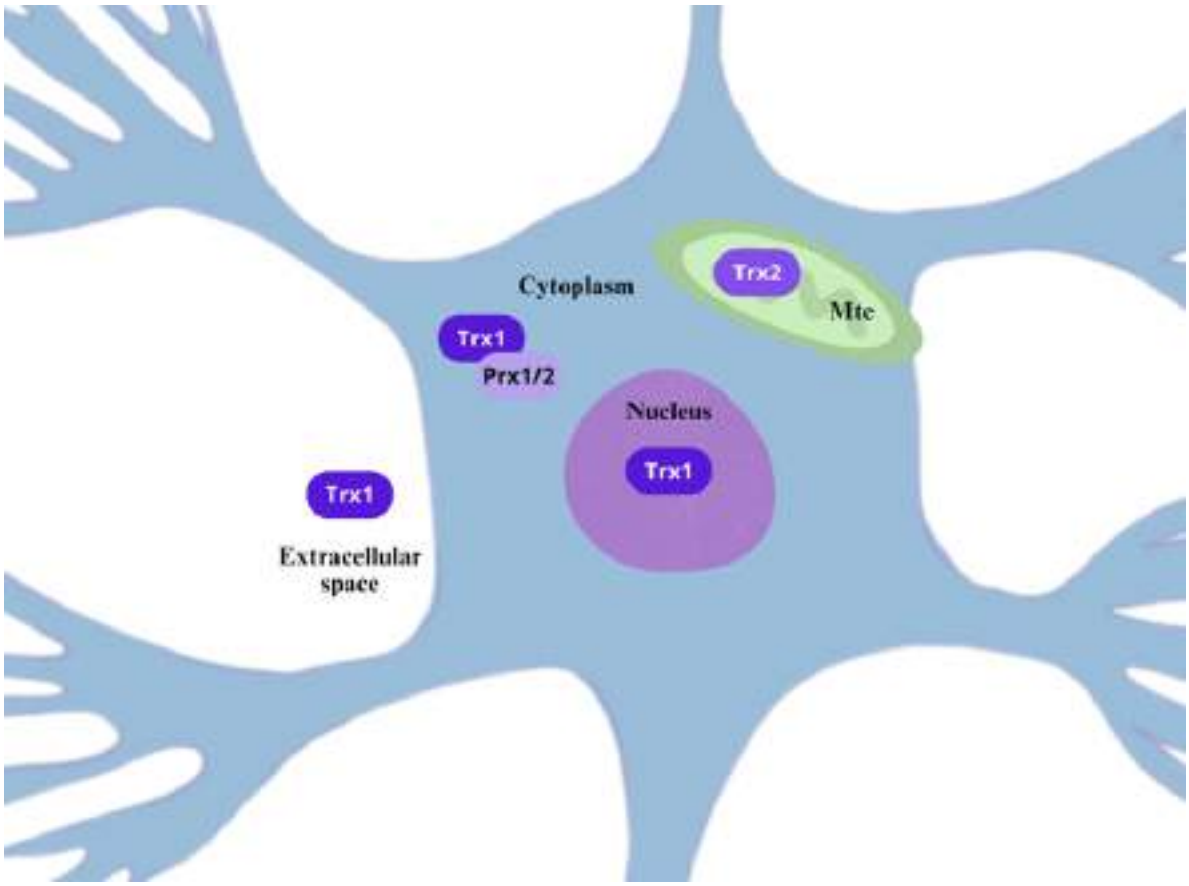


Figure 17 – Thioredoxin (Trx) sites of expression in neuronal cell.

Beyond catalyzing the reduction of the disulfide bond at the Trx active site, TrxR can straightly reduce peroxides like lipid hydroperoxides, H_2O_2, and protein disulfide isomerase; it is also involved in restauration of second – line antioxidant such as ubiquinone, lipoic acid, and dehydroascorbate (JOMOVA et al., 2023). Furthermore, TrxR can maintain a steady state of redox activity detoxifying ROS, directly reducing peroxides, and repairing oxidized proteins, thus, compensating for decreased levels of GPx, which is particularly relevant in the context of reduced levels of selenium (Se), as seen in excitotoxic hippocampal cells in rat models (TAKAGI et al., 2000).

The above-mentioned antioxidant enzymatic proteins play critical roles as regulators of various redox signaling pathways, which profoundly depend on reversible tissue- and target-specific modifications of main thiols in proteins. The CNS, a highly oxidizing environment, is acknowledged as very vulnerable to reactive species effects carried out by nitrosative or

oxidative damages. While each enzyme has a specific role, they work together through synergistic interactions, redundancy, and compartmentalization to ensure a robust, correct defense against reactive species and to guarantee cellular redox equilibrium.

4. NITROXIDATIVE STRESS IN NEURONAL CELLS

Pain can be considered, in a wide sense, as a protective mechanism to alert from actual and imminent danger to tissue integrity, or as a warn to potential lesions, eliciting a myriad of responses, in an emotional, sensorial, social, and cellular scale, but molecular at its very basic set. When the sensory process that provides triggering signals for pain occurs, nociception initiates, comprising the detection of harmful or potentially harmful stimuli by nociceptors, as a normal response to such stimuli. That is, after nerve injury, activation of macrophages, circulating neutrophils and T-cells recruitment to the site of lesion, along with cytokines, chemokines, neurotransmitters and ROS production prompts peripheral and central sensitization (JI; CHAMESSIAN; ZHANG, 2016). In some cases of injury, it often leads to abnormal sensory phenotype expression in the nervous system, causing hypersensitivity due to peripheral nerves, dorsal root ganglia (DRG), or central nervous system damage, ultimately conducing to neuropathic pain (FERNANDES et al., 2018), which is defined as a pain caused by a lesion or a disease of the somatosensory system (JENSEN et al., 2011) estimated to affect approximately 7% - 10% of the global population (BOUHASSIRA, 2019), characterized by paresthesia, dysesthesia (tingling, numbness, pins, and needles sensation), associated to non – painful sensory neurological deficits in the disturbed area, together with motor and cognitive deficits, depending on the affected region (BOUHASSIRA et al., 2008).

Glial cells, are important for homeostasis maintenance in the CNS, providing structural and nutritional assistance for neurons through hydro-electrolyte balance and neuronal synapsis (astrocytes), or surveying and protecting the neuronal milieu against potential disruptions of homeostasis and astrocyte activation (microglia) (JI; BERTA; NEDERGAARD, 2013). Immune cells of the CNS play a critical role in the development and continuance of neuropathic pain, as nerve insult conduces to the neuroinflammation response, characterized by increased local vascular permeability, activation of glial cells and inflammatory mediators release, enhancing pain sensitization (JI; XU; GAO, 2014). Once microglia are activated, suffer phenotypical and functional modifications, leading to proinflammatory cytokines release as TNF-α, IL-1β, and IL-6, cluster differentiation molecule 11b (CD11b), CD68, ionizing calcium binder adapter molecule (Iba1) (JI et al., 2018; ZHOU et al., 2016), contributing early in the

process to the establishment of neuropathic pain, and also causing astrocyte activation, supported by increased expression of glial fibrillary acidic protein (GFAP) and CD11b, indicating a shift in astrocyte function, which in turn activates microglia back, thus, creating a cross-talk for neuropathic pain pathophysiology (CIANCIULLI et al., 2020; GWAK et al., 2012). While microglia contribute initially to the establishment of neuropathic pain via the inflammatory response initiation, astrocytes are accountable for the long-term maintenance of such state (PENG et al., 2016; RAGHAVENDRA; TANGA; DELEO, 2003).

Activated glia release chemokines, RONS, and neurotransmitters that modulates inhibitory and excitatory synaptic transmission, relevant for pain amplification (TEIXEIRA-SANTOS; ALBINO-TEIXEIRA; PINHO, 2020). Glutamate, the primary excitatory neurotransmitter is upregulated in astrocytes in response to inflammatory signals, contributing to central sensitization and pain amplification; aspartate, similarly to glutamate can be influenced by glial activation, contributing to excitatory signaling in neuropathic pain; whilst GABA, the primary inhibitory neurotransmitter, responsible for reducing neuronal excitability and neuronal firing, can be downregulated in neuropathic pain, causing diminished inhibitory signaling, consequently, increasing pain sensitization (JI; XU; GAO, 2014). Additionally, purinergic receptors (P2X, P2Y), TLRs, Src kinase, and MAPK (JNK, ERK, and P38) pathways can be upregulated in conditions of nerve injury. This leads to increased expression of transcription factors in activated glia through various mechanisms, including further GF, proinflammatory cytokine and chemokine, and RONS production, contributing to pain sensitization, as described above (ELLIS; BENNETT, 2013). In this sense, RONS act as redox sensors for signal transduction pathways modulating the neuroinflammatory process in neuropathic pain (GRACE et al., 2016). In case the prompting offense to glial cells is not resolved, chronic activation arises, reflecting in a continuum of glial activation states (TOWN; NIKOLIC; TAN, 2005).

The molecular mechanisms underlying the neuropathic pain shifts the microglial phenotype from a resting, small, highly – branched, compact cell body and surveillance state to an activated bigger cell body, less branched, and ameboid state, causing an altered expression of cell surface proteins, growth factors, intracellular signaling molecules, and redox state consistent with inflammatory mediators release, such as cytokines and proteases contributing to the development of the neuroinflammation and neuropathic pain sensitization (FERNANDES et al., 2018).

Morphologically, three states are described in the microglial cells, similar to that used in peripheral macrophages, M0 phenotype for resting state, M1 phenotype for the classical activation state, and M2 phenotype for alternative activation state (HU et al., 2015). The first state is characterized by expression of genes related to neuronal function and development; whereas M1 and M2 phenotypes are induced by CNS insults and chronic pain, portrayed as mixed temporally changing and lacking equilibrium in the activation continuum. Thus, the M1 state is thought to lead to excessive RONS production and neuroinflammation, and M2 hampers an adequate immune response (HU et al., 2015). Also, astrocytes express antioxidant proteins to maintain overall redox homeostasis in the CNS under normal or pathological conditions (GAN et al., 2012; SCHREINER et al., 2015). In this sense, microglia and astrocytes can influence direct structural and molecular modifications in neuronal synapses, neuron differentiation and axon growth of mature neuronal cells, both physiologically, as part of normal CNS functioning and pathologically to cause oxidative and nitrosative damage and imbalance of redox signaling (VILHARDT et al., 2017).

Microglial cells are of prominent relevance regarding the inflammatory process typical of neuropathic pain (WANG; COUTURE; HONG, 2014), as they release a large number of neuroactive substances, which in turns contribute to the RONS production. Among these RONS, it can be pointed that Nox- and iNOS – derived radicals in neurons, such as $O^{\bullet -}$ and NO, contribute to cell death of dopaminergic neurons in neuropathic pain (GEIS et al., 2017; KIM et al., 2010). Microglia under oxidative stress produces increased inflammatory mediators that can move through the cell membrane, working as signaling molecules to other cells or induce peroxynitrite formation causing DNA fragmentation, lipid oxidation, and neuronal death in a faster manner than that perceived in astrocytes (LIU et al., 2002). In microglia cells producing $O^{\bullet -}$ catalyzed by nitrate and nitrites, O_2 and H_2O_2 levels are rapidly imbalanced, disturbing microglial functions, that in turn facilitates adjacent neuronal cells damage induced by ROS (LEE; CHA; LEE, 2021). Elevated levels of RONS have been proved to increase proinflammatory cytokines in microglial cells, whereas lower RONS levels are associated with anti-inflammatory cytokines upregulation (GEIS et al., 2017). ROS generated by Nox2 contributes to neuropathic pain inducing the activated microglia phenotype, furtherly enhancing the development of chronic glial activation state in the spinal cord, and assisting the interaction of neurons and macrophages in DRG (CHOI et al., 2012; KALLENBORN-GERHARDT et al., 2014; LIJIA et al., 2012), pointing the central and peripheral role in neuropathic pain; whereas Nox4 role is primarily related to peripheral demyelination, causing peripheral nerve

sensitization, therefore adding up to the overall effect of neuropathic pain (GEIS et al., 2017; KALLENBORN-GERHARDT et al., 2012).

Besides microglial cells, other CNS cells can produce $O^{\bullet -}$ and NO, wherever NOS and Nox are expressed, contributing to radical – mediated damage, as seen in β-amyloid – induced neurotoxicity in astrocytes (ABRAMOV; CANEVARI; DUCHEN, 2004). Astrocytes serve key functions in neuronal recovery but can, under certain circumstances, be neurotoxic. When activated, also release NO, TNF and ROS to the extracellular space, which can, negatively affect axon regeneration and nerves development, lead to neuronal injury and death through mitochondrial stress, DNA strand breaks, lipid peroxidation (TEISMANN; SCHULZ, 2004). Moreover, when astrocytes undergo sustained oxidative stress, abiding consequences over glutamate transporters, astrocyte – neuron communication enzymes (MARKIEWICZ; LUKOMSKA, 2006), and connexins occur, which are implicated in the establishment of neuropathic pain (WANG; XU, 2019). Mitochondria dysfunction, NADPH – derived ROS, and RNS in astrocytes are the main accountable factors to rouse oxidative and nitrosative stress, which is distributed across the long and thin processes and cell bodies, thus leading to astrocyte degeneration, SOD1 aggregation, and ischemic/reperfusion injuries (CHEN et al., 2012; SHIH; ROBINSON, 2018).

In a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of cell death of dopaminergic neurons, researchers aimed to document whether chronic exposure to small doses of MPTP were capable of affecting neurons from the dorsal horn of the spinal cord, responsible for the sensory component of pain. Considering that MPTP inhibits mitochondrial complex I, this conduces to augmented production of ROS, oxidative stress, lipoperoxidation, mitochondrial membrane rupture, leading to apoptosis pathways and neuronal damage, evidenced by decreased calbindin D28K, calretinin, and parvabulmin – positive neurons in the laminae II; reduction of SP interneurons and Met – enkephalin – positive fibers in laminae I and II; and finally, increased alpha-synuclein in spared neurons and fibers, stablishing a clear connection between mitochondrial dysfunction, increased ROS production, and neuronal damage (BIAGIONI et al., 2021). Also, Nox – deficient animals in an inflammatory model of dopaminergic cells death were found to be less affected than the wild type of animals, suggesting a direct role of Nox – derived $O^{\bullet -}$ in the mechanisms of microglial cell activation and neurotoxicity (QIN et al., 2004).

Furthermore, TRPA1, TRPM2 and TRPV1 are activated by nitroxidative species, mediating the integration of endogenous and exogenous sensory stimuli, either at the peripheral and central terminals and somas of afferent neurons (GRACE et al., 2016). TRPA1 and TRPV1 are expressed by peptidergic C – fibers, activated directly by peroxidized and nitrated phospholipids and carbonylated proteins, as well as aldehyde formation, due to oxidative stress (ANDERSSON et al., 2015; JULIUS, 2013); TRPA1 is primarily related to nociceptive hypersensitivity development, while TRPV1 is crucial for nociceptive hypersensitivity and thermal hyperalgesia (JULIUS, 2013). TRPM2 is expressed in neurons and microglia, activated directly via H_2O_2 and cytosolic ADP – ribose (CHEN et al., 2013; ÖZDEMIR et al., 2016), which is a by-product of mitochondrial damage and are associated to spinal cord microglia activation, local migration of macrophages after nerve injury, proinflammatory response, promoting nociceptive hypersensitivity and are also crucial in the activation process of the MAPK pathway, causing NF-kB nuclear translocation, consequently increasing the proinflammatory cytokines and chemokines production (CHEN et al., 2013; HARAGUCHI et al., 2012). In this context, TRP play critical roles in the development and maintenance of neuropathic pain through polymodal activation, including chemical, thermal and mechanical signals, inducing ionic changes across neuronal membranes initiating downstream signaling pathways, comprising AMPK, MAPK, NF-kB, and TGF- β, as well as enhancing hyperexcitability via increased expression and activity of TRP, leading to spontaneous pain and disturbed sensitivity (DANGI; SHARMA, 2024). Additionally, such state of increased expression and activity of TRP leads to hyperexcitability, apoptosis, hyperactivation and infiltration of immune cells, amplified levels of proinflammatory cytokines, mitochondria dysfunction, autophagia as seen in models of diabetic neuropathy in peripheral nerves (RODRIGUES; RUVIARO; TREVISAN, 2022), cancer – induced pain (ANTONIAZZI et al., 2019), chemotherapy – induced peripheral pain (MOBASHERI et al., 2017) and trigeminal neuropathic pain (TREVISAN et al., 2016)

It is widely known that proteins can undergo reversible modifications through oxidative actions in amino acids. Amongst the most redox – sensitive amino acids it can be cited the -SH group cysteine, and the aromatic ring of tyrosine (MOLDOGAZIEVA et al., 2018), displaying different predispositions to endure RONS alterations depending on the specific oxidants in the redox reaction, the proportion and distribution of thiol groups, transition metal ions presence as prosthetic groups, motifs and amino acid residues exposure on the molecular surface of the proteins, as well as different levels of chemical modifications ranging from reversible to

irreversible, and specific or non – specific union, which depends on the selective attach to residues and its location, or multiple side – chain and backbone sites affected, respectively (CHONDROGIANNI et al., 2014).

From a redox perspective, reversible and specific oxidation of a few or at least one single redox – sensitive residue in a protein by an electrophile molecule can be enough to induce chemical modification in a switch manner for modulating the protein activity (WINTERBOURN; HAMPTON, 2008). Some of the plausible amino acid residues modifications bearing redox switch capacities can be elicited through cysteine residues S-sulfenylation, S-glutathionylation, S-nitrosylation, S-persulfidation, given that cysteine residues are key redox – sensitive molecules due to the presence of -SH groups, bestowing structural and functional flexibility to proteins **(Figure 18)** (CHANDRAN; BINNINGER, 2023; GO; CHANDLER; JONES, 2015).

Figure 18 – Reversible and irreversible proteins change under ROS/RNS. Reactive species serve as redox – sensitive switch molecules due to amino acids -SH group cysteine, or to aromatic rings of tyrosine, for modulating protein activity. Nevertheless, when RS$^{\bullet}$ interact with NO to form S-nitrosylated cysteine residues in proteins, or undergo hyper-oxidation, to produce sulfinic and sulfonic cysteine after interaction with H_2O_2, $ONOO^-$, and HOCl, changes can become irreversible, impairing antioxidant defense and leading to neuronal damage.

Cysteine residues are usually found in catalytic and regulatory sites of enzymes, signaling pathways effectors, and transcription factors, and they can initially undergo the ionization to thiolate ($R\text{-}S^-$) which is the deprotonated form of -SH, and furtherly undergo one- or two – electron oxidations, to yield thiyl radicals (RS$^{\bullet}$), or cysteine sulfenic acid (-SOH),

respectively, both reversible. Then, $RS^{\bullet}$ can interact with NO to form S-nitrosylated cysteine residues in proteins, or undergo hyper-oxidation, to produce sulfinic and sulfonic cysteine after interaction with H_2O_2, $ONOO^-$, and HOCl, which are irreversible **(Figure 18)** (RAYNER; LOVE; HAWKINS, 2014), as seen in irreversible oxidation of SOD1 Cys111 to its sulfinic and sulfonic states, present in ALS mice models that presented signs of peripheral (FUJIWARA et al., 2007; RUBIO et al., 2016; SHARP; DICK; GREENSMITH, 2005). Accordingly, in spite of ALS being a neurodegenerative disease characterized by upper and lower motoneuron degeneration, it has been recently found to have a sensory neuropathy component observed in up to 20% of ALS human patients, pain among them, affecting unmyelinated small and myelinated large fibers as part of the process of primary neurodegeneration (BOMBACI et al., 2023; FANG; JOZSA; AL-CHALABI, 2017), potentially leading to neuropathic pain, and relevant to understand the full spectrum of ALS, associated with SOD1 mutation. Furthermore, cysteine residues of proteins and peptides exposed to NO and $ONOO^-$ can suffer S-nitrosylation (-SNO groups formation) and S-glutathionylation (SS and GSH mixed groups formation); such reactions are relevant to cell signaling, proteins stability and activity, and redox homeostasis through GST, Grx, and Trx (XIONG et al., 2011).

Tyrosine residues oxidation leading to 3–hydroxytyrosine, 3-nitrotyrosine, halogenated tyrosine, and intramolecular tyrosine cross – links are deeply associated to interference with cellular functions, as seen in endogenous ROS exposure, yielding inter- and intramolecular cross–links in proteins, accompanied by spontaneous fragmentation of the polypeptide chains involved; some of these cross–links, like 3,3'-dityrosine, are biomarkers of oxidative stress, as seen in a study using a neuropathic pain model of chronic constriction injury, where they found dityrosine – containing protein cross – links exposition to activate satellite glial cells (SGCs) in the DRG, and subsequent increase in proinflammatory mediators and ROS, mediated by the NF-kB pathway and the receptors for advanced glycation end products, exacerbating hyperalgesia (TU et al., 2025). As for tyrosine and tryptophane nitration, once they react with $OH^{\bullet}$, and subsequently form tyrosyl and tryptophanyl radicals, both can furtherly react with $NO_2^{\bullet}$ after covalent attachment to produce 3-nitrotyrosine and 6-nitrotryptophan, respectively (NURIEL; HANSLER; GROSS, 2011).

Proteotoxic stress has been linked to neuropathic pain through nitration reactions, that profoundly trigger chemical and structural changes in proteins, leading to function alterations and cell signaling modulation (SALVEMINI et al., 2011; VALKO et al., 2007), as such,

peroxynitrite, a cytotoxic pro-inflammatory and pronociceptive agent is portrayed as a main factor in the development of peripheral and central sensitization associated with neuropathic pain (SALVEMINI et al., 2012; SALVEMINI; NEUMANN, 2010). The mitochondrial MnSOD can be negatively affected by nitration, rendering it inactive, thus contributing to the perpetuation of a vicious cycle of free radical formation. This, in turn, leads to further nitrosative damage to relevant enzymes unable to fight back the development and maintenance of hyperalgesia and central sensitization (SALVEMINI et al., 2011). The accumulation of ROS and RNS, increased during oxidative and nitrosative stress conditions can induce the activation of transcription factors such as AP-1, NF-κB, MAPKs, which can activate and increase the expression and production of COX enzymes and PGs (ILARI et al., 2020a; SALVEMINI; KIM; MOLLACE, 2013).

Whenever antioxidant systems are overpassed by the production of ROS or RNS, the outcome is likely to cause an oxidative or nitrosative stress state, in which subsequent damage to biomolecules like proteins, membrane lipids, and nucleic acids leads to cellular and tissue dysfunction and cell death via necrosis, apoptosis, and autophagy implicated in chronic pain of neuropathic origin (LIAO et al., 2022). Nevertheless, the final outcome or the biological impact of the cross – links between antioxidants and pro-oxidants will depend on the specific type interacting, physicochemical properties, cellular and subcellular location and gradient, rate of formation and degradation, and quantity. Thus, in the late years, not only antioxidants, but also RONS have gained more attention from the scientific community, once the regulatory roles of RONS have emerged as adaptive mechanisms involved in cellular homeostasis, in low steady concentrations, for reversible redox – sensitive modifications in functional motifs of several proteins related to cell signaling.

5. MNP: MECHANISMS OF ACTION AND POTENTIAL THERAPY FOR NEUROPATHIC PAIN

The role of oxidative and nitrosative stress has been stablished for chronic pain of neuropathic origin, through hyper-physiological levels' effects of reactive species over neuronal and glial cells (VALLURU et al., 2012). Antioxidant therapies from diverse origins, aiming the restoration of normal cell functioning has been applied for various chronic pain disorders, with different, sometimes conflicting, and specific pain type – dependent outcomes, either positives, negatives, or not clear (FREDIANI et al., 2024; NTALOUKA; TSIRIVAKOU, 2023). Among the most studied synthetic therapeutic antioxidant compounds, there is a group

of proteins named SOD mimics. By designation, SOD mimics are compounds with the ability to catalyze the oxidation and reduction of $O^{\bullet}$-. Yet, it has been proved that besides reaction with $O^{\bullet}$-, SOD mimics can also catalyze redox reactions with H_2O_2, ONOO-, thiols, and others under specific condition (BATINIC-HABERLE et al., 2014), working as oxidants and reductants.

More specifically, there is a class of metalloproteins of low molecular weights with a central metal ion of manganese contained in the porphyrin ring of synthetic origin, exhibiting different oxidation states of manganese Mn(II), Mn(III), and Mn(IV), which contributes to their redox applications, named manganese porphyrins (MnP) (BONETTA, 2018). MnP were initially, in a very narrow way, seen as simply scavengers of $O^{\bullet}$-, but the new knowledge about redox chemistry applied to cellular physiology proved its worth based on evidence of their potential role as therapeutics, due to their physicochemical properties, longer half – life and cheaper than SOD enzymes, and no – immunogenic reactions in biological systems, hence, affecting signaling pathways and cellular functions such as proliferation, differentiation, and cell death (BATINIC-HABERLE et al., 2012), through modulation of the redoxome (BUETTNER; WAGNER; RODGERS, 2013).

Besides the specific reactive species scavenged by MnP, even more importantly, there are other aspects intrinsic to their structure that must be taken into consideration, when applying MnSOD mimics therapeutical strategies for neuropathic pain. First, the half – wave redox potential ($E_{1/2}$ ($O^{\bullet}$-)), which determines whether a compound can be readily oxidized and/or reduced by the substrate within the redox active molecular targets in the cell. $E_{1/2}$($O^{\bullet}$-) is a reliable measure of physiological relevance, once it compares the electrochemical potency at half of the wave of the compound to that of the SOD enzyme (+300 mV vs normal hydrogen electrode – NHE) in relation to $O^{\bullet}$-; the closer it is, the more biologically compatible the compound is for electron interchange among reactive species and signaling proteins in the cellular context, too high or too low potentials can be toxics or inefficient, respectively (BATINIC-HABERLE et al., 2014). A MnP within a compatible range will also allow a more specific action of the compound over the target molecules, without interfering with other biological processes, and more stability, avoiding fast degradation or inactivation (POLICAR et al., 2022).

Secondly, the intrinsic catalytic activity of the SOD mimic protein (Kcat) for the catalysis of $O^{\bullet}$- is another reliable measure of the therapeutic potential of the compound, once it quantifies the efficiency to catalyze the redox reaction, expressing the number of substrate

molecules converted into the reaction product for each molecule of the compound by unit of time, when the compound is fully saturated with the substrate (i.e. SOD Kcat($O^{\bullet}$-) ~2 × 109 M-1·s-1) (ECKSHTAIN et al., 2009). Such factors stablish not only the catalytic efficiency, but the rate of the reaction, which is relevant for reaching the therapeutic outcomes in a reasonable time, and the compatibility with the cellular redox conditions, that is pH, temperature of the reaction and substrate concentrations (FRANKE et al., 2019). Notwithstanding these factors, in vivo efficacy of metalloporphyrin – based SOD mimics are not only associated to their SOD-like activity, but to other factors like shape, size and bulkiness of the molecule (BATINIĆ-HABERLE; REBOUÇAS; SPASOJEVIĆ, 2010), bioavailability, biodistribution in different cells kinds and subcellular spaces, and reactivity toward other cellular biomolecules (BATINIC-HABERLE; TOME, 2019), which will dictate the overall therapeutic effect of the treatment. Assumed the rich environment of redox – based signaling processes occurring in the cellular metabolism through cellular respiration, glycolysis, cyt P450 detoxifying system, ETC, NO synthesis, and others, an approach targeting these redox-signaling pathways for therapeutical purpose would make more sense when assessing the effects of MnP on redoxome alterations, than the separate scavenging activity toward specific reactive species (BATINIC-HABERLE; SPASOJEVIC, 2014).

It is of main consideration that, under impaired conditions of neurons ability to bear peroxides elimination, excessive accumulation of H_2O_2 as byproducts of the $O^{\bullet}$- dismutation trough MnP, might reach toxic levels. That is, the overall antioxidant power of the synthetic enzyme will only be efficient if H_2O_2 is also removed (BATINIC-HABERLE et al., 2014). Cationic MnP are not potent scavengers of H_2O_2, but once reduced in vivo, may bind O_2 to $O^{\bullet}$- and H_2O_2 (DAY, 2009). Yet, cationic MnP have higher Kcat($O^{\bullet}$-) in comparison to neutral or anionic MnP, since $O^{\bullet}$- is negatively charged, therefore, more likely to be attracted to the positively charged cationic MnP, facilitating a more efficient interaction and better catalytic activity; this translates into a more effective MnP in detoxification and utilization of the $O^{\bullet}$- radical for antioxidant mechanisms (POLLARD et al., 2009; REBOUÇAS; SPASOJEVIĆ; BATINIĆ-HABERLE, 2008). In addition, when MnP holds a highly positive reduction potential, it is very likely that they will tend to accept electrons by cellular reductants, rather than give electrons away; in this process, Mn will be reduced to Mn(II), that will reduce $O^{\bullet}$- to H_2O_2, working as superoxide reductase, rather than superoxide dismutase (FERRER-SUETA et al., 2006).

The electrophilic nature of MnP, especially Mn(III) explains its tendency to react with or bind to anionic substrates, normally electron – rich, such as HOO-, RS-, ClO-,ONOO-, etc. When MnP enters the cell, it can straightaway suffer reduction while inducing oxidation of thiols, thus, working as an oxidant and antioxidant (BATINIC-HABERLE et al., 2014). As proved by previous research, MnP can work as suppressors of glycolysis and mitochondrial respiration (FERRER-SUETA et al., 2006), exhibit significant antihyperglycemic activity and enhance neuronal death (WANG et al., 2011); additionally, MnP can induce NF-κB inactivation (CELIC et al., 2014) and protective responses against oxidative damage to neuronal and glial cells (NIELLA et al., 2024; VALLURU et al., 2012), as well as reduced NF-κB expression, thus a rationale decrease of proinflammatory cytokines such as IL-1β and TNF-α (TSE; MILTON; PIGANELLI, 2004).

MnP with potent log Kcat($O^{\bullet}$-) are also potent ONOO- scavenger. As previously stated, electron – deficient MnP react or bind to electron – rich anionic substrates, such as ONOO-. When Mn(III) is oxidized to Mn(IV), either through $O^{\bullet}$- or ONOO- reaction, it reduces itself back to Mn(III) with cellular reductants present in the electron pool, yielding the highly oxidizing radical nitrogen dioxide ($NO^{\bullet}_2$), or through a two – electronically way to Mn(II) yielding nitrite (NO_2-), being this later reaction the most likely to occur in cells, once MnP are maintained in the Mn(II) state by cellular reductants (FERRER-SUETA et al., 2003). In addition, MnP can interact with deprotonated reductants like GSH, cysteine and protein thiols (BATINIC-HABERLE et al., 2012), in this sense, the MnP works as oxidant, accepting electrons from the substrates, and being reduced to either Mn(III) or Mn(II) lower oxidation states as a result, while oxidizing the reductants.

A significant degree of interaction between MnP and thiols has been reported, specifically towards cysteine residues present in the p50 subunit of the NF-κB protein, resulting in disulfide formation (FERRER-SUETA et al., 1999). The S-glutathionylation of the p65 NF-κB subunit, which is responsible for the κB attachment to certain DNA regions, thereby initiating the transcription of target genes, has been demonstrated in a hyaluronan degradation – induced inflammation in mice (CAMPO et al., 2013). In such case, the MnP functioned as an oxidant, yielding GS radical, that combined with additional GS produces GSSG radicals; GSSG, when oxidized by O_2, produces $O^{\bullet}$-. However, glutathionylation will be only significant if GSH and H_2O_2 are present, and GSSG can be safely removed if glutathione reductase (GR) is present in sufficient levels (CELIC et al., 2014). The antioxidative effect of MnP, as

previously mentioned are only effective when physiological levels of peroxide enzymes are present to remove H_2O_2. Hence, the cycling of MnP with GSH or cysteine would lead to accumulation of H_2O_2, contributing to further oxidation of biomolecules of significant importance in the homeostasis complex process (BATINIC-HABERLE et al., 2014). In a chemotherapy – induced neuropathic pain model in rats, mitotoxicity influenced by reduced ATP production in mitochondria due to respiratory complex I and II inhibition in primary nerve sensory axons was thwarted by administration of Mn(III) 5,10,15,20-tetrakis(N-n-hexylpyridinium-2-yl)porphyrin (MnTE-2-PyP(5+)), without compromising anti-tumor effect and mechanical hypersensitivity was reversed (JANES et al., 2013).

Although manganese – based porphyrins evidence of therapeutic effects over neuropathic pain is scarce, acknowledgeable evidence of iron – based porphyrins exist (ARORA et al., 2008; NEGI; KUMAR; SHARMA, 2010). Manganese and iron porphyrins can both dismutate superoxide anion, although their efficiency and pathway might differ in favor of manganese porphyrins, which have an overall mechanism more promising regarding interaction with cellular reductants and flavoenzymes, and production of $^{\bullet}OH$ after interacting with H_2O_2, once MnP have a very limited capacity to interact with H_2O_2, contrary to iron porphyrins, which can produce $^{\bullet}OH$ in a Fenton – like reaction, contributing to reactive species production in the cell (BATINIĆ-HABERLE; REBOUÇAS; SPASOJEVIĆ, 2010; RAUSARIA et al., 2011b). Yet, it is important to acknowledge that iron porphyrin complexes perform remarkable peroxynitrite decomposition catalyst activity, highlighting the potential of porphyrin – based research for therapies in the context of neuropathic pain.

Structural and functional neuropathic alterations in diabetic type 2 rats were evaluated and the therapeutic potential of peroxynitrite decomposition catalysts Fe(III) tetrakis-2-(N-triethylene glycol monomethyl ether)-pyridyl porphyrin (FP15) and Fe(III) tetra-mesitylporphyrin octasulfonate (FeTMPS) were assessed. Mechanical and sensorial deficits and thermal allodynia, as well as nitrotyrosine and poly(ADP-ribose) levels were restored with both treatments, compared to non – treated animals (DREL et al., 2007). Ischemia/reperfusion – induced neuropathic pain has also been evaluated regarding the peroxynitrite decomposition catalyst effect of intraperitoneal FeTMPyP [5,10,15,20-tetrakis (N-methyl-4'-pyridyl)porphyrinato iron (III)]. Significant enhancement of mechanical withdrawal threshold and phosphorylated NMDA subunit 1 were observed in treated animals, consequently reducing mechanical allodynia and central sensitization (KWAK et al., 2014). The anti-nociceptive and

antioxidant effect of peroxynitrite decomposition catalyst FeTMPyP was tested in chronic constriction of the sciatic nerve – induced neuropathic pain in rats. iNOS, NF-κB, IL-6 and TNF-α high expression levels in sciatic nerves, together with poly (ADP) ribose DRG increased levels were completely reversed after treatment, reducing PARP overactivation, oxidative stress and normalizing behavioral and functional pain parameters (KOMIRISHETTY et al., 2021).

6. FINAL CONSIDERATIONS

This review evaluated the role of reactive species in cell signaling, focusing on their redox-sensitive characteristics and their link to neuropathic pain. Reactive oxygen and nitrogen species, significant for cellular signaling pathways, act as both mediators of cellular damage and modulators of physiological processes. Their dysregulation, especially under oxidative stress, has been increasingly linked to the sensitization of pain pathways, highlighting the potential of targeting redox-sensitive mechanisms for novel therapeutic strategies. Enzymatic antioxidant systems play a crucial role in maintaining redox homeostasis. Enhancing these defenses offers a promising strategy to reduce oxidative and nitrosative damage associated with neuropathic pain. Among emerging therapeutic agents, manganese porphyrins (MnPs) stand out due to their mimetic antioxidant properties, offering significant potential for addressing redox imbalances and neuropathic pain. This review underscores the significance of understanding the intricate dynamics of reactive species, subcellular compartment gradients, and MnP-mediated redox chemistry in the context of neuronal dysfunction. Further research is essential to optimize the design of biocompatible, target-specific MnP molecules and to elucidate their safety and efficacy in clinical settings. Advancing knowledge in this field may pave the way for innovative treatments for neuropathic pain, ultimately improving the quality of life for affected individuals.

5 MATERIAL E MÉTODOS

Este projeto foi submetido ao Comitê de Ética no Uso de Animais (CEUA) da Universidade Estadual de Santa Cruz e aprovado sob protocolo Nº 030/21.

5.1 Porfirinas de manganês (MnP)

A síntese das Porfirinas foi realizada na Universidade Federal de Minas Gerais. A MnP I [MnTE-2-PyP]Cl_5, *meso*-tetrakis(*N*-etilpiridínio-2-yl)porfirinatomanganês(III)cloreto, conhecida como $[MnTE\text{-}2\text{-}PyP]^{5+}$ foi preparada em solução aquosa, sintetizada e purificada como descrita em outros trabalhos prévios (BATINIC-HABERLE et al., 2011; BATINIĆ-HABERLE; REBOUÇAS; SPASOJEVIĆ, 2010; BATINIC-HABERLE; TOVMASYAN; SPASOJEVIC, 2015). A MnP II foi preparada em solução aquosa [MnT(5-Br-3-E-Py)P]Cl_5, *meso*-tetrakis(5-bromo-*N*-etilpiridínio3yl) porfirinatomanganês(III)cloreto, conhecida como $[MnT(5\text{-}Br\text{-}3\text{-}E\text{-}Py)P]^{5+}$. Foi preparada em três fases: primeiramente, a porfirina de base livre $H_2T(5\text{-}Br\text{-}3\text{-}Py)P$ foi sintetizada. Para isso, a purificação foi realizada por cromatografia em coluna, usando óxido de alumínio com fase estacionária e uma mistura de clorofórmio como fase móvel. As frações correspondentes a $H_2T(5\text{-}Br\text{-}3\text{-}Py)P$ foram combinadas, e o solvente foi removido em um evaporador rotativo. A fração $H_2T(5\text{-}Br\text{-}3\text{-}Py)P$ foi caracterizado por espectroscopia de absorção eletrônica UV-Vis e ressonância magnética nuclear de hidrogênio (^1H RMN). Na segunda fase, a porfirina de base livre $H_2T(5\text{-}Br\text{-}3\text{-}E\text{-}Py)PCl_4$, designada como $H_2T(5\text{-}Br\text{-}3\text{-}E\text{-}Py)P^{4+}$ em solução aquosa, foi sintetizada pela alquilação do precursor $H_2T(5\text{-}Br\text{-}3\text{-}Py)P$. A amostra resultante de $H_2T(5\text{-}Br\text{-}3\text{-}E)PyP^{4+}$ foi isolada e purificada como o sal de cloreto, $H_2T(5\text{-}Br\text{-}3\text{-}E)PyPCl_4$, conforme descrito anteriormente (BATINIĆ-HABERLE et al., 1999), e caracterizada por espectroscopia de absorção UV-Vis. Na terceira e última fase, a $H_2T(5\text{-}Br\text{-}3\text{-}E)PyPCl_4$ foi metalizado com manganês. Para isso, foram adicionados 271,35 mg (1,37 mmol) de cloreto de manganês(II) tetra-hidratado, previamente solubilizado em uma quantidade mínima de água. A reação de metalação foi monitorada por espectroscopia de absorção eletrônica UV–Vis. A amostra resultante $[MnT(5\text{-}Br\text{-}3\text{-}E\text{-}Py)P]^{5+}$ foi isolada e purificada na forma de sal de cloreto ($[MnT(5\text{-}Br\text{-}3\text{-}E\text{-}Py)P]Cl_5$) conforme descrito anteriormente (BATINIĆ-HABERLE et al., 1999).

5.2 Animais e condições de alojamento e ambientação

Foram utilizados 144 ratos machos (*Rattus norvegicus*), da linhagem Wistar, com peso entre 225 g ± 25 g, obtidos a partir da Estação de Recria e Manutenção de Animais de Laboratório da UESC. Os animais foram alojados em grupos de quatro , em caixas de polipropileno, sob temperatura controlada (22° C) e ciclo de claro/escuro de 12 horas/12 horas, recebendo alimentação com ração comercial e água *ad libitum*.

Antes do inicio dos experimentos, os ratos passaram por um período de ambientação de uma semana para reduzir as variações decorrente do manejo e das condições ambientais. Após o período de adaptação pré – experimental os animais passaram por avaliação prévia do limiar mecânico nociceptivo basal, que foi usado como referência para comparação estatística.

5.3 Grupos experimentais – lesão constritiva do nervo isquiático (LCI) e tratamentos

Os animais foram submetidos à ligadura do nervo isquiático para indução de dor neuropática, segundo o modelo descrito por (BENNETT; XIE, 1988). Para tal, receberam antibiótico-profilaxia na dose de 60 mg/kg de cefalotina por via intramuscular, e, em seguida, foram levados ao plano anestésico-cirúrgico utilizando 7V% de sevofluorano para indução, seguida de 3,5 – 4V% para a manutenção anestésica, em um sistema semiaberto, avalvular, com fluxo de 200 ml/kg/min e fração de oxigênio inspirado (FiO2) de 0,6. A concentração do sevofluorano foi ajustada conforme necessário para manter o animal em plano anestésico-cirúrgico.

Foi realizada ampla tricotomia da região glútea do membro posterior esquerdo e antissepsia usando técnica de antissepsia consistente em uma sequência de álcool 70%, solução de iodopovidona 10%, e finalmente, álcool 70% **(Fig. 19A)**. Foi feita incisão de aproximadamente 3 cm paralelo e caudal ao fêmur, seguido de dissecção da camada muscular **(Fig. 19B)**, para exposição do nervo isquiático **(Fig. 19C)** Uma vez exposto, foram feitas quatro suturas no nervo isquiático com fio de nylon (5-0) **(Fig. 19D)**. Em seguida, foi feita a síntese do plano muscular e pele com nylon (3-0). Os animais do grupo controle negativo passaram pelos mesmos procedimentos, exceto pela ligadura do nervo isquiático, ao passo que animais do grupo *NAIVE* não foram expostos ao modelo experimental, nem aos tratamentos.

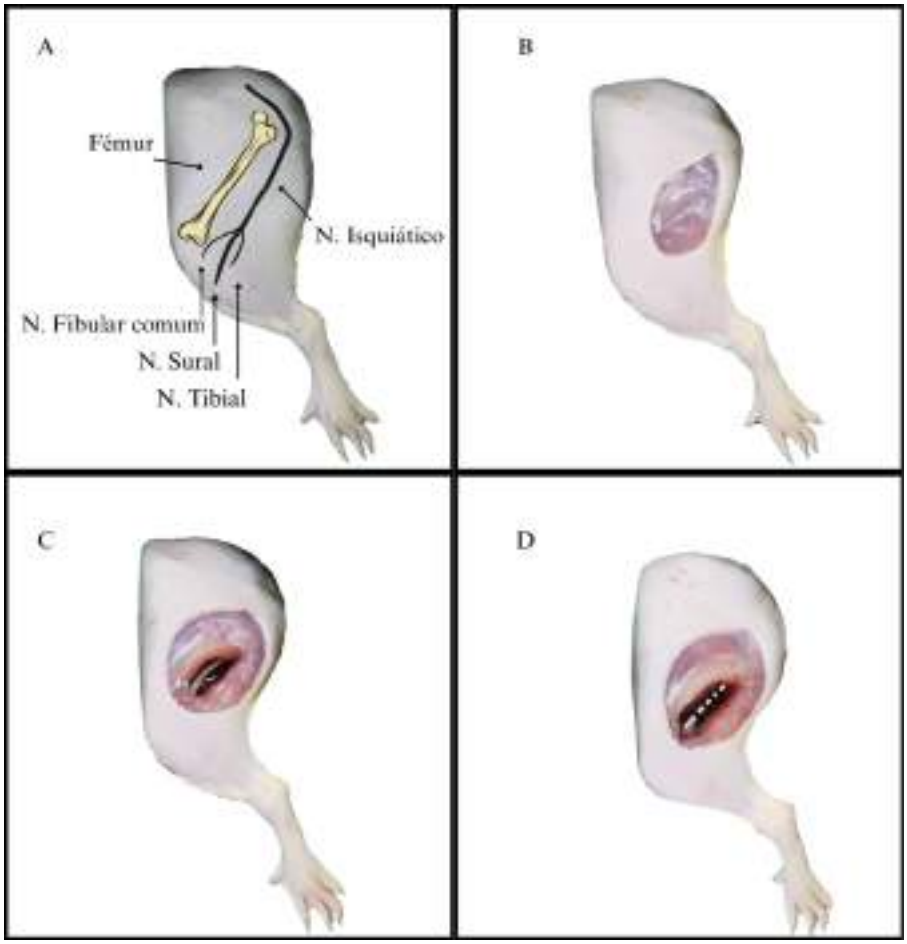


Figura 19 - Modelo de lesão constritiva do nervo isquiático. A. representação esquemática da posição anatómica do nervo isquiático; B. Incisão do plano cutâneo; C. Dissecção do plano muscular, para exposição do nervo isquiático; D. ligaduras do nervo isquiático.

No sétimo dia após procedimento de LCI (dia 1 da neuropatia), os tratamentos foram iniciados nos animais de acordo ao grupo experimental ao que foram designados. Assim, os tratamentos realizados pela via intratecal foram feitos no espaço lombo-sacro, segundo técnica descrita (THOMAS et al., 2016), utilizando agulha hipodérmica 30G, acoplada a seringa de Hamilton. Inicialmente, os animais foram imobilizados quimicamente e levados a estágio III, plano 1 (anestesia leve), usando de 3,5 a 4V% de sevofluorano fluxo de oxigênio 200mL/min e entregue por meio de máscara facial adaptado para ratos **(Fig. 20A)**. No primeiro dia de administração do tratamento, foi realizada tricotomia na região lombo-sacra. Para cada administração do tratamento nos dias subsequentes, foi realizada antissepsia usando uma sequência de álcool 70%, solução de iodopovidona 10%, e finalmente, álcool 70%. O espaço lombo-sacro **(Fig. 20B)** era identificado com o dedo indicador, usando como referência as tuberosidades ilíacas, a seringa de Hamilton posicionada em ângulo de 45º e inserida até o espaço intratecal, confirmado pela presença do reflexo caudal (cauda adotando forma sigmoide)

como indicador da presença no espaço intratecal; seguidamente o tratamento era injetado. Por sua vez, a administração dos tratamentos intraperitoneais **(Fig. 20C)** foi realizada como segue, os animais foram imobilizados fisicamente segurando-os com dedo indicador e polegar, ao nível das escápulas; foi realizada antissepsia da região abdominal com uma sequência de álcool 70%, solução de iodopovidona 10%, e finalmente, álcool 70%; colocando os animais em leve declive de -30º (posição Trendelenburg); em seguida, o tratamento era injetado no quadrante abdominal caudal direito, paralelo a linha média alba, utilizando seringas descartáveis de 3 mL acopladas a agulha hipodérmica descartáveis de calibre 26G.

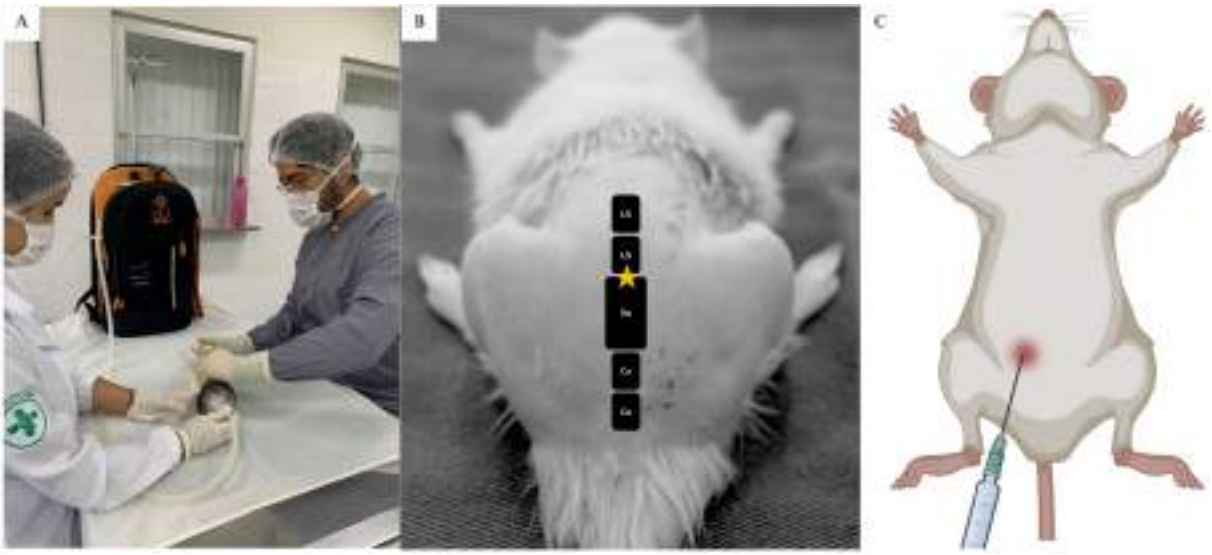


Figura 20 - Métodos e vias de administração dos tratamentos. A. Anestesia inalatória prévio à administração intratecal dos tratamentos. B. Local de injeção intratecal. C. Local de injeção intraperitoneal (imagem C.: Fonte: Adaptado de Dwyer-Nield, 2022).

5.4 Experimento 1: determinação da ED50 e LD50

Inicialmente, foi determinada a curva dose-resposta da MnP I pela via IT, com doses decrescentes do 100% (IT1A), 40% (IT1B), 10% (IT1C), 5% (IT1D), 2,5% (IT1E) e 0,25% (IT1F) da dose intraperitoneal (0,2 mg/kg) usada em trabalhos prévios (DOS ANJOS CORDEIRO et al., 2024), visando otimizar a relação eficácia – segurança, considerando que a administração da MnP acontece de forma direta no tecido alvo. Seguidamente, foram selecionadas as duas doses com melhor efeito antinociceptivo, baseado no LMN ao longo do período de tratamento e usadas como referência para avaliação da MnP II pela via IT.

Os animais foram submetidos ao procedimento de LCI no Dia 0 e permaneceram no Laboratório de Experimentação durante sete dias, sob as condições mencionadas anteriormente, para permitir instauração da neuropatia crônica. Com exceção do grupo *NAIVE*, que não

recebeu tratamento, e do Grupo Controle Negativo, que foi submetido apenas à exposição do nervo isquiático, sem LCI. No 7º dia, foi realizada uma nova avaliação do limiar mecânico nociceptivo (LMN) (considerado o dia 1 da neuropatia) e os tratamentos dos grupos foram iniciados **(Figura 21)**. Após 24 horas da aplicação, foi realizada uma nova avaliação do LMN nos ratos. Foram utilizados 88 ratos, distribuídos aleatoriamente em 11 grupos experimentais (n=8).

- **Grupo Controle Positivo Intratecal (PCG):** os ratos receberam 10 µL/sítio/24h de água destilada intratecal;
- **Grupo Controle Negativo (NCG):** os ratos receberam água destilada 10µL/sítio/24h;
- **Grupo MnP Ia – Intratecal dose A (IT1A)**: dose de 0,2 mg/kg/24h MnP I intratecal;
- **Grupo MnP Ib – Intratecal dose B (IT1B):** dose de 0,08 mg/kg/24h I intratecal;
- **Grupo MnP Ic – Intratecal dose C (IT1C):** dose de 0,02 mg/kg/24h MnP I intratecal;
- **Grupo MnP Id – Intratecal dose D (IT1D)**: dose de 0,01 mg/kg/24h MnP I intratecal;
- **Grupo MnP Ie – Intratecal dose E (IT1E):** dose de 0,005 mg/kg/24h MnP I intratecal;
- **Grupo MnP If – Intratecal dose F (IT1F):** dose de 0,0005 mg/kg/24h MnP I intratecal;
- **Grupo MnP IIa – Intratecal dose A (IT2A):** dose de 0,01 mg/kg/24h MnP II intratecal;
- **Grupo MnP IIb – Intratecal dose B (IT2B):** dose de 0,005 mg/rato/24h MnP II intratecal;
- **Grupo *NAIVE*:** Animais que não foram submetidos ao modelo de lesão nem receberam qualquer tratamento.

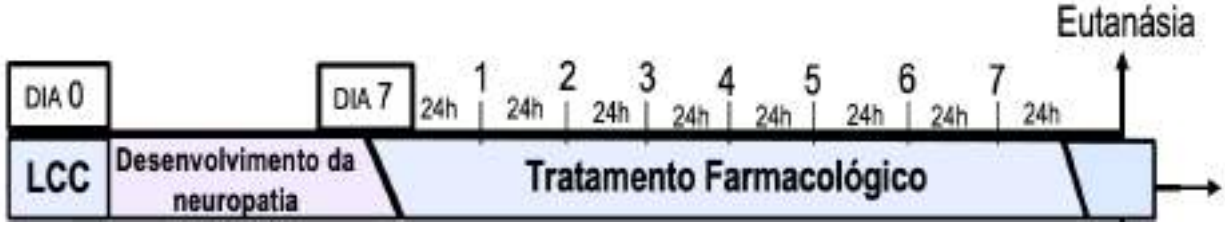


Figura 21 – Esquema da cronologia do experimento

No oitavo dia após LCI, os animais foram eutanasiados com sobredose anestésica de propofol (150mg/kg) pela via intraperitoneal (IP). A coleta da medula espinhal foi realizada pelo método asséptico por meio de hidropropulsão descrita por MOGHADDASI et al., (2007). **(Figura 22)**

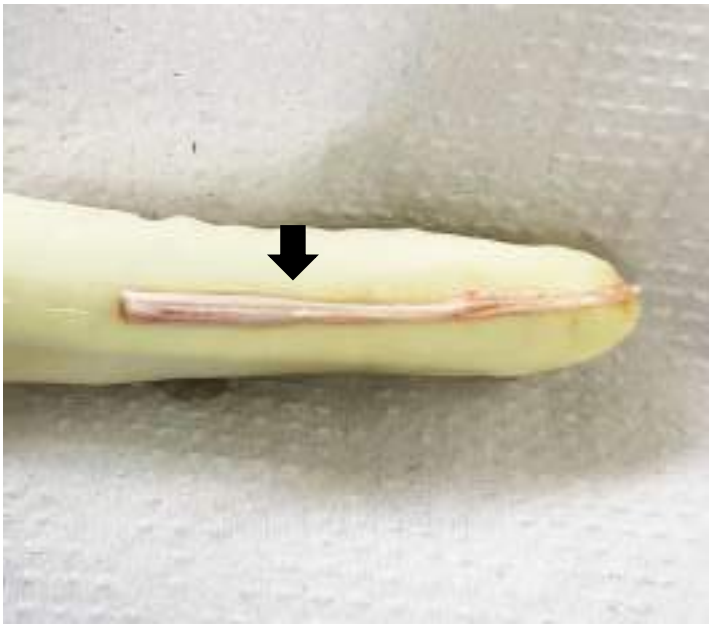


Figura 22 - Segmento lombossacro da medula espinhal coletado por hidropropulsão.

5.5 Experimento 2

Os animais foram submetidos ao procedimento de LCI, mantidos e manejados sob as mesmas condições citadas no experimento 1. Foram utilizados 63 ratos, distribuídos aleatoriamente em sete grupos experimentais (n=9), para comparação das MnP I e MnP II pelas vias IT e IP:

- **Grupo Controle Positivo (PCG) -** os ratos receberam 5 ml/kg/24h de solução salina intraperitoneal;
- **Grupo Controle Negativo (NCG) -** os ratos receberam 5 ml/kg/24h de solução salina intraperitoneal;
- **Grupo MnPI – Intraperitoneal (IP1)** – dose de 0,1 mg/kg/24h, MnP I intraperitoneal;
- **Grupo MnPI - Intratecal (IT1) –** dose de 0,01 mg/kg/24h, MnP I intratecal, a partir do resultado obtido no experimento 1;
- **Grupo MnPII – Intraperitoneal (IP2):** dose de 0,1 mg/kg/24h, MnP II intraperitoneal;
- **Grupo MnPII – Intratecal (IT2):** dose de 0,01 mg/kg/24h, MnP I intratecal, a partir do resultado obtido no experimento 1;
- **Grupo *NAIVE*** Animais que não foram submetidos ao modelo de lesão nem receberam qualquer tratamento.

As doses intraperitoneais foram determinadas a partir de estudos prévios (DOS ANJOS CORDEIRO et al., 2024); ao passo que as doses intratecais das porfirinas foram determinadas no experimento 1. Visando a redução do número total de animais utilizados, animais do IT1 e IT2 corresponderam àqueles com os melhores resultados baseados na avaliação do LMN. Para ambos os experimentos, os tratamentos foram aplicados nos animais a cada 24 horas durante os sete dias consecutivos após o desenvolvimento da neuropatia, e depois de 24 horas da aplicação era realizada a avaliação do LMN. Os animais foram eutanasiados e os fragmentos de medulas espinhais, coletados sob as mesmas condições dos animais no experimento 1.

5.6 Avaliação do limiar mecânico nociceptivo

Para avaliação do limiar mecânico nociceptivo (LMN) foi utilizado um analgesímetro digital **(Fig. 23A)** que consiste em um transdutor de pressão, conectado a um contador de força onde expõe em gramas o resultado, calibrado com uma precisão de 2-100 g. Uma ponteira descartável de polipropileno com 0,5 mm de diâmetro foi colocada em contato com o animal **(Fig. 23B)**, no local de maior intensidade de dor. Foi considerada resposta positiva a retirada da pata do contato com o filamento ou comportamento de sacudir ou lamber a pata imediatamente ou após a estimulação. A avaliação de dor foi realizada todos os dias após o início dos tratamentos em todos os animais.

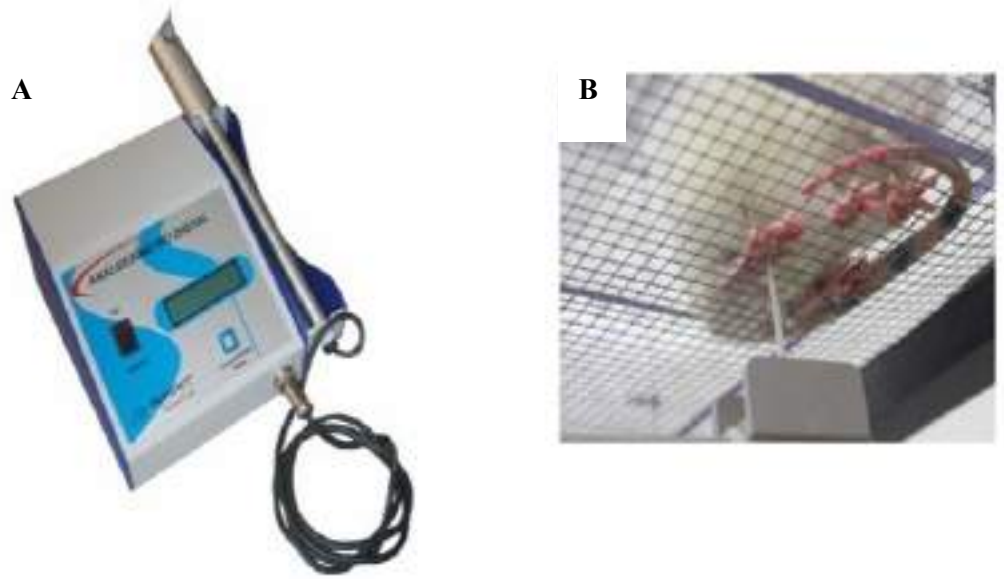


Figura 23 - Analgesímetro digital (A) e método de avaliação do LMN (B)

5.7 Coleta e processamento da medula espinhal

Imediatamente após eutanásia, a coluna vertebral foi dissecada no segmento lombos-sacro, com auxílio de cisalha Liston reta e tesoura de Líster. A medula espinal foi exteriorizada

do canal vertebral por hidropropulsão, insertando ponteira de polipropileno estéril, livre de RNAase, DNase, acoplada a seringa de 20 mL, em sentido caudo-craneal. Seguidamente, a medula espinhal foi seccionada em quatro fragmentos, de cranial a caudal, mesmos que foram padronizados e processados da forma a seguir:

Primeiro fragmento: destinado à avaliação de expressão gênica de mRNA, por meio de RT qPCR. As amostras foram armazenadas em microtubos tipo 1,5 mL, livres de RNAase e DNase, previamente preenchidos com 150 µL de trizol e imediatamente congelado em nitrogênio líquido a -196ºC, e armazenado, posteriormente, a –80ºC até processamento das amostras.

Segundo fragmento: destinado à avaliação de expressão proteica de SOD1, GPx1. As amostras foram fixadas em solução de paraformaldeído a 4% durante 24 horas, processado pela técnica de rotina para inclusão em parafina e submetido a cortes transversais seriados de 5 µm.

Terceiro fragmento: destinado à avaliação da produção de espécies reativas de oxigênio e peroxinitritos. As amostras foram armazenadas em microtubo de 1,5 mL libre de RNase e DNase e homogeneizada no sonicador (3 ciclos de 4s cada) com tampão Tris-HCl35 10 mM, pH 7,4 na proporção de 1:10. O homogenato foi centrifugado a 1.000 g, 10 minutos a 4ºC. O sobrenadante foi colhido e congelado a – 80ºC para realização das análises correspondentes.

5.8 qPCR tempo real

Foram avaliadas as vias de ativação de estresse oxidativo e estresse do reticulo endoplasmático – RE, por meio da técnica semiquantitativa de RT-qPCR. A extração de mRNA das amostras foi feita usando trizol, seguindo instruções do fabricante (Invitrogen, Life Technologies, Carlsbad, CA, USA). O método consistiu em uma etapa inicial de lise e homogeneização por maceração manual, com auxílio de pistilo estéril. Uma vez homogeneizado, o volume do trizol é levado a 1000 µL e incubado por 5 minutos em temperatura ambiente.

Para separação do mRNA, foram adicionados 200 µL de clorofórmio ao microtubo de 1,5 mL contendo a amostra, seguido de 15 segundos de agitação manual e 3 minutos de incubação em temperatura ambiente. Em seguida, foi centrifugação a 12.000g por 15 minutos (4ºC). A centrifugação gerou a separação de três fases, onde a fase transparente superficial continha o mRNA, sendo esta a de interesse.

Já na fase de precipitação do mRNA, foram transferidos 100 µL da fase transparente para um novo tubo, com a adição de 500 µL de álcool isopropílico e incubação por 30 minutos (-80ºC), seguido de descongelamento no gelo e posterior centrifugação a 12.000g por 10 minutos a (4ºC). O sobrenadante foi transferido para segunda precipitação num outro microtubo, seguindo as mesmas condições de primeira precipitação enquanto o pellet foi acondicionado no gelo.

Os pellets foram lavados com 1 mL de etanol a 75%, homogeneizados e centrifugados a 10.500g por 5 minutos (4ºC). Após o descarte e secado do etanol, o pellet de RNA foi solubilizado em 10 µL de água livre de RNase e DNase. Os microtubos foram colocados em termobloco a 56ºC por 10 minutos para solubilizar o mRNA e imediatamente estocado a -80ºC, para posterior dosagem e processamento. A análise da concentração e da qualidade do RNA do tecido de cada amostra foi feita utilizando nanodrop 2000 Spectrophotometer (Thermo scientific).

Após determinação da melhor extração para cada amostra, em função da sua concentração e grau de pureza (razão 260/280), foram realizadas as sínteses de cDNA ajustado ao número de genes alvo. Foi utilizado 1 µg de mRNA para cada cDNA e 1,5 µL de cDNA para cada gene alvo. As reações de transcrição reversa foram realizadas utilizando o kit comercial GoTaq® qPCR and RT-qPCR Systems (A6010, PROMEGA).

Os genes alvo foram quantificados pela qPCR utilizando o equipamento Applied Biosystems® 7500 Real-Time PCR System. Para as reações, foi utilizado 1,5 µL de cDNA, 100 nM de cada iniciador e 12,5 µL do reagente GoTaq® qPCR Master Mix, 2X em um volume final de 20 µL de reação. Como controle negativo utilizou-se água livre de nuclease. Os iniciadores para *Gapdh, Cat, Sod1, Sod2, Gpx1, Caspase-3, Caspase-9, Hif-1a, Chop, Il-1b, Il-6, CX3CL1, CX3CR1, Grp78, Atf6, Perk, Nrf2, Ho-1 e Gdnf* foram delineados baseados na sequência do mRNA de *Rattus norvegicus*.

O número de cópias foi inferido de acordo com o número mínimo de ciclos necessários para atingir um sinal de fluorescência significativamente superior à fluorescência basal (CT). A expressão gênica relativa foi calculada pelo método $2^{-\Delta\Delta CT}$ e os resultados obtidos para cada grupo foram contrastados estatisticamente após normalização baseada na expressão de GAPDH *Rattus norvegicus*. Os primers usados para delineamento dos genes alvo foram **(Tabela 1)**:

Tabela 1 - Genes e sequências de primers utilizados *forward* e *reverse* utilizados

Gene	**Sequência do Primer (5' - 3')**	
Gapdh	F: 5'-GCGCTACAGCGGATTTTTGA-3'	NM_031797.2
	R: 5'-GAAGGCATACACGGTGGACT-3'	
Cat	F: 5'- CTGACTGACGCGATTGCCTA-3'	NM_012520.2
	R: 5'- GTGGTCAGGACATCGGGTTT-3'	
Sod1	F: 5'-GAAAGGACGGTGTGGCCAAT-3'	NM_017050.1
	R: 5'-CTCGTGGACCACCATAGTACG-3'	
Sod2	F: 5'-CGGGGGCCATATCAATCACA-3'	NM_017051.2
	R: 5'-GCCTCCAGCAACTCTCCTTT-3'	
Gpx1	F: 5'-GCGCTACAGCGGATTTTTGA-3'	NM_030826.4
	R: 5'-GAAGGCATACACGGTGGACT-3'	
Caspase-3	F: 5′-GAGCTTGGAACGCGAAGAAA-3'	NM_012922.2
	R: 5′-AGTCCATCGACTTGCTTCCA-3'	
Caspase-9	F: 5′-TCCCCACTGATCAAGTCTCCT-3'	NM_031632.2
	R: 5′-CCAGGCTCACTTAGCAAGGAA-3'	
Hif-1a	F: 5′-AGCAATTCTCCAAGCCCTCC-3'	NM_024359.1
	R: 5′-TTCATCAGTGGTGGCAGTTG-3'	
Chop	F: 5'-TGGCACAGCTTGCTGAAGAG-3'	NM_001109986.1
	R: 5'-TCAGGCGCTCGATTTCCT-3'	
Il-1b	F: 5′-GCACAGTTCCCCCAACTGGTA-3'	NM_031512.2
	R: 5′-TGTCCCGACCATTGCTGTTT-3'	
Il-6	F: 5′-GACTTCCAGCCAGTTGCCTTR-3'	NM_053595.2
	R: 5′-AAGTCTCCTCTCCGGACTTGT-3'	
CX3CL1	F: 5′-GGTGGCAAGTTTGAGAAGCG-3'	NM_134455.2
	R: 5′-ACTCGGCCAAATGGTGGTAG-3'	
CX3CR1	F: 5′-TCTTCACGTTCGGTCTGGTG-3'	NM_133534.2
	R: 5′-GTTGCACTGTCCGGTTGTTC-3'	
Grp78	F: 5'-TGAAGGGGAGCGTCTGATTG-3'	NM_013083.2
	R: 5'-TCATTCCAAGTGCGTCCGAT-3'	
Atf6	F: 5′-CCAGCAGAAAACCCGCATTC-3'	XM_017598829.1
	R: 5′-CAGAATTCCTGATGCTAGTGGTT-3'	
Perk	F: 5′-GGCTGGTGAGGGATGGTAAA-3'	NM_031599.2
	R: 5′-TTGGCTGTGTAACTTGTGTCATC-3'	
Nrf2	F: 5′-TCTGACTCCGGCATTTCACT-3′	NM_031789.2
	R: 5′-GGCACTGTCTAGCTCTTCCA-3′	
Ho-1	F: 5′-CAGCATACGTAAAGCGTCTCCA-3'	NM_012580.2
	R: 5′-CATGGCCTTCTGCGCAATCTTCTT-3'	
Gdnf	F: 5′-CAAGGTAGGCCAGGCATGTT-3'	NM_001401780.1
	R: 5′-CACACCGTTTAGCGGAAT-3'	

5.9 Imuno-histoquímica

Cortes histológicos da medula espinhal foram coletados, e utilizados para realização de análise imuno-histoquímica para identificação da expressão proteica de SOD1/2 e GPx1/2. Os anticorpos utilizados foram anti-SOD1/2 (1:20,000, sc-101523), anti-GPx1/2 (1:100, sc-133160) da Santa Cruz Biotechnology, CA, USA.

A técnica de estreptavidina-biotina-peroxidase foi utilizada pelo sistema de detecção Dako (EnVision™ FLEX+, Mouse, High pH, (Link)). A recuperação antigênica foi realizada utilizando solução de citrato (0,54 mol/L; pH 6,0) em banho-maria a 98ºC, 20 minutos. Os cortes foram imersos por 30 minutos em solução de peróxido de hidrogênio 3% em metanol para bloqueio de peroxidase endógena, e em seguida mantidas em solução de soro bloqueio por 30 minutos. Seguidamente, as lâminas foram incubadas em câmara úmida durante 18h com anticorpo primário e cortes controles utilizando solução tamponada de fosfato (PBS). As lâminas foram incubadas com anticorpo secundário conjugado a estreptavidina peroxidase (EnVision™ FLEX/HRP; ref. SM802), por 30 minutos. O cromógeno utilizado foi a diaminobenzidina (DAB Substrate system, Lab Vision Corp., Fremont, CA. USA). Finalmente, as secções foram contra-coradas com hematoxilina de Harris.

Foi determinada a área de imunomarcação utilizando o software WCIF ImageJ® (Media Cybernetics Manufacturing, Rockville, MD, USA) em fotomicrografias aleatórias feitas em 4 regiões da medula em microscópio Leica DM 2500 utilizando a câmera digital Leica DFC 295 (Leica Microsytems, Germany). Foram realizadas as análises das imagens usando a função '*color deconvolution*' e '*thresholding*'. Os dados de cada tecido foram expressos como área de imunomarcação em pixels e contrastados entre os grupos experimentais (SILVA; OCARINO; SERAKIDES, 2014).

5.10 Avaliação da produção de espécies reativas de oxigênio (ROS) e peroxinitritos (PRN)

Foi realizada avaliação fluorimétrica da produção endógena de ROS e PRN, a partir de fragmento de medula espinhal. O ensaio foi realizado usando sondas específicas para ROS (diacetato de 2',7'-diclorofluoresceína; DCFH-DA) e peroxinitrito (diidrorodamina 123). Para tal, iniciou-se homogeneização dos tecidos congelados, em Tris/HCl 50 mM pH 7.4, dissolvendo 0.788 g de Tris/HCl em 100 ml de água destilada. A homogeneização foi feita com auxílio de sonicador.

Seguidamente, foi centrifugado por 10 min a 2500 rpm (S_1). Para a dosagem de ROS (DC-DA): preparou-se solução estoque de diclorofluoresceína-diacetato (DCF-DA) em DMSO na concentração final de 10mM e a solução de trabalho, diluindo 5µL da solução estoque em 395 µL (1:80) de tampão tris-HCl com pH = 7,4 (125µM DCF-DA). Posteriormente, foram pipetados 20 µL de amostra em cada poço da placa de 96, em triplicata. A continuação, utilizando a pipeta multicanal, foram pipetados 80 µL de solução de trabalho de DCF-DA (100 µM DCF-DA). A placa foi incubada por 30 min a 37ºC. E finalmente lidos no fluorímetro nos **λ**=488 (exc) e 525 (em). Os dados foram expressos como unidades arbitrárias (AU) de fluorescência + SEM. Os testes foram realizados em duplicata.

5.11 Análise estatística

Para a distribuição dos animais nos diferentes grupos experimentais, foi utilizado o delineamento inteiramente ao acaso. As análises estatísticas foram realizadas utilizando o software estatístico (IBM SPSS Statistics 21). O teste de normalidade de Shapiro-Wilk foi aplicado para determinar a distribuição dos dados das variáveis estudadas. As variáveis paramétricas foram submetidas à análise de variância (ANOVA), com comparação de médias pelo teste de Student Newman Keuls (SNK). Para avaliação das variáveis com distribuição não paramétrica, os dados foram analisados utilizando teste de Kruskal-Wallis para comparação das medianas entre grupos e o teste de Friedman para comparação das medianas dos tempos em cada grupo. O nível de significância adotado foi $p < 0,05$.

6 RESULTADOS

6.1 Limiar mecânico nociceptivo (LMN), Dose efetiva 50 (ED50) e dose letal 50 (LD50)

Foi realizada avaliação comparativa do efeito antinociceptivo das MnP I e MnP II pelas vias intratecal e intraperitoneal, em ratos da linha Wistar com lesão neuropática por constrição do nervo isquiático. Observou-se uma marcada diminuição do LMN nos animais submetidos ao modelo de constrição do nervo isquiático no 7° dia pós-cirúrgico (dia 1 da lesão), exceto em animais do NCG ($p < 0,05$) **(Figura 24A)**. Os animais tratados com MnP I via IT dos grupos IT1A (0,2 mg/kg), IT1B (0,08 mg/kg) e IT1D (0,01 mg/kg) apresentaram um aumento significativo do LMN ao 6° e 7° dia de tratamento pela via intratecal, quando comparados ao PCG ($p < 0,05$) **(Figura 24A)**. Baseado na curva dose resposta, foi observado que a dose letal para 50% da população (DL50) testada foi de 0,08 mg/kg pela via intratecal (**Figura 24C)** e a dose efetiva para 50% (ED50) para MnP I foi de 0,004 mg/kg **(Figura 24D)**.

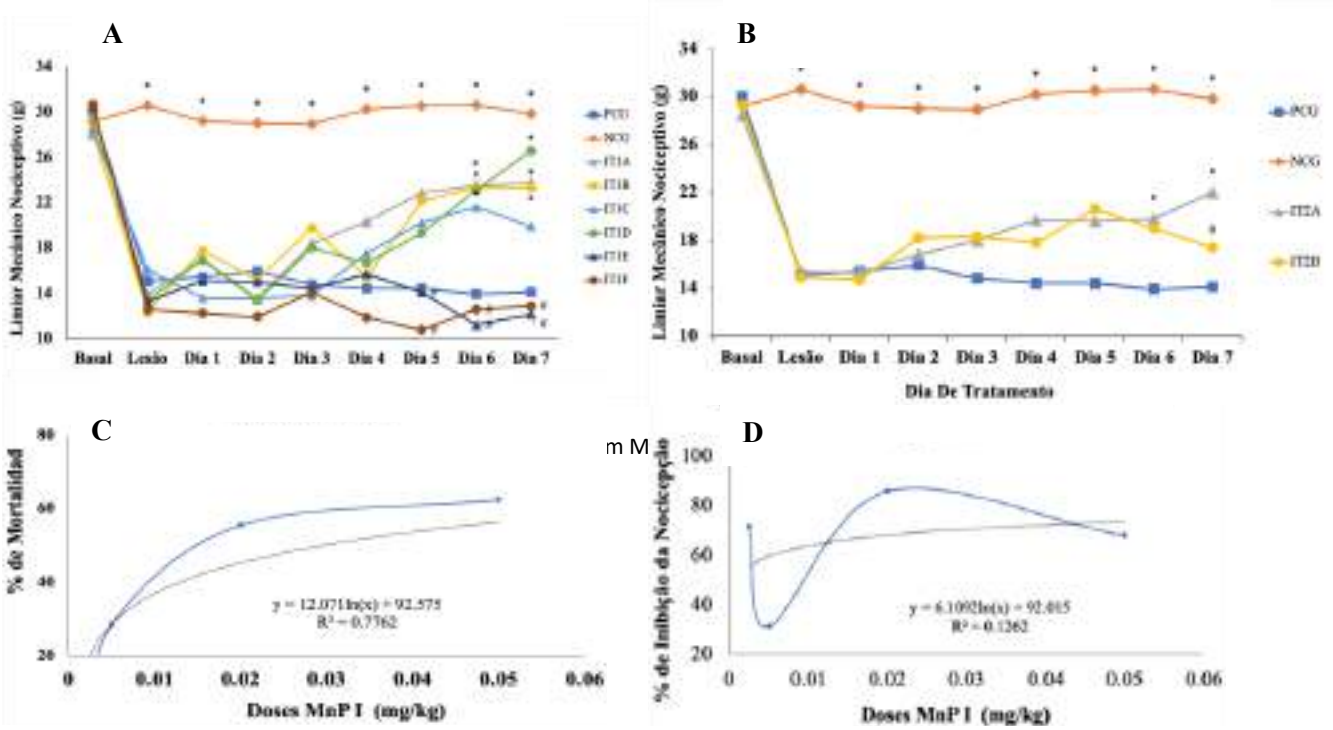


Figura 24 – Limiar mecânico nociceptivo (LMN) por meio de analgesímetro digital, em modelo de neuropatia constritiva do nervo isquiátivo em ratos Wistar tratados com MnPI (A) e MnP II (B), DL50 (C) e DE50 (D) de MnP I. Os tratamentos foram administrados durante sete dias após lesão pela via intratecal (IT). PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo Controle Negativo (10 µL água destilada); ITA: Grupo MnP I, dose A (0,2 mg/kg, via IT); ITB: Grupo MnP I, dose B (0,08 mg/kg, via IT); ITC: Grupo MnP I, dose C (0,02 mg/kg, via IT); IT D: Grupo MnP I, dose D (0,01 mg/kg, via IT); ITE: Grupo MnP I, dose E (0,005 mg/kg, via IT); GIF: Grupo MnP I, dose F (0,0005 mg/kg, via IT); IT2A: Grupo MnP II, dose A (0,01 mg/kg, via IT); IT2B: Grupo MnP II, dose B (0,005 mg/kg, via IT). Os valores são expressos como medianas. *: $p < 0,05$ comparado ao PCG, #: $p < 0,05$ comparado ao NCG. DL50 e DE50 calculado pelos métodos de Probit e Logit.

As duas doses com melhor efeito terapêutico decorrentes da avaliação da MnP I pela via IT foram utilizadas como referência para avaliação da MnP II pela mesma via **(Figura 24B)**. Ao se avaliar o LMN, foi constatada uma redução significativa no 7° dia pós-cirúrgico (dia 1 da lesão) em comparação aos animais do NCG ($p < 0,05$). Além disso, os animais do grupo IT2A (0,01mg/kg) apresentaram um aumento significativo do LMN em relação ao PCG ($p < 0,05$) no 6º e 7° dia de tratamento, indicando uma tendência de retorno aos valores basais. Em contraste, os animais do IT2B (0,005mg/kg) não apresentaram melhora significativa do LMN e permaneceram estatisticamente diferentes dos animais do NCG ($p < 0,05$) no 7º dia de tratamento **(Figura 24B)**.

Adicionalmente, foram comparados os efeitos antinociceptivo da administração de MnP I (IP1) e MnP II (IP2) pela via intraperitoneal, na dose 0,1mg/kg para ambos os grupos de tratamento. Assim, todos os animais, com exceção do NCG, apresentaram diminuição do LMN, condizente com desenvolvimento da neuropatia, a partir do dia 7° após procedimento cirúrgico (dia 1° da lesão) ($p < 0,05$). Os animais tratados com a MnP II (IP2) apresentaram um aumento significativo do LMN no 6° e 7° dia de tratamento, com valores estatisticamente diferentes de animais do PCG ($p < 0,05$), ao passo que os animais tratados com a MnPI (IP1) não apresentaram uma diferença estatisticamente significativa **(Figura 25A)**.

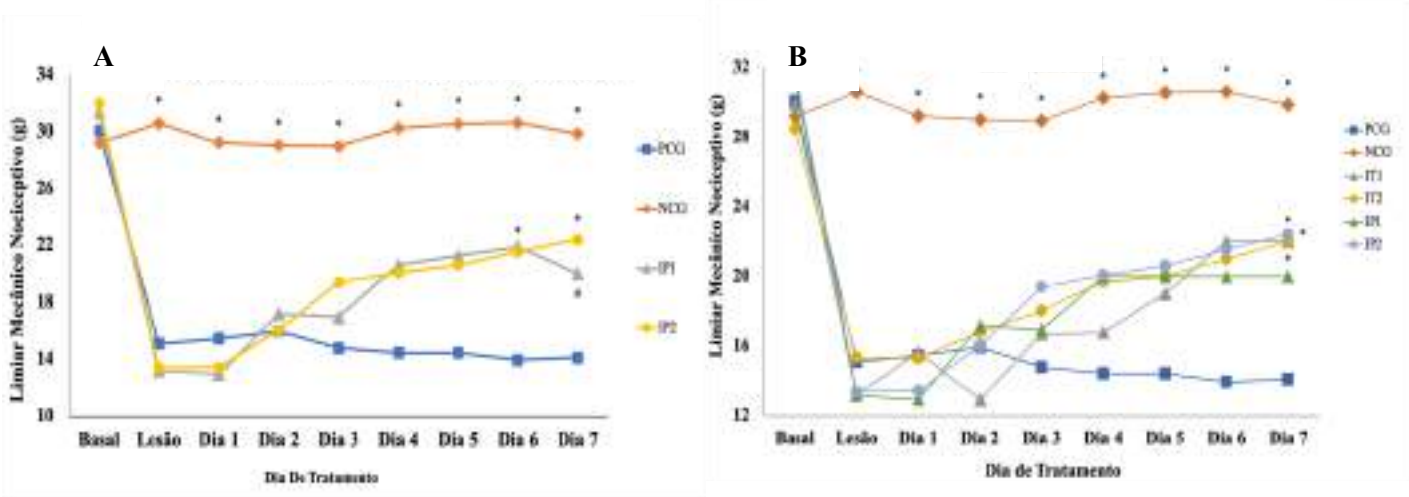


Figura 25 – Avaliação do limiar mecânico nociceptivo (LMN) por meio de analgesímetro digital, em modelo de neuropatia constritiva do nervo isquiático em ratos Wistar tratados com MnP I e MnP II pela via intraperitoneal (IP) (A), e comparação das doses mais eficientes de MnP I e MnP II pelas vias intratecal (IT) e intraperitoneal (IP) (B). Os tratamentos foram administrados durante sete dias após lesão. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo Controle Negativo (10 µL água destilada); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo Mn I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP) Os valores são expressos como medianas. * $p < 0,05$ comparado ao PCG, # $p < 0,05$ comparado ao NCG.

A partir dos resultados supracitados, foram selecionadas como referências aquelas doses com melhor efeito terapêutico, para comparação da antinocicepção, contrastando as diferentes vias de administração das MnP I e MnP II, pela via intratecal e intraperitoneal **(Figura 25B)**. O desenvolvimento da lesão constritiva foi confirmado no 7° dia após procedimento cirúrgico (dia 1° da lesão), evidenciado pela diminuição do LMN em todos os animais, com exceção dos animais do NCG ($p < 0,05$). A partir do dia 5° de tratamento, os animais dos grupos IT1 (MnP I 0,01 mg/kg, via IT), IT2 (MnP II 0,01 mg/kg, via IT) e IP2 (MnP II 0,1mg/kg, via IP) apresentaram um aumento significativo de LMN, atingindo o ponto máximo no dia 7° dia de tratamento, com diferenças estatisticamente significativas em relação ao PCG ($p < 0,05$. **Figura 25B**).

6.2 MnP reduzem a produção de espécies reativas de oxigênio (ROS) e peroxinitritos (PRN) em ratos com dor neuropática

Constatou-se uma redução estatisticamente significativa na produção de espécies reativas de oxigênio (ROS) e peroxinitritos (PRN) **(Figura 26)** nos animais dos grupos IT1, IT2, IP1 e IP2, quando confrontados ao PCG ($p < 0,05$), isto é, aqueles que desenvolveram a lesão constritiva do nervo isquiático e que não receberam qualquer tratamento. Contudo, animais dos grupos IT1, IP1 e IP2, expressaram valores ainda inferiores ao NCG, quanto à produção de ROS e PRN.

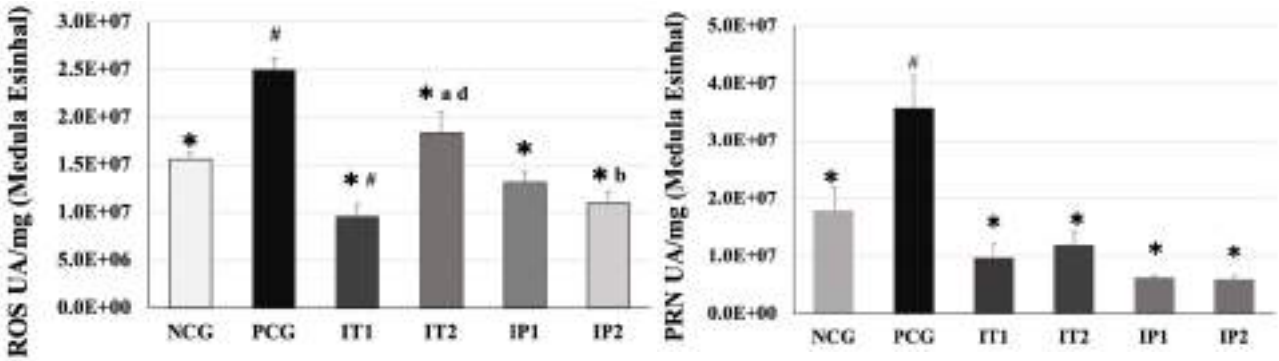


Figura 26 – Produção de espécies reativas de oxigênio (ROS) e peroxinitritos (PRN) em modelo de neuropatia constritiva do nervo isquiático em ratos Wistar tratados com MnP I e MnP II pelas vias IT e IP. As medulas espinais foram coletadas após sete dias de tratamento. PCG: Grupo controle positivo (10µL água destilada); NCG: Grupo controle negativo (10µL água destilada, sem LCC); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP). Os valores são expressos como média + EP. * $p \leq 0,05$ comparado ao PCG; # $p < 0,05$ comparado ao NCG, a $p < 0.05$ comparado ao IT1; b $p < 0.05$ comparado ao IT2; c $p < 0.05$ comparado ao IP2.

6.3 MnP modulam a expressão medular de enzimas antioxidantes em ratos com dor neuropática

As MnP, conhecidas pelos seus efeitos moduladores do meio redox intracelular, foram capazes de afetar a regulação gênica e expressão proteica de enzimas antioxidantes. A análise de expressão gênica diferencial do gene *Cat* demonstrou uma *down-regulation* significativa no grupo IT2 em comparação ao grupo NAIVE ($p < 0,05$). Os grupos IT1 e IP2 apresentaram *up-regulation* significativa de *Cat* em relação ao IT2 ($p < 0,05$), mas não diferiram significativamente entre si ou em comparação aos grupos NCG, PCG e *NAIVE* ($p > 0,05$) **(Figura 27A)**.

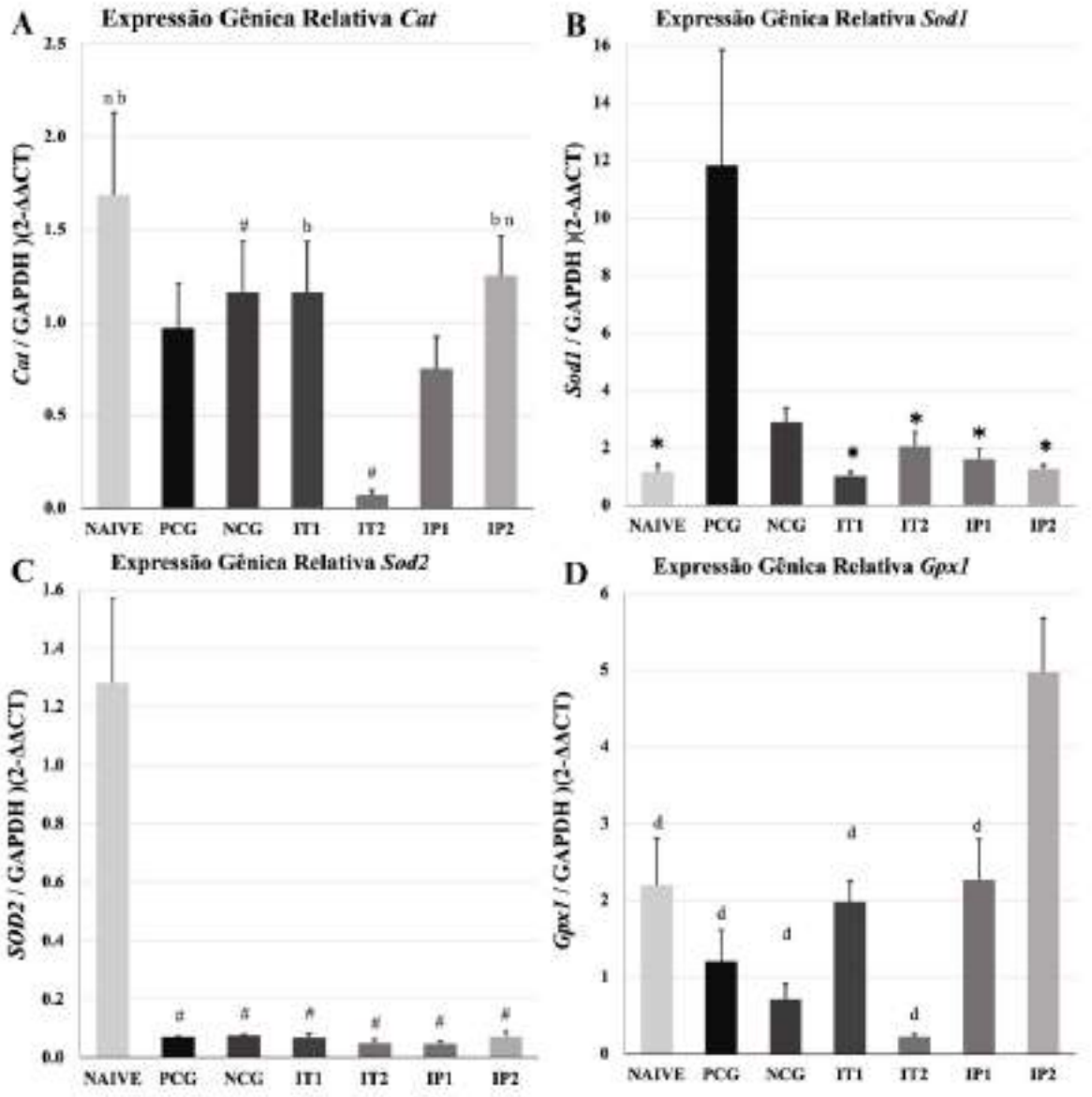


Figura 27 - Expressão Gênica Relativa de genes antioxidantes *Cat*, *Sod1*, *Sod2* e *Gpx1* em modelo de neuropatia constritiva do nervo isquiático em ratos tratados com MnP I e MnP II pelas vias IT e IP. As medulas foram coletadas após sete dias de tratamento. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo controle negativo (10 µL água destilada, sem LCC); *NAIVE*: Grupo *NAIVE* (sem modelo de LCI e sem tratamento); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01

mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP). Os valores são expressos como média + EP. * p < 0,05 comparado ao PCG, # p < 0,05 comparado ao *NAIVE*, b p < 0.05 comparado ao IT2, d p < 0,05 comparado ao IP2, n p < 0,05 comparado ao NCG.

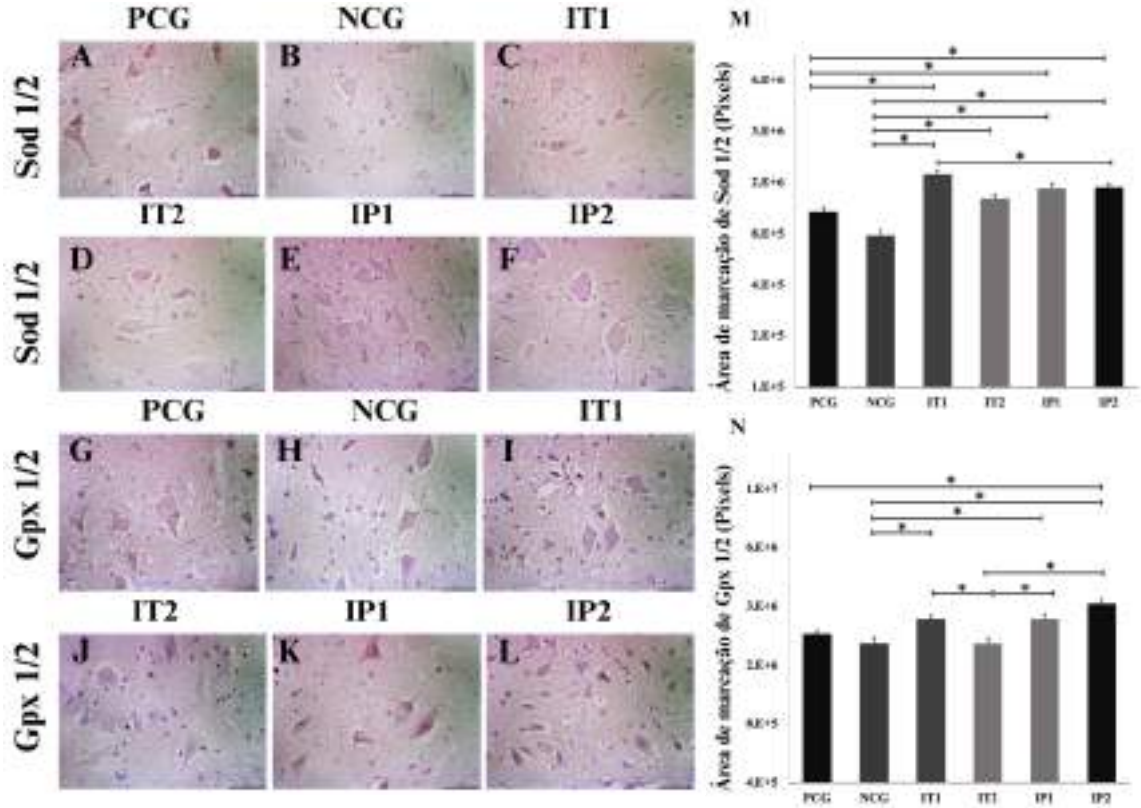


Figura 28 - Efeitos do tratamento com MnP na expressão de enzimas antioxidantes SOD1/2 e GPx1 em modelo de neuropatia constritiva do nervo isquiático em ratos tratados com MnP I e MnP II pelas vias IT e IP. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo controle negativo (10 µL água destilada, sem LCC); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP). (A-F): Fotomicrografias da imunomarcação de SOD1/2; (G-L): Fotomicrografias da imunomarcação de GPx1/2; M: Área de imunomarcação de SOD1/2 expresso em pixels; N: Área de imunomarcação de GPx1/2 expresso em pixels, os valores são expressos como média + EP; * p < 0,05. Aumento de 40x. Barra = 50 µm

A análise da expressão gênica de *Sod1* revelou uma diminuição significativa em todos os grupos experimentais (IT1, IT2, IP1, IP2) em comparação ao grupo PCG (p < 0,05). Embora o grupo NCG também tenha apresentado uma redução na expressão de *Sod1* em relação ao GCP, essa diferença não atingiu significância estatística (p > 0,05). Os níveis de expressão de *Sod1* nos grupos experimentais foram similares aos observados no grupo *NAIVE* (sem lesão nem tratamento) **(Figura 27B)**. Ao passo que a expressão proteica de SOD1/2 foi significativamente maior nos grupos IT1, IP1 e IP2, em comparação ao PCG **(Figura 28M).**

A expressão do gene *Sod2*, responsável pela síntese da superóxido dismutase 2, localizada na mitocôndria foi significativamente reduzida em todos os grupos experimentais

(IT1, IT2, IP1, IP2, PGC, NCG) quando comparados ao grupo *NAIVE* ($p < 0,05$). **(Figura 27C)**. A análise da expressão gênica *Gpx1*, que codifica a glutationa peroxidase 1, de ação antioxidante citosólica, revelou que o grupo IP2 apresentou uma expressão significativamente maior em comparação a todos os outros grupos experimentais ($p < 0,05$) **(Figura 27D)**. Enquanto a expressão proteica de GPx1/2 foi significativamente superior apenas no grupo IP2 em contraste com o PCG ($p < 0,05$) **(Figura 28N)**.

6.4 Tratamento com MnP modula expressão de mediadores do estresse oxidativo e estresse do retículo endoplasmático em ratos com dor neuropática

A análise da expressão gênica de *Grp78*, marcador de estresse do retículo endoplasmático, revelou uma diminuição significativa nos grupos IT1, IT2, IP1 e IP2 em comparação ao PGC ($p < 0,05$). Notavelmente, os níveis de expressão de *Grp78* nos grupos tratados foram similares aos observados no grupo *NAIVE*, indicando uma atenuação do estresse do RE **(Figura 29A)**.

A análise da expressão gênica de *Atf6*, codificador da proteína ATF6, um componente essencial da resposta de proteína não dobrada (UPR) no RE, revelou uma redução significativa nos grupos IT2, IP1 e IP2 em comparação ao grupo PGC ($p < 0,05$) e ao grupo NCG ($p < 0,05$). Por outro lado, os grupos PCG, NCG e IT1 apresentaram níveis de expressão de *Atf6* significativamente maiores do que o grupo *NAIVE* ($p < 0,05$). **(Figura 29B)**.

A expressão do gene *Perk*, um elemento chave da UPR no RE, foi significativamente maior no grupo PGC em comparação ao grupo *NAIVE* ($p < 0,05$). Embora os grupos IT1, IT2, IP1, IP2 e NCG tenham apresentado uma diminuição na expressão de *Perk* em relação ao PCG, essa diferença não atingiu significância estatística ($p > 0,05$ **Figura 29C**). A análise de expressão gênica de *Nrf2* demonstrou uma *down-regulation* significativa nos grupos IT1, IT2, IP1, IP2 e NCG em comparação aos animais do grupo PCG ($p < 0,05$), com níveis de expressão similares aos observados nos animais do grupo *NAIVE* **(Figura 29D)**.

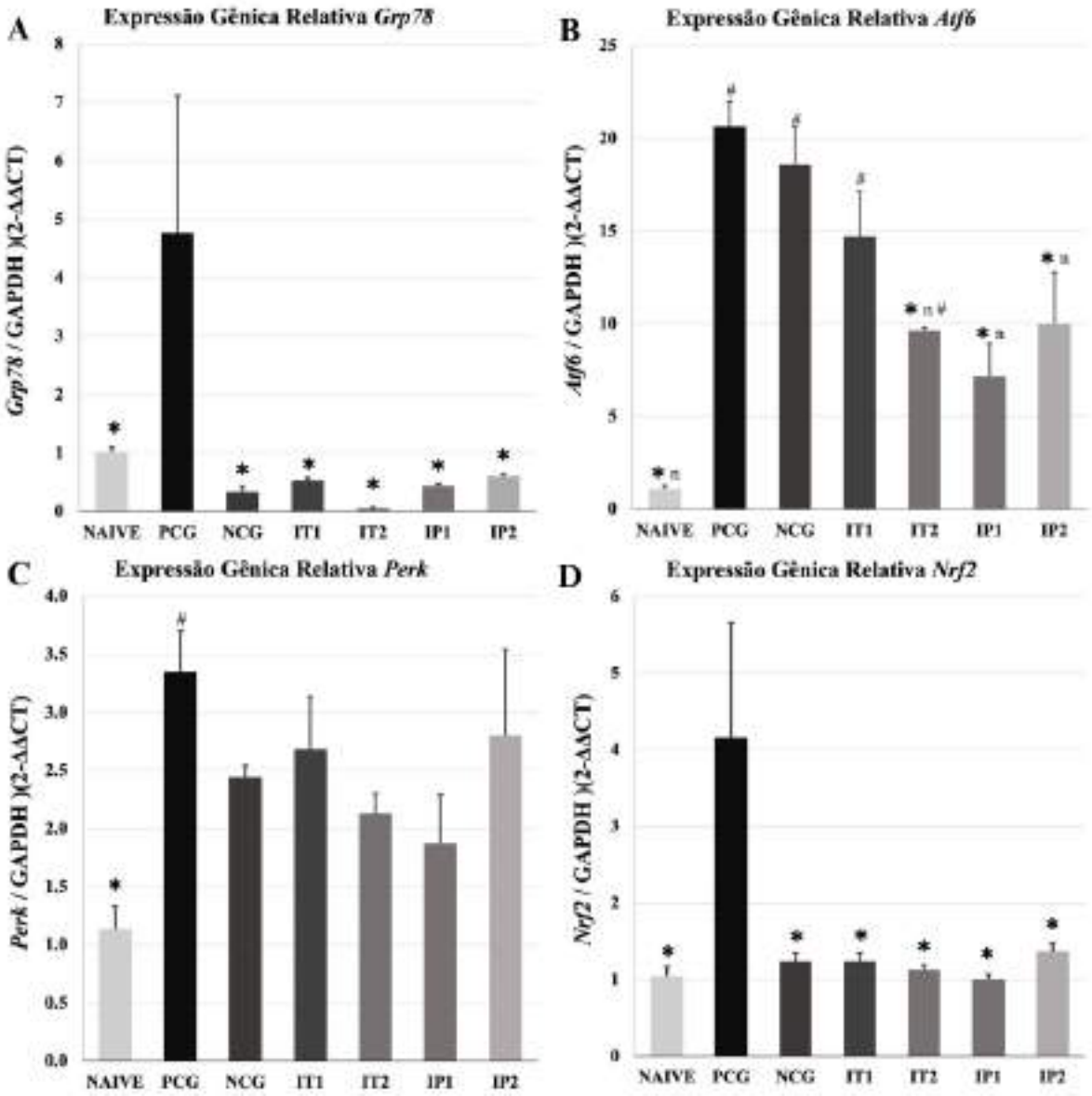


Figura 29 – Expressão gênica relativa de genes moduladores do estresse do retículo endoplasmático: *Grp78, Atf6, Perk e Nrf2* em modelo de neuropatia constritiva do nervo isquiático em ratos tratados com MnPI e MnPII pelas vias IT e IP. As medulas foram coletadas após sete dias de tratamento. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo controle negativo (10 µL água destilada, sem LCC); *NAIVE*: Grupo *NAIVE* (sem modelo de LCI e sem tratamento); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP).Os valores são expressos como média + EP. * $p < 0,05$ comparado ao PCG, # $p < 0,05$ comparado ao *NAIVE*, n $p < 0,05$ comparado ao NCG.

6.5 MnP modulam expressão gênica medular de *Caspase 3* induzida por lesão neuropática em ratos

A análise da expressão gênica de *Chop*, um marcador inicial de apoptose induzida por estresse do retículo endoplasmático (RE), revelou que os grupos experimentais (PCG, NCG, IT1, IT2, IP1 e IP2) apresentaram níveis de expressão significativamente menores em comparação ao grupo NAIVE ($p < 0,05$). No entanto, não foram observadas diferenças

significativas na expressão de *Chop* entre os grupos lesionados (IT1, IT2, IP1 e IP2) e o grupo PCG ($p > 0,05$) **(Figura 30A)**.

A análise da expressão gênica da *Caspase 9*, uma caspase iniciadora na via apoptótica intrínseca, revelou que os grupos PCG, NCG, IT2 e IP2 apresentaram níveis de expressão significativamente maiores em comparação ao grupo *NAIVE* ($p < 0,05$). Os grupos IT1 e IP1, por sua vez, apresentaram níveis de expressão de *Caspase 9* menores em relação ao grupo PCG, e maiores em relação ao grupo *NAIVE,* embora sem diferença estatística ($p > 0,05$) **(Figura 30B)**.

A análise da expressão gênica da *Caspase 3*, uma caspase executora de apoptose, revelou uma redução significativa nos grupos experimentais tratados com MnP (IT1, IT2, IP1 e IP2) em comparação ao grupo PCG ($p < 0,05$). Notavelmente, os níveis de expressão de *Caspase 3* nesses grupos foram similares aos observados no grupo *NAIVE*, indicando um efeito protetor das MnP contra a apoptose induzida pela lesão **(Figura 30C)**.

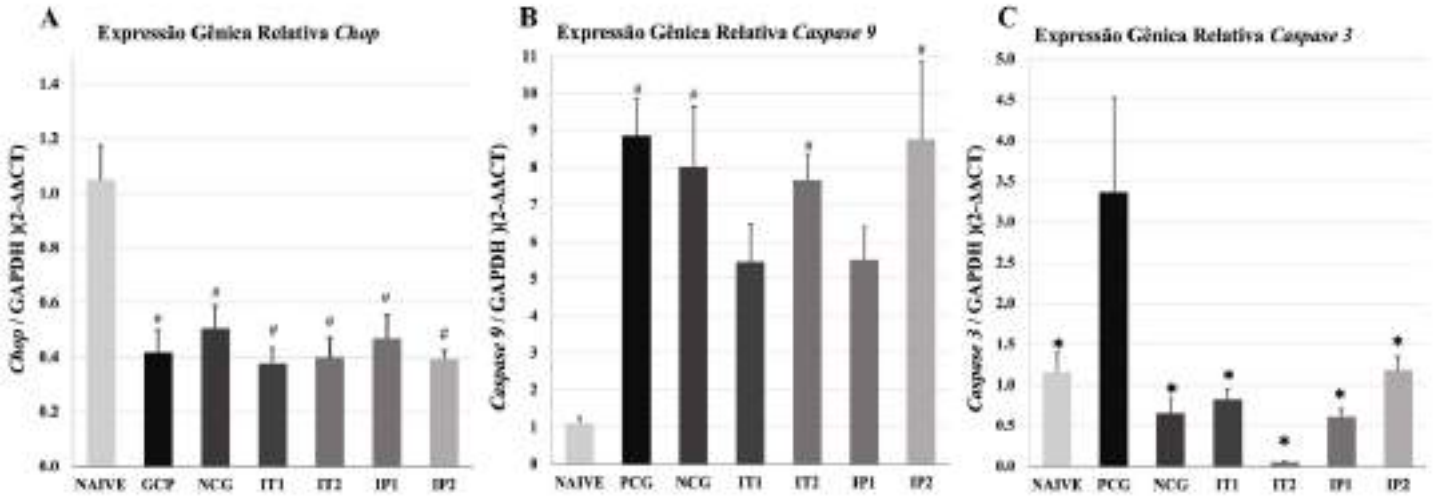


Figura 30 – Expressão Gênica Relativa de genes implicados em apoptose e morte celular *Caspase 9*, *Caspase 3* e *Chop* em modelo de neuropatia constritiva do nervo isquiático em ratos tratados com MnPI e MnPII pelas vias IT e IP. As medulas foram coletadas após sete dias de tratamento. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo controle negativo (10 µL água destilada, sem LCC); *NAIVE*: Grupo *NAIVE* (sem modelo de LCI e sem tratamento); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP). Os valores são expressos como média + EP. * $p < 0,05$ comparado ao PCG, # $p < 0,05$ comparado ao *NAIVE*.

6.6 Tratamento com MnP induz modulação da expressão medular de receptor de fractalkina em ratos com dor neuropática

A análise da expressão gênica das citocinas pró-inflamatórias *Il-1b* e *Il-6* não revelou diferenças estatisticamente significativas entre os grupos experimentais estudados ($p > 0,05$) (**Figura 31A e B)**. Quanto à análise de expressão gênica do gene *CX3CR1* (receptor de fractalkina), expresso principalmente em micróglia, demonstrou uma *up-regulation* significativa nos grupos PCG, NCG, IT1 e IP2 em comparação ao grupo *NAIVE* ($p < 0,05$). Os grupos IT2 e IP1 apresentaram *down-regulation* significativa de *CX3CR1* em relação ao grupo PCG ($p < 0,05$), com valores similares aos animais do grupo *NAIVE* **(Figura 31C)**. Ressalta-se que todos os grupos experimentais apresentaram uma diminuição estatisticamente significativa na sua expressão quanto à expressão gênica de *CX3CL1* (fractalkina), em contraste com o grupo *NAIVE*, cuja expressão foi significativamente maior ($p < 0,05$) **(Figura 31D)**.

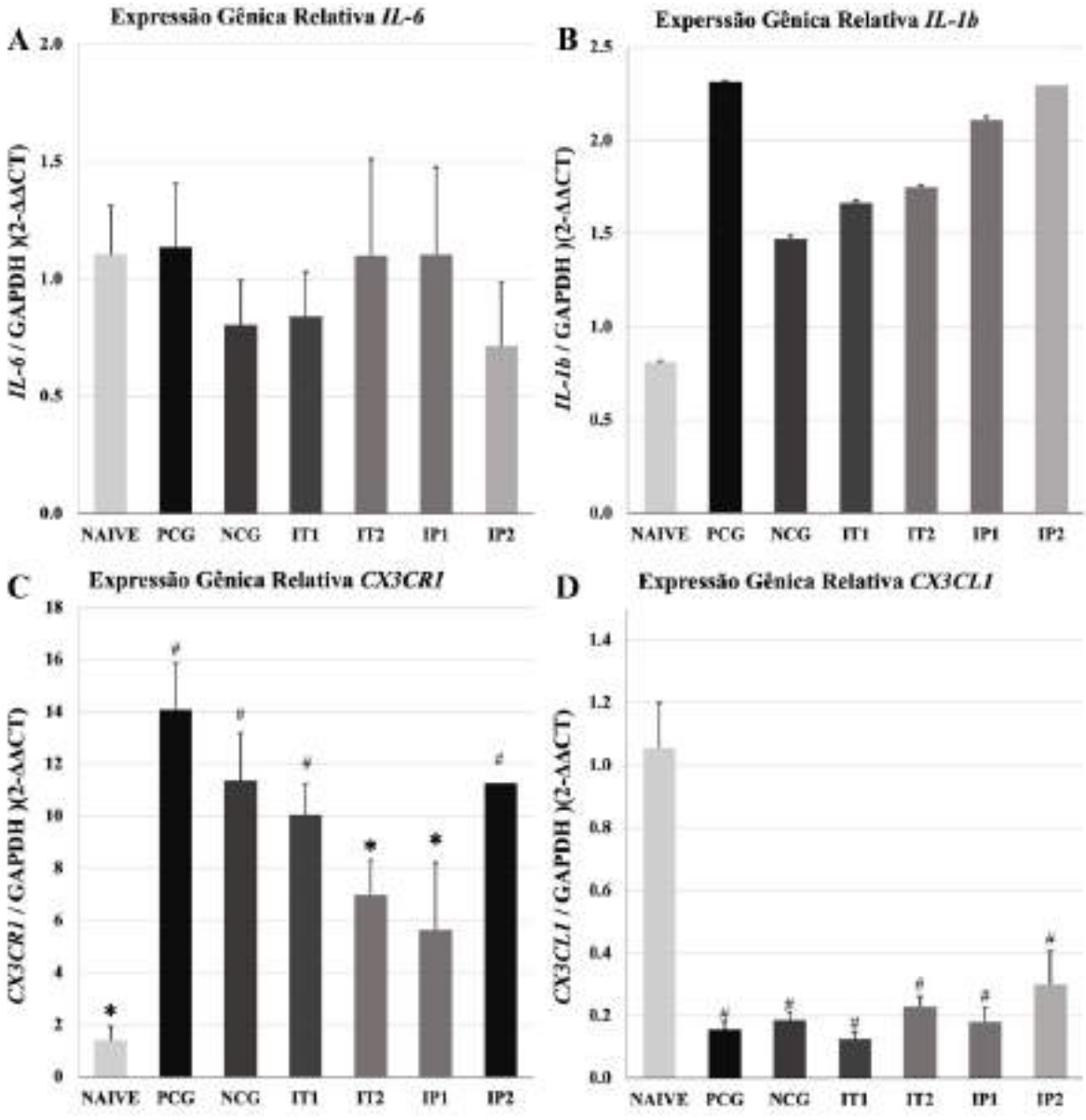


Figura 31 - Expressão Gênica Relativa de genes moduladores da inflamação: *Il-1b, Il-6, CX3CR1 e CX3CL1* em modelo de neuropatia constritiva do nervo isquiático em ratos tratados com MnP I e MnP II pelas vias IT e IP. As medulas foram coletadas após sete dias de tratamento. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo controle negativo (10 µL água destilada, sem LCC); *NAIVE*: Grupo *NAIVE* (sem modelo de LCI e sem tratamento); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP). Os valores são expressos como média + EP. * $p < 0,05$ comparado ao PCG, # $p < 0,05$ comparado ao *NAIVE*.

6.7 MnP modula expressão de genes neuroprotetores e em ratos com dor neuropática

A análise da expressão relativa do gene *Hif-1a,* mostrou que animais do grupo *NAIVE* apresentaram valores significativamente maiores comparadas aos grupos de tratamento e grupos de controle (PCG, NCG, IT1, IT2, IP1 e IP2) ($p < 0,05$) **(Figura 32A).** Já a análise da expressão gênica de *Gdnf*, um fator neurotrófico essencial para a sobrevivência e regeneração neuronal, revelou uma diminuição significativa nos grupos IT2, IP1 e IP2 em comparação ao grupo PGC ($p < 0,05$). Notavelmente, os níveis de expressão de *Gdnf* nos grupos tratados foram similares aos observados no grupo *NAIVE*, indicando uma modulação da expressão gênica de *Gdnf*. **(Figura 32B)**

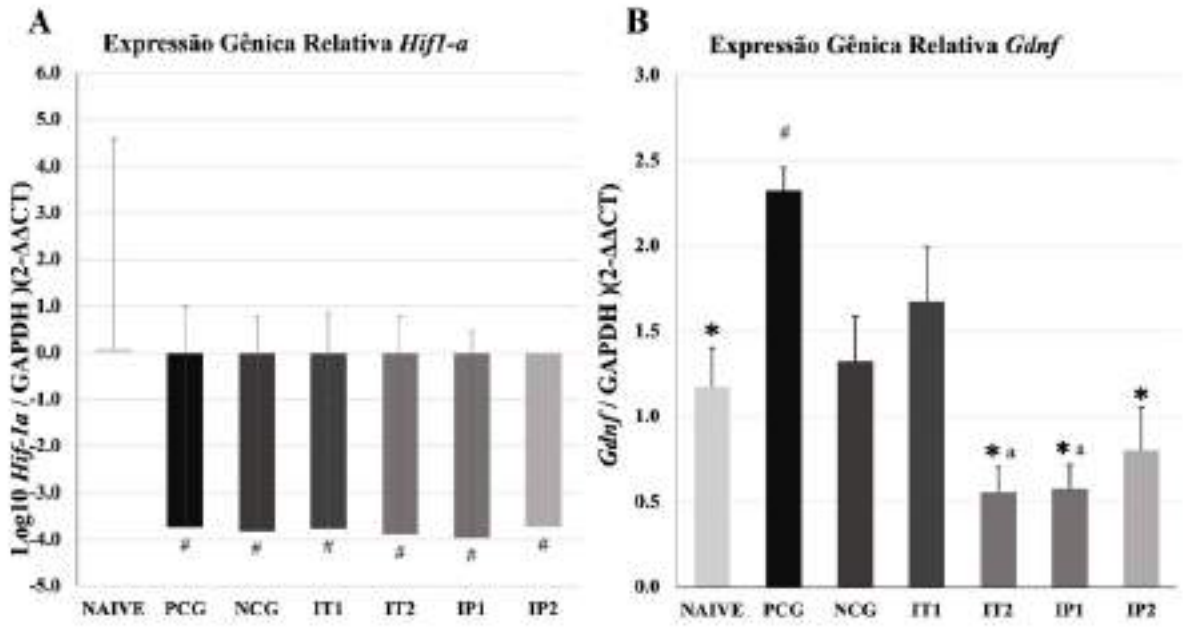


Figura 32 - Expressão gênica relativa de genes neuroprotetores: *Hif1-a* e *Gdnf* em modelo de neuropatia constritiva do nervo isquiático em ratos tratados com MnPI e MnPII pelas vias IT e IP. As medulas foram coletadas após sete dias de tratamento. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo controle negativo (10 µL água destilada, sem LCC); *NAIVE*: Grupo *NAIVE* (sem modelo de LCI e sem tratamento); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP). Os valores são

expressos como média + EP. * p < 0,05 comparado ao GCP, # p < 0,05 comparado ao *NAIVE*, a p < 0,05 comparado ao IT1.

6.8 MnP modula expressão de *Ho-1* em ratos com dor neuropática

A análise da expressão gênica de *Ho-1*, um modulador tardio de resposta antioxidante e anti-inflamatória, revelou que os grupos PCG, NCG, IT1 e IP2 apresentaram um aumento significativo na expressão de *Ho-1* em comparação ao grupo *NAIVE* (p < 0,05). Em contraste, os grupos IT2, IP1 e IP2 demonstraram níveis de expressão de *Ho-1* significativamente menores que animais do grupo PCG (p < 0,05) **(Figura 33).**

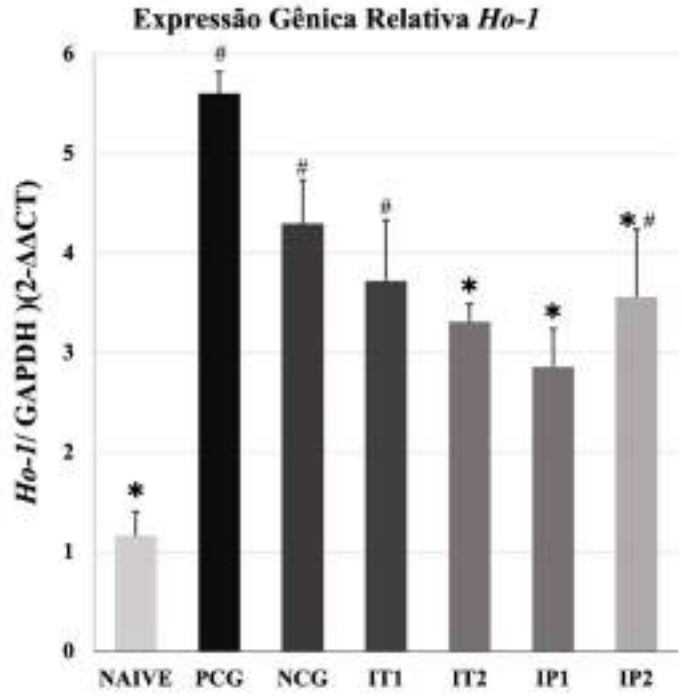


Figura 33 - Expressão gênica relativa do gene *Ho-1* em modelo de neuropatia constritiva do nervo isquiático em ratos tratados com MnPI e MnPII pelas vias IT e IP. As medulas foram coletadas após sete dias de tratamento. PCG: Grupo Controle Positivo (10 µL água destilada); NCG: Grupo controle negativo (10 µL água destilada, sem LCC); *NAIVE*: Grupo *NAIVE* (sem modelo de LCI e sem tratamento); IT1: Grupo MnP I, (0,01 mg/kg, IT); IT2: Grupo MnP II, (0,01 mg/kg, Via IT); IP1: Grupo MnP I, (0,1 mg/kg, via IP); IP2: Grupo MnP II, (0,1 mg/kg, via IP). Os valores são expressos como média + EP. * p < 0,05 comparado ao GCP, # p < 0,05 comparado ao *NAIVE*, a p < 0,05 comparado ao IT1.

7 DISCUSSÃO

Este estudo visou avaliar os efeitos antinociceptivo, antioxidante, anti-inflamatório de MnP I [MnIIITE-2P-PyP]$^{5+}$ e MnP II [MnIIIT(5-Br-3-E-Py)P]$^{5+}$ na dor neuropática induzida por constrição do nervo isquiático em ratos e as possíveis vias de modulação do estresse do RE. Os resultados demonstraram que MnP I e MnP II reduziram o LMN de ratos com LCI, diminuíram a formação de ROS e PRN no segmento lombo-sacro da medula espinhal, modularam a expressão de genes relacionados ao estresse oxidativo (*Cat*, *Sod1* e *Gpx1*), modularam a resposta ao estresse do RE (*Grp78*, *Atf6* e *Nrf2*), além de genes relacionados à inflamação e antioxidação tardia e dor (*CX3CR1*, *Ho-1*). Também restauraram os níveis basais na expressão de genes neuroprotetores (*Hif-1a* e *Gdnf*), que promovem a sobrevivência e a regeneração neuronal, e protegeram contra a expressão do gene pro-apoptótico *Caspase 3*, gerado pelo modelo experimental. Este é o primeiro estudo a avaliar os efeitos antinociceptivo, antioxidante, anti-inflamatório em modelo experimental de dor neuropática por constrição do nervo isquiático, considerando as vias de administração intratecal e intraperitoneal.

O modelo de LCI induziu uma diminuição significativa do LMN no sétimo dia após procedimento cirúrgico, evidenciada pelo aumento da sensibilidade ao toque no membro locomotor pélvico acometido, indicando o desenvolvimento da neuropatia nos ratos. Em contraste, no NCG, onde o nervo isquiático foi apenas exposto, esse estímulo não foi suficiente para o desenvolvimento de hiperalgesia. O aumento significativo no LMN nos animais tratados com a MnP I pela via IT (IT1A, IT1B e IT1D) demonstra seu efeito antinociceptivo da MnP I ao reverter parcialmente a hiperalgesia mecânica induzida pela lesão. A eficácia do tratamento pela via IT sugere que a MnP I pode atuar diretamente na medula espinhal para modular o processo de desenvolvimento da dor neuropática, em uma relação dose-dependente, resultado este em concordância com outros trabalhos que avaliaram a capacidade antinociceptiva de metaloporfirinas em modelos de dor neuropática (JANES et al., 2013; RAUSARIA et al., 2011a).

A determinação da DL50 e da ED50 foi essencial para avaliar a eficácia e a segurança das MnP. A DL50 de 0,08 mg/kg pela via IT, indica toxicidade lenta para 50% dos animais, enquanto a ED50 de 0,004 mg/kg, indica que 50% dos animais apresentaram efeito antinociceptivo. Essa relação entre DL50 e ED50 permite definir uma janela terapêutica eficaz sem efeitos significativamente tóxicos.

Com base nos valores obtidos para a MnP I, os dados foram extrapolados para avaliação do efeito antinociceptivo da MnP II, pela via IT. De forma semelhante, os animais tratados com

0,01 mg/kg (IT2A) apresentaram um retorno significativo aos valores basais de LMN, em comparação ao PCG, no 6º e 7º dia de tratamento, demonstrando eficácia na reversão da hiperalgesia mecânica. Por outro lado, aqueles tratados com a dose 0,005 mg/kg (IT2B) não apresentaram melhora significativa no LMN e permaneceram significativamente diferentes do NCG no 7º dia de tratamento, sugerindo que a dose de 0,005 mg/kg de MnP II foi insuficiente para um efeito antinociceptivo relevante. Assim, a eficácia da MnP II mostrou-se dependente da dose, com melhores resultados em 0,01 mg/kg.

A MnP II também demonstrou efeito antinociceptivo significativo pela via IP, promovendo aumento do LMN nos animais tratados e aproximando-se dos valores basais, sendo eficaz na reversão da hiperalgesia mecânica induzida pela neuropatia. Em contraste, a MnP I não apresentou efeito antinociceptivo estatisticamente significativo pela via IP, indicando diferenças notáveis entre as duas MnP quanto a biodisponibilidade e mecanismo de ação possivelmente devido a diferenças estruturais e de interação com alvos moleculares distintos (BATINIĆ-HABERLE et al., 1999, 2009a).

O estresse oxidativo, um dos principais fatores na dor neuropática, foi avaliado por meio da mensuração de ROS/PRN em medula espinhal. A redução significativa dessas espécies reativas nos grupos tratados (IT1, IT2, IP1 e IP2) em comparação ao PCG confirma o efeito antioxidante das MnP. Esse resultado indica que elas neutralizam ROS/PRN e interrompem cascatas oxidativas, resultado coerente com estudos anteriores (BATINIĆ-HABERLE et al., 2009b; BATINIC-HABERLE et al., 2014). Os grupos IT1, IP1 e IP2 apresentaram valores de ROS/PRN inferiores aos do NCG, sugerindo que as MnP podem modular a produção dessas espécies para além dos níveis basais, reforçando seu potencial neuroprotetor. A MnP I possui grupo *meso*-tetrakis(*N*-etilpiridínio), localizado na 2ª posição do anel de piridina, o que altera a distribuição total de cargas, melhorando a sua solubilidade em meio aquoso, contribuindo de forma geral ao seu perfil de solubilidade, relevante para sua atividade biológica. A sua estabilidade molecular lhe permite ter uma função de neutralização de ROS e PRN, crucial para a sua atividade redox (REBOUÇAS; SPASOJEVIĆ; BATINIĆ-HABERLE, 2008). No entanto, a MnP II possui um substituinte 5-Br e um grupo *N*-etilpiridínio na 3ª posição do anel de piridina, o que pode lhe conferir uma capacidade catalítica de reações redox, uma solubilidade aquosa e uma capacidade de transferência de elétrons ainda maior que a MnP I, aumentando sua distribuição nos sistemas biológicos, o que melhora sua interação com membranas biológicas, potencialmente beneficiando sua reatividade com ROS/PRN no contexto da dor neuropática (DOS ANJOS CORDEIRO et al., 2024; PINTO et al., 2013).

O estresse oxidativo depende amplamente do funcionamento dos sistemas enzimáticos antioxidantes, cujo papel é fundamental no desenvolvimento da dor neuropática (SINGH et al., 2020; ZAMANI et al., 2025). Desta forma, foi avaliado o efeito modulador das MnP I e MnP II sobre a expressão de genes de enzimas antioxidantes (*Cat*, *Sod1*, *Sod2* e *Gpx1*), bem como a expressão proteica de SOD1/2 e GPx1/2.

As MnP demonstraram capacidade de afetar a regulação gênica de enzimas antioxidantes. Em relação à expressão gênica de *Cat,* observou-se uma redução significativa no grupo IT2 e um aumento nos grupos IT1 e IP2 em relação ao IT2, sugerindo uma modulação complexa da catalase, possivelmente devido a diferenças na biodisponibilidade ou mecanismo de ação das MnP. A catalase produzida em resposta à exposição ao ânion superóxido (O•-), é responsável pela conversão de duas moléculas de H_2O_2 em H_2O e O_2 (AMIR ASLANI; GHOBADI, 2016). Expressa em todas as células do sistema nervoso central, incluindo neurônios e células da glia, a catalase desempenha um papel essencial na detoxificação do H_2O_2 (USUI et al., 2009). A diferença observada na expressão de *Cat* pode estar relacionada às diferenças estruturais entre as MnP. Embora ambas sejam altamente polares devido à presença do grupo *N*-etilpiridínio, a MnP II contém um substituinte de Br, que pode influenciar as propriedades eletrônicas do anel de porfirina, aumentando sua capacidade de alterar o estado redox do centro de Mn, essencial para a neutralização de O•- (DOS ANJOS CORDEIRO et al., 2024). A alta taxa de dismutação do O•- pode gerar níveis elevados níveis de H_2O_2, superando a capacidade celular da catalase e desencadeando um mecanismo de regulação negativa na expressão de *Cat,* de modo a prevenir uma produção excessiva da enzima, o que poderia comprometer outros processos celulares (DAY et al., 2012; NING; MO; LAI, 2010; VENKATESAN et al., 2007). Já a MnP I pode ter um efeito mais acentuado sobre a expressão de vias específicas que regulam enzimas antioxidantes, como NRF2, discutido adiante. Além disso, MnP catiônicas não são potentes miméticos da atividade catalase (BATINIĆ-HABERLE et al., 1999; DAY, 2009).

Adicionalmente, MnP I e II, independentemente da via de administração, foram capazes de afetar positivamente a expressão gênica de *Sod1*, evidenciado pela redução significativa em todos os grupos experimentais (IT1, IT2, IP1, IP2) em comparação com animais com lesão neuropática e solução salina, retornando a níveis basais, similares ao grupo *NAIVE* (sem lesão nem tratamento). A SOD1 catalisa a dismutação do ânion superóxido em peróxido de hidrogênio, um processo essencial na regulação do estresse oxidativo, prevenindo a sensibilização central e periférica e reduzindo a neuroinflamação (JOMOVA et al., 2024). O aumento da expressão de *Sod1* em animais com dor neuropática foi relatado em estudos prévios,

refletindo sua relevância na redução de ROS no contexto de dor neuropática (DAVIS; PENNYPACKER, 2017; NIELLA et al., 2024). No entanto, a diminuição da expressão de *Sod1* observada neste estudo é condicente com uma menor concentração de espécies reativas após o tratamento com MnP, demonstrando o seu potencial para restaurar o estado de equilíbrio redox na medula espinhal (DOS ANJOS CORDEIRO et al., 2024). O aumento da expressão proteica de SOD1/2 sugere um possível mecanismo de *feedback* negativo, onde a presença da proteína regula a expressão do respetivo mRNA. No contexto da dor neuropática, níveis elevados de ROS e PRN podem oxidar e nitrar MnSOD, inativando a enzima e impedindo sua ação na neutralização de espécies reativas, contribuindo para a manutenção do estado hiper-oxidativo na medula espinhal, e favorecendo a sensibilização central e periférica (BENNETT; DOYLE; SALVEMINI, 2014; SALVEMINI et al., 2011). A expressão do gene *Sod2*, responsável pela síntese da superóxido dismutase 2, localizada na mitocôndria, foi significativamente reduzida em todos os grupos experimentais (IT1, IT2, IP1, IP2, PGC, NCG) quando comparados ao grupo *NAIVE*; sugerindo que as MnP não apresentaram impacto direto sobre a expressão da SOD2.

A família GPx desempenha múltiplas funções fisiológicas, sendo sua principal atuação a redução enzimática de H_2O_2 e hidroperóxidos, convertendo a glutationa reduzida (GSH) em sua forma oxidada (GSSH) por meio de um ciclo dependente da oxidação e subsequente redução do GPx (WEAVER; SKOUTA, 2022). No presente estudo, a expressão gênica de *Gpx1* e a enzima GPx1 de ação antioxidante citosólica revelou que o grupo IP2 apresentou uma expressão significativamente maior em comparação a todos os outros grupos experimentais, sendo esta diferença particularmente saliente ao se comparar com o grupo IT2 ($p < 0,05$). Essa diferença pode estar associada a mecanismos de retroalimentação negativo, similares aos descritos para *Cat*, visto que níveis elevados de H_2O_2 podem interromper a função do sistema NRF2 (NING; MO; LAI, 2010).

A regulação da expressão de GPx1está intrinsecamente relacionada ao H_2O_2. Embora GPx1 contribua na redução de H_2O_2, em moléculas menos reativas, quantidades excessivas desta espécie reativa podem sinalizar uma diminuição na expressão de GPx1 como mecanismo de compensação para evitar a atividade excessiva das defensas antioxidantes. Essa regulação interage com a catalase, desempenhando um papel essencial no controle do estresse oxidativo celular (BAUD et al., 2004; PEI et al., 2023). A capacidade de atravessar a barreira hematoencefálica, no caso da via de administração IP, pode ser limitada devido a presença de grupos *N*-metilpiridíneo e do substituinte de Br, aumentando sua polaridade e reduzindo sua

permeabilidade através de membranas lipídicas. Além disso, a depuração mais rápida no líquido cefalorraquidiano pode influenciar o tempo de permanência da MnP ao tecido alvo, impactando a eficácia da via IP. Estudos em modelos de dor crônica demonstraram que GPx é regulada positivamente, em condições neuropáticas, melhorando a recuperação neuronal para o restabelecimento do limiar nociceptivo a valores basais (DRUMMOND et al., 2024; HAZZAA et al., 2021).

As MnP I e II também modularam a expressão de genes de resposta ao estresse do retículo endoplasmático (RE). No contexto da dor neuropática, o estresse do RE e níveis elevados de ROS desempenham um papel relevante no desenvolvimento e manutenção da cronicidade da dor. Esse estresse é desencadeado por desequilíbrios dos sistemas redox, hipóxia, alterações na regulação de cálcio, bem como aumento do tráfego de proteínas mal dobradas no interior do RE (GONG et al., 2017; MALHOTRA; KAUFMAN, 2007). Em tais condições, respostas protetoras são iniciadas (UPR), visando restaurar a função do RE e a homeostase neuronal; quando estas circunstâncias se tornam crônicas, a célula é levada a sinalização celular alterada, neuroinflamação, neurotoxicidade, disfunção celular e morte (NIELLA et al., 2024; SOKKA et al., 2007).

Neste estudo, a análise da expressão gênica de *Grp78*, *Atf6* e *Nrf2* mostrou uma redução significativa em animais tratados com MnP I e MnP II em comparação com aqueles com lesão sem tratamento. Esses achados estão alinhados com estudos que investigaram o impacto do estresse oxidativo em marcadores do estresse do RE na dor neuropática (BASU et al., 2022; CHEN et al., 2019; NIELLA et al., 2024), reforçando o efeito protetor das MnP contra o estresse do RE na medula espinhal de animais neuropáticos.

Neste estudo, foi avaliada a expressão gênica de *Nrf2*, observando-se uma redução em comparação com o PCG. No entanto, a expressão proteica aumentada de NRF2 relatada em outros estudos (DOS ANJOS CORDEIRO et al., 2024; NIELLA et al., 2024), paralelo à diminuição na expressão gênica, pode ser explicada pela hipótese de que, MnP atuam diretamente na atenuação de espécies reativas, reduzindo a necessidade de expressão de NRF2 (BATINIC-HABERLE; TOVMASYAN; SPASOJEVIC, 2018). Além disso, as MnP podem agir diretamente na via de sinalização de NRF2, catalisando a oxidação do Keap1; tal reação ativa o NRF2 e regula positivamente as defensas antioxidantes endógenas, o que resulta em um aumento da expressão proteica de NRF2, enquanto a expressão do gene *Nrf2* se mantém reduzida (SHARMA et al., 2023). Evidência crescente suportam a ideia de que MnP agem na

modificação estrutural e funcional de proteínas via *S*-glutationilação de resíduos de cisteína por meio de uma reação MnP/H_2O_2/GSH (BATINIC-HABERLE; TOVMASYAN; SPASOJEVIC, 2018).

Dado que níveis elevados de ROS/RNS, estresse oxidativo, estresse do RE, neuroinflamação e a disfunção mitocondrial podem induzir a ativação de vias apoptóticas, por meio de vias de sinalização celular no sistema nervoso, contribuindo com a patogênese da dor neuropática (LIAO et al., 2022; ZHANG et al., 2022a), investigamos possíveis alterações pro-apoptóticas na medula espinhal dos modelos experimentais. As análises da expressão gênica de *Chop*, um marcador inicial de apoptose induzida por estresse RE, e do gene *Caspase 9,* uma caspase iniciadora na via apoptótica intrínseca, não mostraram qualquer diferença entre os grupos experimentais. Já a *up-regulation* da *Caspase 3* induzida pela lesão constritiva do nervo isquiático, foi revertida pelas MnP I e MnP II independente da via de administração. Ainda, os níveis de expressão de *Caspase 3* nesses grupos foram similares aos observados no grupo *NAIVE*, indicando um efeito protetor das MnP contra a apoptose induzida pela lesão. Este resultado encontra-se em concordância com aqueles obtidos por estudos avaliando vias apoptóticas em modelos de dor neuropática (MO et al., 2018; TURTLE et al., 2017; WU et al., 2012; YANG et al., 2018; ZHANG et al., 2025b).

É importente destacar que ROS/RNS desempenham um papel relevante na ativação de vias pro-apoptóticas no sistema nervoso central, por meio de vários mecanismos, incluindo vias MAPK/ERK e PI3K/Akt, promovendo a ativação de vias mitocondriais, aumentando o estresse oxidativo, induzindo a ativação de proteínas inflamatórias como caspases (WANG et al., 2018a). A apoptose, um dos fatores desencadeantes da ativação da glia, pode impactar significativamente a dor neuropática, entre outros motivos, devido à perda de suporte celular, aumento da vulnerabilidade dos neurônios e intensificação da transmissão de sinais dolorosos (INQUIMBERT et al., 2018). Ademais, a apoptose de células da glia pode desencadear a liberação de citoquinas e quimosinas que ativam células da glia e neurônios circundantes, exacerbando a resposta inflamatória (OSMANLIOĞLU; NAZIROĞLU, 2025; YANG et al., 2023).

No contexto da dor neuropática, um aumento na expressão da *Caspase 3*, considerada uma caspase efetora, sem o correspondente incremento da *Caspase 9*, uma caspase iniciadora, sugere que fatores como o estresse oxidativo e neuroinflamação, podem estar ativando preferencialmente a via extrínseca da Caspase 3 , em vez da via intrínseca da Caspase 9. Esse

achado também sugere que a *up-regulation* da *Caspase 3* reflete uma resposta ao estado doloroso, e não necessariamente um marcador direto da sinalização apoptótica da Caspase 9 (ZHANG et al., 2022a). A clivagem da caspase 3 na medula espinhal é necessária para a degeneração axonal, disfunção mitocondrial, estresse oxidativo e apoptose na patogênese da dor neuropática (CHEN; WANG; SONG, 2020; ZHANG et al., 2022a). Dessa forma, a redução na expressão de *Caspase 3* após tratamento com MnP pode reduzir a apoptose neuronal na medula espinhal e regular negativamente a hiper-reatividade de nociceptores, melhorando os comportamentos associados a dor neuropática.

A análise de expressão gênica de *CX3CR1*, mostrou que animais dos grupos IT2 e IP1 apresentaram uma redução significativa na expressão desse gene em comparação ao PCG, com valores similares aos observados em animais hígidos. Além disso, animais com lesão, com ou sem tratamento e animais com exposição do nervo, sem tratamento e sem lesão, apresentaram uma diminuição estatisticamente significativa na expressão de *CX3CL1*, em contraste com o grupo *NAIVE*, que apresentou os maiores níveis de expressão. A fractalkina é liberada dos neurônios em resposta a sinais inflamatórios. Um estudo prévio demonstrou que seus níveis permaneceram estáveis, mesmo em condições neuropáticas, sugerindo que o envolvimento na neuropatia pode estar relacionado a outros mecanismos, como clivagem do seu domínio quimiotático, facilitando a atração da micróglia para o local da lesão. Assim, a fractalkina remove o sinal inibitório na micróglia, promovendo sua ativação. A maior expressão de *CX3CR1* reflete o estado ativado da micróglia em resposta à dor neuropática (MILLIGAN et al., 2004; VERGE et al., 2004). Logo, animais hígidos (*NAIVE*) apresentam valores normais de CX3CR1, mantendo a homeostase celular e a micróglia em estado de vigilância, enquanto animais lesionados apresentam níveis aumentados de CX3CR1 pela ativação da micróglia, em comparação aos animais hígidos (*NAIVE*). Esses achados sugerem que a fractalkina pode não ser regulada para cima, mas seu rol de sinalização é crítico no contexto da dor neuropática. Desta forma, MnP I e II foram eficazes na redução da expressão de *CX3CR1,* promovendo a restauração da homeostase neuronal, pela diminuição da ativação da micróglia.

O fator induzível por hipóxia HIF-1a, é uma proteína que desempenha um papel fundamental na sobrevivência dos neurônios em condições de hipóxia, induzindo a expressão de genes que protegem os neurônios contra o estresse oxidativo e a apoptose, no entanto em condições de hipóxia grave e prolongada, pode tornar-se deletério (HSIEH et al., 2012). Em

nosso estudo foi avaliada sua expressão gênica (*Hif-1a*), não sendo observada qualquer diferença entre os grupos experimentais e os respectivos controles.

O GDNF é um fator neurotrófico essencial para a sobrevivência e regeneração neuronal, no processo da dor neuropática. É capaz de contribui no alívio desta condição, por meio da supressão de citocinas pro-inflamatórias e vias de sinalização de dor (TLR2/MyD88/Nf-kB). Essa supressão é essencial para evitar a exacerbação da dor neuropática (ZHANG et al., 2024b). Neste estudo, a análise da expressão gênica revelou uma diminuição significativa nos grupos IT2, IP1 e IP2 em comparação ao grupo PGC, sendo similares aos observados no grupo *NAIVE*. O aumento observado em animais com LCI e solução salina, sugere uma tentativa intrínseca de modulação da função de neurônios sensoriais, e da liberação de citocinas pro-inflamatórias alteradas nesta condição, promovendo a recuperação neuronal e restauração da função normal (BOUCHER et al., 2000). Embora GDNF tenha um papel reconhecido na recuperação neuronal e restauração da função normal de neurônios na dor neuropática (ZHANG et al., 2024b), a normalização dos seus níveis em animais tratados com MnP, atingindo valores similares ao dos animais hígidos, indica uma possível função neuroprotetora das MnP.

A heme oxigenasse-1 (Ho-1) desempenha um papel crítico na resposta ao estresse oxidativo e na proteção celular, pela sua capacidade de degradar o grupo heme em biliverdina, se contrapondo à formação de ROS. Sua indução ocorre em resposta ao estresse oxidativo, neuroinflamação e hipóxia, sendo regulada pela via NRF2 (LEE et al., 2013). No contexto da dor neuropática, Ho-1 tem sido associada à proteção contra eventos inflamatórios exacerbados, reduzindo a liberação de TNF-α, IL-1β e IL-6, atenuando a hiperalgesia e alodinia (CHEN et al., 2019). Neste estudo, a análise da expressão gênica de *Ho-1*, um modulador tardio de resposta antioxidante e anti-inflamatória, revelou que os grupos IT2, IP1 e IP2 apresentaram níveis de expressão de *Ho-1* significativamente menores que animais com lesão LCI. Em condições fisiológicas, Ho-1 é expressa em níveis relativamente baixos, indicando que os neurônios não requerem dos efeitos protetores contra o estresse oxidativo e inflamação, como acontece em estados patológicos como dor neuropática (CHEN et al., 2019). Assim, a *down-regulation* em animais tratados com MnP sugere que o tratamento foi eficaz na redução da resposta inflamatória e do estresse oxidativo, minimizando a necessidade de indução compensatória dessa enzima. Isto sugere que, além de reduzir a hiperalgesia, as MnP podem atuar na resolução da neuroinflamação subjacente.

Os achados deste estudo sugerem que as MnP desempenham um papel fundamental como potentes moduladores do estresse oxidativo e da inflamação neurogênica, contribuindo para a redução da hiperalgesia por meio da regulação da expressão de genes antioxidantes e anti-inflamatórios. Entretanto, algumas limitações devem ser consideradas. A biodisponibilidade das MnP e sua distribuição nos tecidos ainda precisam ser melhor investigadas, assim como sua eficácia a longo prazo e possíveis efeitos adversos. A falta de correlação direta entre a expressão gênica e a expressão proteica de algumas vias sinalizadoras sugere a necessidade de estudos adicionais para elucidar os mecanismos de regulação pós-transcricional. Para pesquisas futuras, recomenda-se explorar os mecanismos detalhados das MnP em diferentes tipos de dor crônica, incluindo modelos de dor inflamatória e neuropática em diferentes espécies, além de investigações sobre sua aplicação clínica. Outra possibilidade seria o desenvolvimento de formulações que otimizem a biodisponibilidade das MnP, especialmente para administração sistêmica, garantindo maior eficácia terapêutica.

CONCLUSÃO

Foi verificada a janela terapêutica de MnP I a partir da DL50 de 0,8 mg/kg e DE50 de 0,004 mg/kg, pela via intratecal. As doses de 0,1 mg/kg pela via intraperitoneal e 0,01 mg/kg pela via intratecal de MnP I e MnP II, apresentam efeitos antinociceptivo, antioxidante, anti-inflamatório, anti-apoptótico e neuroprotetor em modelos de neuropatia induzida por constrição do nervo isquiático em ratos. As vias de administração e as características estruturais das MnP I e MnP II parecem influenciar sua eficácia. As porfirinas de manganês representam uma abordagem inovadora no tratamento da dor neuropática, destacando-se como potenciais candidatas para intervenções clínicas futuras em condições dolorosas crônicas.

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