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New modalities for treating neuropathic pain and associated emotional disorders

Xue Bai

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Report of the doctoral thesis presented by Xue Bai to qualify for the degree of doctor in Neurosciences by the Autonomous University of Barcelona

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PhD student — Xue Bai

Thesis director/tutor — Olga Pol Rigau

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List of Abbreviations

2-AG: 2-arachidoylglycerol
4-HNE: 4-hydroxynonenal
5-HT: 5-hydroxytryptamine
AC: adenylate cyclase
AEA: anandamide
AKT: protein kinase B
AMG: amygdala
ARE: antioxidant response element
ATP: adenosine triphosphate
BAX: Bcl2-associated X
BDNF: brain-derived neurotrophic factor
cAMP: cyclic adenosine monophosphate
CB1R: cannabinoid 1 receptor
CB2R: cannabinoid 2 receptor
CCI: chronic constriction of the sciatic nerve
CNS: central nervous system
CO: carbon monoxide
COX-2: cyclooxygenase-2
CPSP: chronic post-surgical pain
DADS: diallyl disulfide
DAS: diallyl sulfide
DATS: diallyl trisulfide
DOR: δ-opioid receptor
DRG: dorsal root ganglia
ERK: extracellular signal-regulated kinase
FAAH: fatty acid hydrolase
GABA: γ-aminobutyric acid
GPCR: G protein-coupled receptors
GSTM: glutathione S-transferase subunits
GYY4137: morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate
H_2S: hydrogen sulfide
HIP: hippocampus
HO-1: heme oxygenase-1
IASP: International Association for the Study of Pain
Iba1: ionized calcium-binding adaptor molecule-1
IKK: IκB kinase
IL-10: interleukin-10

IL-1β: interleukin-1β
IL-6: interleukin-6
JAK: Janus kinase
JNK: c-Jun N-terminal kinase
KATP: ATP-sensitive K+
KOR: κ-opioid receptor
LC: locus coeruleus
MAGL: monoacylglycerol lipase
MAPK: mitogen-activated protein kinases
MOR: μ-opioid receptor
MS: medial septum
NA: noradrenaline
Na_2S: sodium sulfide
NADPH: nicotinamide adenine dinucleotide phosphate
NaHS: sodium hydrosulfide
NF-κB: nuclear factor-κB
NGF: nerve growth factor
NMDA: N-methyl-D-aspartate
NO: nitric oxide
NOS: nitric oxide synthase
NOX: NADPH oxidase
NQO1: quinone oxidoreductase 1
Nrf2: nuclear factor erythroid 2-related factor
NSAIDs: non-steroidal anti-inflammatory drugs
P2X4R: P2X purinoceptor 4 receptor
PAG: periaqueductal gray matter
PFC: prefrontal cortex
PI3K: phosphoinositide 3-kinase
PKA: protein kinase A
PNS: peripheral nervous system
ROS: reactive oxygen species
RVM: rostral ventromedial medulla
SC: spinal cord
SCDH: spinal cord dorsal horn
SOD: superoxide dismutase
TNF-α: tumor necrosis factor-α
TRKB: TrkB tyrosine kinase
vHIP: ventral hippocampus

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1. Abstract

Neuropathic pain is an unbearable chronic pain that is often accompanied by emotional and cognitive disorders such as the anxiety, depression, memory deficits, and also insomnia. Due to its complexity, its clinical treatment is still unsatisfactory. The current therapies not only have multiple side effects, but also fail to inhibit the anxiety and depression associated with neuropathic pain, being necessary, the development of new therapeutic strategies. Therefore, in male mice with neuropathic pain induced by chronic constriction of the sciatic nerve, we investigated the effects of diallyl disulfide (DADS) and morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex (GYY4137), two slow-releasing hydrogen sulfide (H_2S) donors, on pain and its accompanying emotional disorders. We also examined the possible improvements of the analgesic and/or anxiolytic and antidepressant effects of opioids and cannabinoids induced by H_2S donors. Our results showed that: 1) the administration of DADS and GYY4137 inhibited the allodynia and hyperalgesia, as well as the anxiety- and depressive-like behaviors accompanying neuropathic pain, 2) GYY4137 is more potent than DADS inhibiting pain, 3) the effects of DADS and GYY4137 are mainly mediated by inhibiting the oxidative, nociceptive, inflammatory and apoptotic responses caused by nerve injury, and by regulating the expression of the neurotrophic factor BDNF in the central and/or peripheral nervous system, 4) the participation of the Kv7 potassium channels and heme oxygenase-1 enzyme in the analgesic actions of DADS and GYY4137, 5) the improvement of the analgesic effects of specific agonists, morphine (μ-opioid receptor; MOR), UFP-512 (δ-opioid receptor; DOR) and JWH-133 (cannabinoid 2 receptor; CB2R) induced by slow-releasing H_2S donors, 6) the anxiolytic and antidepressant effects of JWH-133 combined with GYY4137, 7) these last actions might be explained by the up-regulation of MOR and DOR, the maintenance of the high levels of CB2R and the activation of the endogenous opioid and cannabinoid systems induced by DADS and GYY4137 during neuropathic pain. In conclusion, this study reveals: i) the potential use of slow-releasing H_2S donors, principally GYY4137, alone and combined with opioids, specially DOR, as a new strategy for treating neuropathic pain, and ii) the combined administration of GYY4137 with a CB2R agonist, as a potential effective therapy for neuropathic pain caused by peripheral nerve-injury and the accompanying emotional disorders, with few side effects.

2. Introduction

2.1 Pain

Pain is an unpleasant sensation that indicates real or potential tissue damage, being as an alert and protective mechanism of the organism. Depending on the duration of the pain, it can be classified as either acute or chronic. Acute pain usually late less than one month and tends to disappear with the resolution of the cause. Although, it can be converted into chronic pain if it is not properly managed (Sinatra, 2010). According to the latest definition from the International Association for the Study of Pain (IASP) and World Health Organization, chronic pain is defined as pain that persists or recurs for more than three months (Treede et al., 2019). Chronic pain is a health issue that should not be overlooked. It affects approximately 30% of the global population, around 20.4% in the United States, and approximately 13-50% in the United Kingdom (Cohen et al., 2021; Mills et al., 2019). The IASP classifies chronic pain into the following seven categories in the international classification of diseases: chronic primary pain; chronic cancer related pain; chronic postsurgical or posttraumatic pain; chronic secondary musculoskeletal pain; chronic secondary visceral pain; chronic neuropathic pain; chronic secondary headache or orofacial pain (Treede et al., 2019; Cohen et al., 2021).

Neuropathic pain is caused by a lesion or disease of the somatosensory nervous system. This type of pain is typically characterized by recurrent or persistent episodes and is therefore considered as chronic pain. The IASP classifies neuropathic pain into central or peripheral based on the location of the nerve damage and/or of the disease (Scholz et al., 2019). Central neuropathic pain includes pain provoked by brain or spinal cord injury, the post-stroke pain, and pain caused by multiple sclerosis. While peripheral neuropathic pain encompasses trigeminal neuralgia, pain caused by peripheral nerve lesions, painful polyneuropathy, postherpetic neuralgia, and painful radiculopathy. Cohen et al. (2021) have summarized the clinically common causes of neuropathic pain (Figure 1).

The prevalence of neuropathic pain in the general population can range from 6.9% to 10% (Scholz et al., 2019) while in Europe, it is estimated to be between 7% and 8% (Liedgens et al., 2016). The hallmark characteristics of neuropathic pain are the following: I) spontaneous pain such as, cutting, burning, stabbing, or tearing pain; II) hyperalgesia, as a stimulus-evoked decreased pain threshold or increased pain perception in response to a mechanical or thermal stimuli; III) allodynia, as the nociceptive response caused by non-noxious stimuli, such as touch or pressure and IV) dysesthesias or sensory deficits, among others (Bouhassira, 2019).

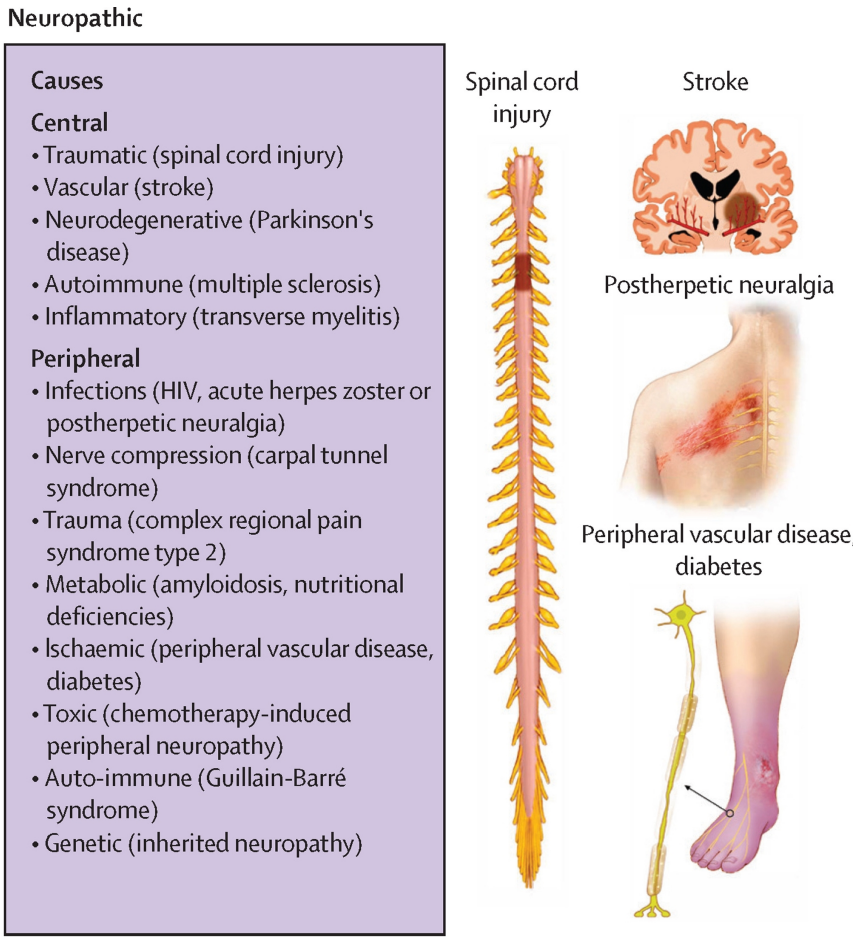

Figure 1*. Causes of central and peripheral neuropathic pain. (Cohen et al., 2021).*

Patients' mood, physical activity, sleep, and social relationships are severely disturbed by neuropathic pain, and some patients may even experience a loss of work capacity as a result. Furthermore, neuropathic pain can lead to decreased attention, loss of appetite, and even emotional disorders such as insomnia, anxiety, and depression (Yalcin et al., 2014). Neuropathic pain significantly impacts the patient's quality of life and has become an urgent health issue, being not only a burden on the patients themselves, but also on society in terms of economic (McDermott et al., 2006; Liedgens et al., 2016).

2.1.1 Ascending pathway

Primary afferent neurons (or known as first order neurons) have their cell bodies located in the dorsal root ganglia (DRG) and trigeminal ganglia, with their incoming fibers widely distributed in tissues such as the skin, muscles, and joints. After receiving harmful stimulation, the peripheral nociceptors produce signals that are then integrated within the DRG and subsequently projected to the spinal cord dorsal horn (SCDH). Substance P is released in the SCDH and transmits signals

from first order neurons to second order neurons. Subsequently, the second order neurons directly project the impulses through the spinothalamic tract to the thalamus. At the end, the third-order neurons convey the impulses to their ultimate destination, the somatosensory cortex (Schaible, 2015; Boadas-Vaello et al., 2016; Yam et al., 2018; Larson et al., 2019).

2.1.2 Descending pathway

The periaqueductal gray matter (PAG) plays a key role in the control of pain, is not only a critical structure in the central descending pain modulation system, but also is a primary target for analgesic drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs) and opioid medications (Vanegas et al., 2010). The PAG receives glutamatergic neuronal projections from the amygdala (AMG), hypothalamus, and cortex, mainly the prefrontal cortex (PFC), and transmits neural impulses to the rostral ventromedial medulla (RVM) and locus coeruleus (LC). Furthermore, the RVM and LC activate the descending noradrenergic pathway and releases key neurotransmitters in the descending inhibitory pathway, 5-hydroxytryptamine (5-HT) and noradrenaline (NA), to the SCDH (Calvino and Grilo, 2006). These neurotransmitters inhibit the release of substance P and activate the interneurons to release enkephalins, an endogenous opioid peptide, which can also inhibit the release of substance P and suppressing pain (Vanegas and Schaible, 2004; Pertovaara and Almeida, 2006; Morgan et al., 2008) (Figure 2).

It is worth mentioning that in the transmission and regulation of pain, the PFC is primarily responsible for pain perception (Ong et al., 2019), and the AMG has the function of conveyance of descending inhibitory signals and modulation of pain (Li et al., 2017; Raver et al., 2020). These two regions are closely anatomically connected and the basolateral AMG-PFC-PAG-spinal cord (SC) pathway is essential for pain regulation (Huang et al., 2019). Likewise, both AMG and PFC are also closely related to the negative emotions, such as fear and anxiety regulation (Hiser and Koenigs, 2018; Neugebauer et al., 2020), and can project neurons to other brain regions capable of regulating pain and emotions, for example the medial septum (MS) (Takeuchi et al., 2021).

Different investigations have proved that the activation of the γ-aminobutyric acid (GABA) system in the MS suppresses the nociceptive sensation in animals (Lee et al., 2011; Ariffin et al., 2018). Moreover, the MS projects to the hippocampus (HIP), in which the ventral part of the hippocampus (vHIP) is highly involved in the regulation of the anxiety (Fanselow and Dong, 2010; Hu et al., 2022).

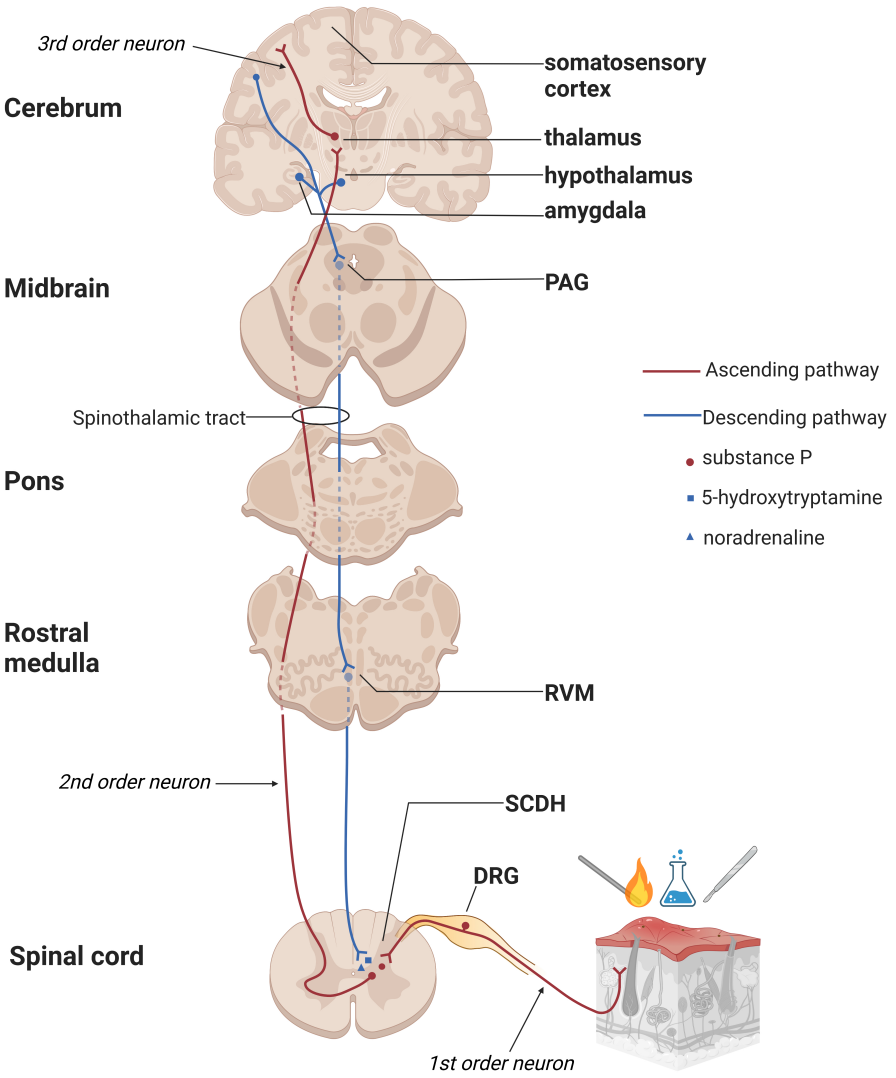

Figure 2. *The ascending and descending pain pathways.*

2.2 Nerve injury-induced neuropathic pain

After nerve injury, the expression of ion channels in the damaged neural fibers undergoes changes, such as an increase in sodium channels, resulting in a decrease in the stimulation threshold of the damaged neurons. This causes excessive excitability and ectopic discharges, resulting in stimulus-independent pain. Nerve injury also leads to a reduction of the inhibitory neurotransmitters in the SCDH, such as GABA and glycine, as well as of opioid receptors which block the ascending of nociceptive signals, resulting in the occurrence of spontaneous pain (Woolf and Mannion, 1999; Jensen and Finnerup, 2014).

Moreover, axons may become damaged and unmyelinated, leading to a decrease in the insulation of nerve fibers. When a particular nerve fiber is activated, depolarizing potentials can trigger discharge in surrounding resting fibers, resulting in the activation of high threshold nociceptive C and Aδ fibers by the low threshold non-nociceptive Aβ fibers. These activated fibers lead to the release of substance P, calcitonin gene-related peptide, and adenosine triphosphate (ATP) in the SCDH, as well as activation and phosphorylation of N-methyl-D-aspartate (NMDA) receptors, that contribute to the hyperexcitability of the peripheral (PNS) and central nervous system (CNS) (Basbaum et al., 2009; Jensen and Finnerup, 2014). On the other hand, the cells activated by nerve injury, such as microglia, astrocytes, Schwann cells and neutrophils, provoke the phosphorylation of mitogen-activated protein kinases (MAPK), the release of the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), several pro-inflammatory factors such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), among others (Trang et al., 2012; Ji et al., 2013; Zhou et al., 2019; Kuffler, 2020) that act on the undamaged or damaged nerve fibers and neurons increasing their excitability (Ji et al., 2018). All these above-mentioned factors are implied in the development of central sensitization, which is clinically manifested as spontaneous pain, hyperalgesia, allodynia, and other neuropathic pain characteristics (Woolf and Mannion, 1999; Jensen and Finnerup, 2014).

2.3. *Inflammatory responses*

Microglia are extensively distributed in the CNS (brain and SC) and play a crucial role in maintaining the homeostasis in these areas. Numerous studies performed in animals with neuropathic pain caused by peripheral or central nerve injury and associated with diabetes, cancer, and/or chemotherapy demonstrated that spinal microglia are strongly involved in the occurrence of neuropathic pain (Inoue and Tsuda, 2018; Pottorf et al., 2022). The activation of microglial cells-induced by nerve injury has harmful effects on the neuroprotective system due to the release of numerous pro-inflammatory mediators such as cytokines and inducible nitric oxide synthase (NOS), the inducement of reactive oxygen species (ROS), and the activation of the nuclear factor-κB (NF-κB), leading to neuroinflammation even apoptosis, maintaining chronic pain and the development of neurodegenerative diseases (Ji et al., 2013; Shabab et al., 2017; He et al., 2022). Nonetheless, the appropriately activated microglia can have beneficial effects in repairing damaged tissue through the phagocytosis of cellular debris and the secretion of anti-inflammatory cytokines such as IL-10 (Hu et al., 2015; Tu et al., 2021). CD11/bc and the ionized calcium-binding adaptor molecule-1 (Iba1) are the main markers of microglia. The expression of CD11/bc increases with the activation of microglia and the inhibition of microglial activation significantly contributes to the reduction of neuropathic and inflammatory pain (Miranpuri et al., 2021) (Figure 3).

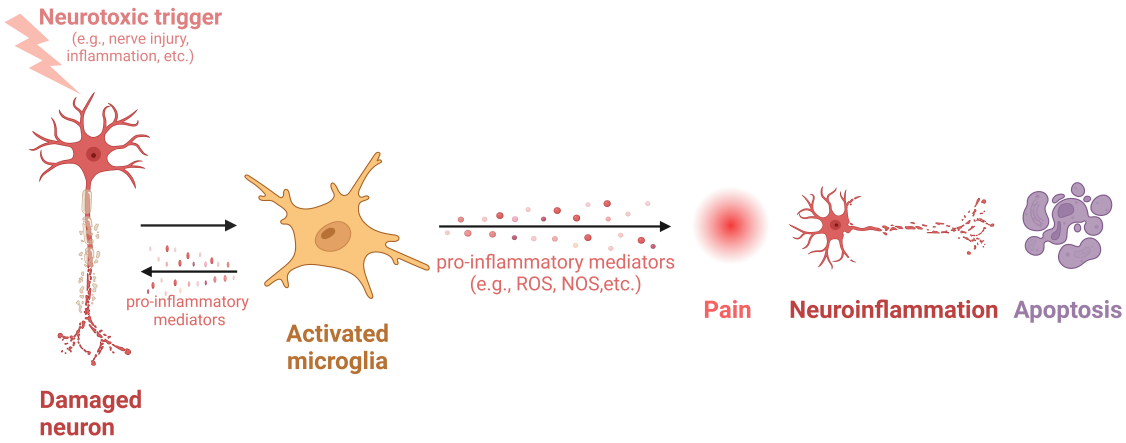

Figure 3. *Summary of the progress of inflammatory, nociceptive, and apoptotic responses induced by nerve injury.*

2.4. MAPK

The MAPK is a family of serine/threonine protein kinases that includes the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and P38. MAPK are activated by nerve injury-provoked activation of glial cells and Aδ, C nociceptors (Basbaum et al., 2009; Ji et al., 2009). The regulatory role of these proteins in the progression of neuropathic pain has been extensively studied and confirmed. Indeed, following nerve injury, the levels of phosphorylated forms of ERK (p-ERK), P38 (p-P38) and JNK (p-JNK) are significantly elevated in the SC, but their distribution in glial cells varies. That is, p-JNK is predominantly expressed in astrocytes, while p-P38 in microglia. The distribution of p-ERK changes over the time after nerve injury, thus in the early stages is mainly activated in the microglia, transitioning to the astrocytes during the later stages (Zhuang et al., 2005; 2006). The activation of MAPK leads to increase the synthesis of several pro-inflammatory mediators such as cyclooxygenase-2 (COX-2), NOS2, TNF-α and IL-1β, contributing to nerve damage (Ji et al., 2009; Zhang et al., 2023). In addition, the expression of p-ERK is also an important factor leading to central sensitization (Chen et al., 2018). An increasing number of studies have confirmed that the inhibition of MAPK phosphorylation in the spinal cord and DRG effectively alleviates neuropathic pain in rodents (Zhuang et al., 2006; Ramesh, 2014; Huang et al., 2022). Furthermore, Riego et al. (2018) have found that treatment with antioxidants, a heme oxygenase-1 (HO-1) inducer, and a carbon monoxide (CO) donor, alleviated neuropathic pain occasioned by sciatic nerve injury in mice by attenuating MAPK phosphorylation in certain brain regions: AMG, PFC, and/or HIP. In addition, due to the longer duration of the expression and wider distribution of p-ERK in the SC and DRG than those of p-P38 and p-JNK, it seems that p-ERK inhibition is the most effective for treating persistent neuropathic pain (Ma and Quirion, 2005).

2.5. NF-kB/p-IKBα

During the cellular resting state, the NF-κB combines with an inhibitory protein IκB (mainly IκBα, IκBβ, etc.) forming a complex structure, remains in an inactive state and primarily localizes in the cytoplasm. The activation of IκB kinase (IKK) leads to the phosphorylation (i.e., p-IKBα and p-IKBβ) and degradation of IκB, resulting in the dissociation of NF-κB from IκB and its translocation into the nucleus (O'Dea and Hoffmann, 2009). At this stage, NF-κB is activated and induces the expression of pro-inflammatory mediators (IL-1β, IL-6, TNF-α, NOS2, etc.) and oxidative stress, thereby impairing mitochondrial function and aggravating neuronal damage (Liu et al., 2017; Chu et al., 2020; Zhang et al., 2023).

Previous studies have shown that after SC lesion and sciatic nerve injury, NF-κB is activated and involved in microglia-induced inflammation (Liang et al., 2022; Shi et al., 2023) and its inhibition contributes to pain relief. Studies have demonstrated that blocking the NF-κB signaling pathway by administering NF-κB inhibitors or IκBα repressors inhibited the allodynia caused by sciatic nerve inflammation, as well as the allodynia and hyperalgesia provoked by sciatic nerve injury (Ledeboer et al., 2005; Meunier et al., 2007). In addition, the therapy with arctigenin, a bioactive component of Fructus arctii, also suppresses depressive-like behaviors in mice through reducing microglial activation, IκBα and NF-κB phosphorylation in the PFC (Xu et al., 2020). Furthermore, several drugs possessing anti-inflammatory and antioxidant properties (such as astaxanthin or oltipraz) attenuated neuropathic pain caused by nerve injury in mice by inhibiting NF-κB activation in glial cells or the IκBα phosphorylation in the spinal cord (Díaz et al., 2019; Zhao et al., 2021).

2.6. PI3K/AKT

In addition to the MAKP family, microglial activation also activates the intracellular phosphoinositide 3-kinase/serine-threonine kinase/protein kinase B (PI3K/AKT) signaling pathway through G protein-coupled receptors (GPCR) and the phosphorylated Janus kinase (JAK) (Guo et al., 2022). AKT is a crucial downstream target of PI3K, which is phosphorylated by PI3K activated, resulting in p-AKT, that induces the expression of IKKa, the upstream activator of NF-κB, as mentioned before (Shabab et al., 2017). Previous research indicates that the activation of the MAPK and PI3K/AKT pathways at the spinal cord level is involved in the hyperalgesia induced by inflammatory and neuropathic pain, and that there is a significant increase of the PI3K and p-AKT levels in the spinal cord of animals with nerve injury (Carvalho et al., 2011; Liu et al., 2018). Furthermore, these signaling pathways are also implicated in bone cancer pain, opioid tolerance, etc. (Chen et al., 2017). In concordance, the PI3K/AKT inhibitors have been confirmed to have analgesic properties in preclinical pain models. Thus, the administration of PI3K inhibitors

reversed the over expression of p-AKT In the spinal cord and alleviated the mechanical allodynia provoked by sciatic nerve injury in rats (Liu et al., 2018) and further reduced the p-AKT levels in the DRG and SC as well as the thermal hyperalgesia and mechanical allodynia observed in mice with postoperative pain (Xu et al., 2014). In addition, the AKT inhibitor IV also exhibited anti-allodynic effects in rats with inflammatory pain (Choi et al., 2010).

2.7. BDNF

The BDNF is a neurotrophic factor with three different forms: pre-pro-BDNF, pro-BDNF and mature BDNF (mBDNF). The initial form of BDNF, the pre-pro-BDNF splits quickly into pro-BDNF, which subsequently decomposes into mBDNF. Pro-BDNF and mBDNF have different functions, then whereas the pro-BDNF promotes apoptosis and inhibits synapses in the HIP, the mBDNF induces neuron survival and synaptic plasticity (Cappoli et al., 2020). After peripheral nerve injury, extracellular ATP activates P2X purinoceptor 4 receptor (P2X4R) on spinal microglia, thereby promoting calcium influx into the cells. These Ca^{2+} activate the P38 MAPK pathway, which mediates the release of BDNF from microglia. Ultimately, this leads to an elevation of BDNF levels in the spinal cord (Trang et al., 2012). The mBDNF by binding to the TrkB tyrosine kinase (TRKB) receptors on postsynaptic neurons produces a series of actions closely related to the central sensitization of neuropathic pain such as the activation of NMDA receptors in the SCDH further increasing intracellular Ca^{2+} influx and the disinhibition of the GABAergic system leading to the hyperexcitability of neurons (Coull et al., 2005; Inoue and Tsuda, 2018). In addition, the activation of PI3K and ERK induced by BDNF is also involved in the progression of neuropathic pain (Ji et al., 2009; Cappoli et al., 2020). All the above-mentioned changes/alterations contribute to the occurrence of central sensitization and the maintenance of neuropathic pain. On the other hand, a positive feedback loop between BDNF and microglia was identified, which means that BDNF can also activate microglia (Zhang et al., 2014; Khan et al., 2015).

Changes in the expression of mBDNF are also associated with the depression and many neurodegenerative diseases (Zhang et al., 2016). In animals with nerve injury, while the expression of BDNF increased in the sciatic nerves, SC and DRG (Obata and Noguchi, 2006; Lee et al., 2018; Tan et al., 2020), it was decreased in the PFC and HIP (Fang et al., 2020). In this line, Ge et al. (2019) demonstrated that dihydromyricetin alleviated allodynia and hyperalgesia and suppressed depressive-like behaviors by normalizing the elevated or decreased BDNF levels in the DRG, SC or HIP of rats with diabetic neuropathy. In accordance, the levels of BDNF in the HIP of animals with chronic post-surgical pain (CPSP) and depressive-like behaviors were lower than in CPSP rats without depressive-like behaviors, and ketamine relieved the depressive-like behaviors in these rats through the re-establishment of the BDNF levels in the HIP (Yang et al. (2020). These studies suggest the important role played by BDNF in the depressive-like behaviors

associated with pain in the HIP. However, given the complex role of BDNF in the different regions of the nervous system, further research is necessary to better understand its functions.

2.8. Oxidative stress and apoptosis

Oxidative stress is also a crucial component in the development of neuropathic pain (Naik et al., 2006; Jia et al., 2012). Inflammation resulting from neural injury can lead to oxidative stress, and a key characteristic of oxidative stress is the excessive production of ROS by over activated microglia and astroglia (Sheng et al., 2013). It is well known that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and mitochondria are the main sources of ROS . While moderate ROS levels are beneficial to the organism as they can help kill pathogens, excessively high levels can attack and damage mitochondria, promoting apoptosis (Brieger et al., 2012). Neuronal death mediated by the Bcl-2 protein promotes neuropathic pain and neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, Parkinson's disease (Wu et al., 2019). Moreover, the Bcl2-associated X (BAX), a pro-apoptotic protein belonging to the Bcl-2 family, is a marker of apoptosis and its expression is increased in the brains of animals with neuropathic pain (Mokhtari et al., 2023).

The role of ROS promoting pain is also closely related to other signaling pathways, such as the activation of NMDA receptors that induces mitochondrial dysfunction by increasing Ca^{2+} influx, leading to the release of large amounts of ROS (Wu et al., 2019). Excessive ROS can also exert a disinhibitory effect by inhibiting the GABAergic system and activating the MAPK pathway. On the other hand, NOX can also induce the activation of NF-κB, which together with activated MAKP trigger the release of inflammatory mediators, favoring the development of neuroinflammation, apoptosis, and pain (Teixeira-Santos et al., 2020). In addition, high ROS levels also lead to lipid peroxidation, which increases the synthesis of 4-hydroxynonenal (4-HNE), which contributes to the development of peripheral sensitization by activating the transient A1 receptor ion channel potentials, and can also incite apoptosis (Trevisani et al., 2007; Dalleau et al., 2013; Ji et al., 2014).

Another important characteristic of oxidative stress is the reduction or the relative insufficiency of endogenous antioxidants. The nuclear factor erythroid 2-related factor (Nrf2) plays a pivotal role in the regulation of endogenous antioxidant enzymes. Under normal physiological state, Nrf2 is sequestered within the cytoplasm. During oxidative stress, it is liberated and translocated into the nucleus, where it binds to the antioxidant response element (ARE), forming the Nrf2/ARE pathway which modulates the expression of numerous antioxidant enzyme genes, for instance HO-1, quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD) and glutathione S-transferase subunits (GSTM) (Fujita et al., 2012; Marengo et al., 2016). The antioxidant responses mediated by the activation of the Nrf2 pathway are crucial to protecting

the organism from inflammation and propitiating the neuronal survival (Zhou et al., 2017; Wang et al., 2019; Upadhayay and Mehan, 2021). Numerous studies have shown that treatment with antioxidants or drugs with antioxidant properties not only reduces ROS levels, but also decreases inflammatory reactions and suppresses neuropathic pain associated with diabetes or chemotherapy, and that caused by nerve damage (Jia et al., 2012; Abbaszadeh et al., 2018; Lu et al., 2018; Li et al., 2019; Shim et al., 2019; Cabarga et al., 2020; Düzova et al., 2021). These effects may also be achieved through the inhibition of NF-κB factor (Kim et al., 2010; Park and Kim, 2020). In summary, antioxidants have effective neuroprotective and therapeutic actions during neuropathic pain (Figure 4).

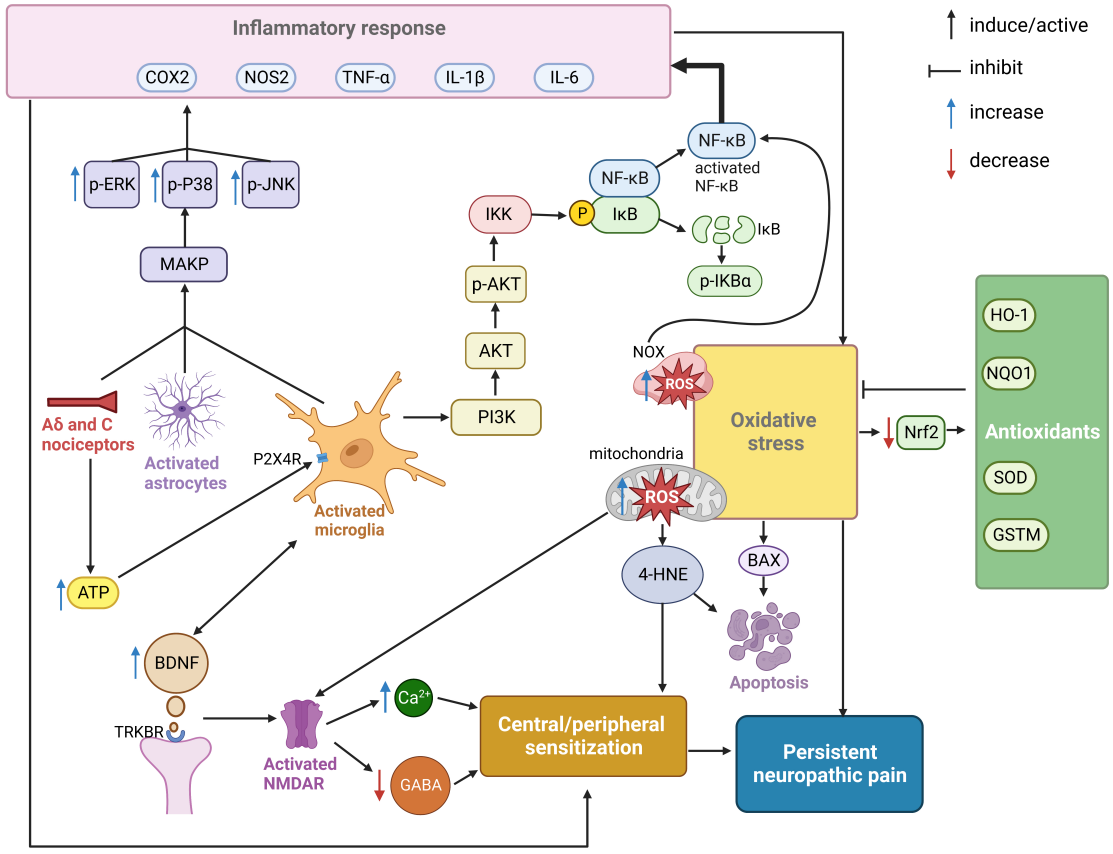

***Figure 4**. Schematic representation of the main mechanisms involved in the development neuropathic pain.*

2.9. Pharmacological treatments for neuropathic pain

At present, the clinical treatment of neuropathic pain is still mainly based on drug therapy. The current consensus first-line drugs are gabapentinoids, tricyclic antidepressants, serotonin–norepinephrine reuptake inhibitors. The second-line therapies are opioids and topical treatment. The third line of treatments includes strong opioids (such as morphine) and neurotoxin. Despite

the wide range of medication options, most of them carry important side effects that cannot be ignored (Cavalli et al., 2019; Marcianò et al., 2023)

Gabapentinoids are a class of anticonvulsant drugs that relieve neuropathic pain by modulating Ca^{2+} channels. Their side effects include dose-dependent lethargy and vertigo, and the dosage should be reduced in patients with renal insufficiency. Furthermore, they inhibited neuropathic pain and improved the accompanying anxiety-like behaviors but do not avoid the associated depressive-like behaviors (La Porta et al., 2016; Alles and Smith, 2018). Other anticonvulsant such as carbamazepine and oxcarbazepine are efficient to treat the trigeminal neuralgia, but they use also elicit remarkable side effects including sedation, vertigo, abnormal liver enzymes, and hyponatremia (Gilron et al., 2015). Tricyclic antidepressants, belonging to the antidepressant medications, produce analgesic and antidepressant effects by blocking the histamine, norepinephrine, acetylcholine, and sodium channels (non-selective blockade), thereby giving rise to numerous potentially dangerous side effects such as anticholinergic effects, orthostatic hypotension, cardiac arrhythmias, etc. Hence, careful attention should be paid to their cardiotoxicity when administered them because they are strictly contraindicated in patients with cardiac arrhythmias (Gilron et al., 2015; Bates et al., 2019). Additionally, serotonin–norepinephrine reuptake inhibitors antidepressants are also frequently associated with nausea, dry mouth, sedation, and anxiety as a secondary effects (Gilron et al., 2015).

Opioids, especially μ-opioid receptor (MOR) agonists, have shown low efficacy on severe neuropathic pain and many undesirable effects such as vomiting, constipation, respiratory depression, and tolerance (Smith, 2012; Stein, 2018). Moreover, and due to the associated addictive behaviors to its misuse, opioids are only recommended as a second or third-line treatment option (Cavalli et al., 2019)

As mentioned earlier, the mechanisms of neuropathic pain are quite complex. When dealing with severe neuropathic pain, targeting a single mechanism alone may not achieve the desired therapeutic outcome. In the advancements of pain therapy, apart from improving the chemical structure of existing drugs and exploring new compounds targeting known or newly discovered mechanisms. The discovering of the analgesic effects of drugs used for other diseases, or the combination of existing drugs to reduce their side effects, are also worth exploring and focusing on (Burgess and Williams, 2010). However, and considering the positive feedback between chronic pain and associated affective deficits, and that mood disorders can also negatively affect the sensation of pain (Doan et al., 2015; Sheng et al., 2017), it is crucial to find a treatment modality that can alleviate not only neuropathic pain but also the accompanying emotional disorders such as anxiety and depression.

2.10. Hydrogen sulfide

Hydrogen sulfide (H_2S) is a colorless, water-soluble gas with a distinct smell of rotten eggs. It is one of the three major gasotransmitters, along with CO and nitric oxide (NO). It was regarded as a toxic gas, as high concentrations of H_2S can be lethal. However, in the past decade, beneficial effects of this gas have been increasingly discovered (Powell et al., 2018).

Endogenous H_2S is produced through enzymatic or non-enzymatic pathways, although the enzymatic pathways play a major role in its synthesis. Therefore, several enzymes are involved in the synthesis of endogenous H_2S, including cystathionine-β-synthase, cystathionine-γ-lyase, 3-mercaptopyruvate sulfurtransferase, cysteine aminotransferase and D-amino acid oxidase. These enzymes synthesize H_2S in various organs, including the liver and kidneys, in the cardiovascular and nervous system, and in the gastrointestinal tract, and their dominant role in the H_2S synthesis may vary among different organs (Panthi et al., 2016). The non-enzymatic way to generate H_2S depends on food sources, gut microbiota, thiosulfate, vitamin B6, and Fe^{3+}. The metabolism of H_2S involves the mitochondria, and it is excreted through the kidneys after being oxidized to sulfate (Panthi et al., 2016; Cirino et al., 2023). The H_2S is widely distributed in the body and is involved in the regulation of different functions in the brain, heart, lungs, liver, kidneys, gastrointestinal tract, and reproductive organs, among others. It also can modulate neurologic, immune, and vascular functions (Dilek et al., 2020; Cirino et al., 2023). In addition, and depending on its concentration, H_2S can promote cell survival, differentiation, or apoptosis (Szabo and Papapetropoulos, 2017).

The H_2S does not act isolated to achieve its physiological regulatory functions. Several studies have proven that H_2S is capable of: 1) inhibiting inflammatory proteins, for example TNF-α and IL-1β, as well as the NF-κB pathway to achieve its anti-inflammatory properties, 2) inhibiting oxidative stress through direct reaction with oxidants or activating the Nrf2 transcription factor and the subsequent synthesis of antioxidant enzymes, 3) inducing the synthesis of CO via Nrf2-induced HO-1, and activating ATP-sensitive K^+ (K_{ATP}) channels of vascular smooth muscle exerting vasodilation (Szabo and Papapetropoulos, 2017). In addition, H_2S can also regulate the homeostasis of intestinal flora (Głowacka et al., 2020).

Lately, the neuroprotective effects of H_2S have been further revealed in neurodegenerative diseases such as in Alzheimer's and Parkinson's due to its anti-inflammatory and antioxidant properties (Panthi et al., 2016). Furthermore, in distinct animal pain models provoked by knee osteoarthritis, paw inflammation or opioid withdrawal, the exogenous administration of low-dose, slow-releasing H_2S donors have been shown to alleviate hyperalgesia and/or allodynia in animals (Di Cesare Mannelli et al., 2017; Batallé et al, 2020; Porta et al., 2021). These analgesic effects are not only produced through its aforementioned anti-inflammatory and antioxidant

capacities or by activating the K_{ATP} channels, but they are also related to the capacity of this gas to open the Kv7 potassium channels thus inhibiting the nociceptive responses (Di Cesare Mannelli et al., 2017; Guo et al., 2020). Furthermore, Cabarga et al. (2020) demonstrated that the administration of isocyanates (H_2S slow-releasing donors) alleviated pain and the accompanying depressive- but not the anxiety-like behaviors in mice with sciatic nerve injury-induced neuropathic pain, thus the search of a treatment that can inhibits not only the depressive but also the anxiety-like behaviors associated with neuropathic is indispensable.

In contrast to these results, the administration of fast-releasing H_2S donors such as sodium hydrosulfide (NaHS) and sodium sulfide (Na_2S), that liberate a large amount of H_2S in a short time, does not inhibit pain or even can increase it (Tsubota-Matsunami et al., 2012; Velasco-Xolalpa et al., 2013; Zheng et al., 2018). These effects could be related to the difficulty of these compounds to control the concentration of H_2S at steady-state, and to the toxicity, inflammation, and cell damage caused by this gas when it exceeds physiological levels. Thus, it is worth focusing on and investigating the effects of slow H_2S-releasing donors (Szabo and Papapetropoulos, 2017; Zheng et al., 2018). In this study, we evaluated the effects of two slow-releasing H_2S donors (natural and synthetic) on the allodynia and hyperalgesia provoked by sciatic nerve injury as well as on the anxiodepressive-like behaviors associated with neuropathic pain.

2.10.1 DADS

Garlic is a natural source of H_2S. The main components in garlic responsible for H_2S production are: diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). DADS exhibits a slower rate of H_2S release than DATS and generates a higher quantity of H_2S than DAS, lying at an intermediate level among the three. DADS has liposoluble and hydrosoluble properties and acts as a slow-releasing H_2S donor (Szabo and Papapetropoulos, 2017).

Previous works demonstrated the beneficial effects of DADS such as: 1) protects the gut by modulating the microbiota through its anti-Helicobacter pylori properties (Głowacka et al., 2020; Hu et al., 2022); 2) exhibits antitumor activity by inhibiting the growth of tumor cells (Yin et al., 2014); 3) presents anti-inflammatory actions by inhibiting NF-κB and reducing the production of inflammatory mediators, it also induces a shift in microglial cells towards their anti-inflammatory phenotype (Liu et al., 2018; Xu et al., 2020; Zhang et al., 2020) and 4) demonstrates antioxidant properties by activating the Nrf2 signaling pathway and reducing ROS levels (Kim et al., 2014; Liu et al., 2018). Furthermore, it is noteworthy that DADS suppressed depressive-like behavior induced by unpredictable chronic mild stress in rats by increasing the expression of 5-HT and BDNF in the frontal cortex (Huang et al., 2019), suggesting the possible utility of DADS as an antidepressant. Nevertheless, the effects of DADS in treating neuropathic pain and the accompanying anxiety- and depressive-like behaviors, have not yet been investigated.

2.10.2 GYY4137

In 2008, Li et al. reported a novel type of H_2S slow-releasing donor, morpholin-4-ium 4 methoxyphenyl(morpholino) phosphinodithioate (GYY4137). It is an artificial synthesized H_2S donor and soluble in water, that can slowly release this gas, providing a more sustained supply of H_2S at a steady rate. Additionally, unlike NaHS, the administration of GYY4137 at similar doses, does not induce cell apoptosis, thus exhibiting its beneficial physiological effects (Li et al., 2008). Extensive investigations have shown that GYY4137 exhibits similar effects to other slow-releasing H_2S donors, including the vasodilation, the anti-inflammatory actions (inhibiting the NF-κB and inflammatory mediators), antioxidant effects (up-regulating the Nrf2 and antioxidant enzymes levels), and protective actions (preventing cell apoptosis) (Zhao et al., 2015; Dilek et al., 2020).

Moreover, GYY4137 exhibits additional positive abilities, which contain the antiviral (Bazhanov et al., 2017) and anti-tumoral effects (Lee et al., 2011); the prevention of osteoporosis (Hao et al., 2021) and the anti-platelet and anti-thrombotic actions (Grambow et al., 2017). Furthermore, other studies also revealed the anti-nociceptive effects of GYY4137. Indeed, treatment with GYY4137 reduced the hyperalgesia and allodynia caused by chemotherapy through the activation of the K_{ATP} potassium channels (Qabazard et al., 2020) and alleviated neuropathy in diabetic rats by suppressing the spinal glial activation and the expression of several pro-inflammatory agents (Shayea et al., 2020). These findings suggest that GYY4137 may exert its analgesic effect through the activation of K_{ATP} potassium channels, together with its anti-inflammatory effects. However, the effect of GYY4137 on the affective disorders linked with nerve injury-induced neuropathic pain still requires further investigation (Figure 5).

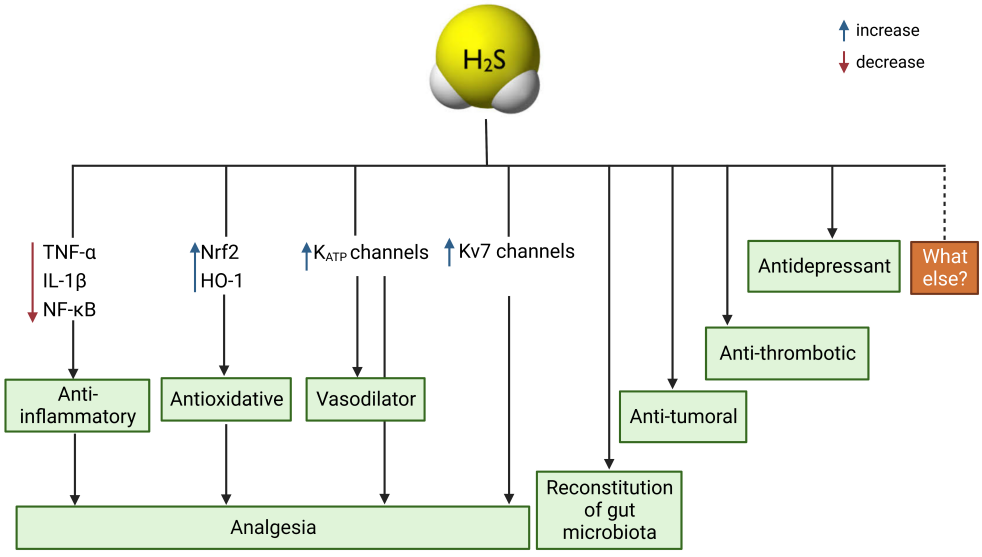

***Figure 5**. Beneficial effects of H_2S: antidepressant, anti-thrombotic, anti-tumoral, reconstitution of gut microbiota, and analgesia induced by its anti-inflammatory, antioxidative, and via activating the K_{ATP} and Kv7 potassium channels.*

2.11. Opioids

The effects of opioids are primarily mediated by the activation of three classical opioid receptors: μ (MOR), δ (DOR), and κ (KOR), all coupled to G protein. While MOR are mainly distributed in the cerebral cortex, brain stem, midbrain, cerebellum, SC, and DRG, and in peripheral organs, for instance the adrenal glands, pancreas, and intestine; DOR are primarily present in the cortex, DRG, SC, and are expressed to a lesser extent in the adrenal glands, pancreas, and skeletal muscles and finally, KOR are mainly located in the HIP, hypothalamus, and temporal lobe, and lesser in the peripheral tissues (Benarroch, 2012; Peng et al., 2012). The differences in their distribution also explain why different receptors, upon activation, manifest not only analgesic effects but also distinct secondary effects. For example, both MOR and DOR have sedative side effects, but due to the distribution of MOR in the brainstem, adrenal glands, and intestines, it can also cause respiratory depression, hypotension, and constipation (Stein, 2018). On the other hand, KOR, primarily located in the hippocampus, does not lead to significant respiratory depression but can result in restlessness or psychotomimetic effects (Ji, et al., 2021; Khan et al., 2022).

The most common endogenous opioid peptides are β-endorphins, enkephalins and dynorphins which activate of MOR, DOR, and KOR, respectively. The opioid peptides are widely distributed in the CNS and PNS, as well as in immune cells (including T cells, B cells, and macrophages). In the nervous system, β-endorphin are primarily located in the hypothalamus, nucleus tractus solitarius, and pituitary; enkephalins in the striatum, cortex and SC; dynorphins in the striatum and hypothalamus (Fichna et al., 2007; Benarroch, 2012).

Opioids participate in the ascending and descending pathways of pain to achieve their analgesic effects. In the event of nerve injury, endogenous opioid peptides (such as enkephalins) are released by interneurons in the SC that active opioid receptors. This activation inhibits cAMP through the G-protein coupling, which suppresses the release of substance P and reduces Ca^{2+} influx, attenuating the ascending transmission of pain signals. Whereas the inwardly rectifying potassium channels are activated leading to an inhibition of the neurotransmitter release (Corder et al., 2018; Stein, 2018). On the other hand, in the descending inhibitory pathway of pain, opioid agonists stimulate MOR on the presynaptic membrane as well as MOR and DOR on the postsynaptic membrane of the synapses. This directly or indirectly suppresses the inhibitory effect of GABAergic on the PAG-RVM projection, thereby facilitating the transmission of descending analgesic signals (Bagley and Ingram, 2020).

The most common opioid agonists include: DAMGO and morphine (MOR agonists); DPDPE and SNC 80 (DOR agonists); U-50488 and U-69593 (KOR agonists) (Stein, 2016). It is well known that MOR agonists exert powerful analgesic effects, but their side effects such as tolerance,

hyperalgesia and addiction are also evident, leading to significant physical and mental health issues for patients and imposing an important societal burden (Wang et al., 2019). Moreover, the fact that MOR agonists, especially morphine, are more effective for inhibiting inflammatory than neuropathic pain (Obara et al., 2009; Stein and Lang, 2009) makes that these opioids are only considered as second- or third-line treatments for neuropathic pain. It was also showed that KOR agonists exhibited lower efficacy in treating neuropathic than inflammatory pain, like to MOR agonists, and that only DOR agonists exhibited similar effectiveness in both types of pain, suggesting the potential use of DOR agonists for the treatment of chronic pain (Obara et al., 2009; Polo et al., 2019).

Therefore, how to maximize the analgesic properties of opioids during neuropathic pain and reduce their side effects, as much as possible, requires further investigations. Different studies revealed positive interactions between H_2S and opioids. In this lane, H_2S alleviates withdrawal-induced pain caused by MOR agonists by inhibiting spinal calcitonin gene-related peptide production (Yang et al., 2014) and the analgesic effect of H_2S in rats with visceral pain was associated with MOR activation (Distrutti et al., 2010). More recent studies, performed in animals with inflammatory pain, show that the induction of H_2S potentiates the painkiller actions of DOR agonists (Porta et al., 2021). These findings provide new insights for exploring novel treatments for neuropathic pain by using the co-administration of H_2S and opioids.

2.12. Cannabinoids

Like opioids, cannabis has a longstanding history of being utilized for analgesic purposes. Currently, the positive effects of the main component of cannabis, known as cannabinoids, in the treatment of neuropathic pain, are being amply studied (Lee et al., 2018; Soliman et al., 2021). The endogenous cannabinoid system contains the endogenous ligands anandamide (AEA) and 2-arachidoylglycerol (2-AG), that have high-affinity to cannabinoid 1 (CB1R) and cannabinoid 2 (CB2R) receptors and that are degraded by the enzymes fatty acid hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively.

Both, CB1R and CB2R, are coupled to the G protein, acting to inhibit the adenylate cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, reducing neuronal excitability (Rezende et al., 2023). Thus, the activation of cannabinoid receptors modulates pain by inhibiting the ascending transmission of pain signals at the peripheral, spinal, and supraspinal levels, as well as by facilitating the descending analgesic pathways through GABAergic disinhibition (Maldonado et al., 2016). Current studies also reveal the outstanding role performed by the endocannabinoid system in the microbiota–gut–brain axis, through which controls the gastrointestinal, affective, and cognitive disorders (Guida et al., 2018).

The CB1R are widely and abundantly distributed in both the CNS and PNS and its activation not only participates in pain relief but also lead to side effects such as psychotomimetic, tolerance, anxiety, and depression (Kibret et al., 2022). CB2R was once thought to exist only in the immune system, including lymphocytes, macrophages, pancreas, spleen, liver, and other immune-related cells and organs (Xu et al., 2023). However, it has been later confirmed that CB2R also exists in the CNS, as in the microglia and neurons from HIP, PFC, AMG, brainstem, spinal cord, and DRG, under pathological conditions (Beltramo et al., 2006; Kibret et al., 2022; Grabon et al., 2023). Nevertheless, the fact that the distribution of CB2R in the CNS is not as extensive and abundant as that of CB1R, results in relatively fewer side effects, particularly the lack of psychotropic effects (Kibret et al., 2022).

There are natural cannabinoids extracted from cannabis plants such as delta-9-tetrahydrocannabinol and cannabidiol, and synthetic cannabinoids such as WIN55212-2, HU-210 and JWH-018 as CB1R agonists and JWH-015, JWH-133 and GW-405833 as CB2R agonists (Starowicz and Finn, 2017; Coronado-Álvarez et al., 2021). The analgesic effects of CB2R agonists in inhibiting neuropathic pain have been confirmed in several animal experiments (Maldonado et al., 2016; Hossain et al., 2020; Wilkerson et al., 2020; Xu et al., 2023). These effects are principally mediated via inhibiting the PI3K/AKT pathway (Kibret et al., 2022), modulating the MAPK activation as well as by blocking the inflammatory signaling pathways through promoting the transformation of microglia into the anti-inflammatory phenotype in the SC (Wilkerson et al., 2020; Xu et al., 2023). Then, the noticeable anxiogenic- and depressive-like behaviors observed in mice with the CB2R gene deleted clarifies the impact of CB2R in modulating the emotions (Ortega-Alvaro et al., 2011). However, the administration of CB2R agonists in animal models displaying anxiety- and depression-like behaviors exhibits some opposite actions, such the induction of anxiogenic or anxiolytic effects, the alleviation of the depressive-like behaviors and/or the lack of a significant impact in these affective states (Kibret et al., 2022). Therefore, there are still controversies regarding the role of CB2R agonists in modulating emotional disorders and, more specifically, in those related to chronic neuropathic pain caused by the peripheral nerve injury which need to be more investigated.

Finally, the fact that the administration of a CB1R antagonist attenuated the pain reliever effects of ATB-352 (a H_2S donor) in the gastrointestinal tract of mice demonstrates a marked enhancement of the potency and effectiveness of ATB-352, due in part, through the involvement of the endogenous cannabinoid system (Costa et al., 2020). Thus suggesting a possible link between the endocannabinoid and H_2S systems in pain controlling. Nevertheless, further research and exploration are still needed of both systems to discover novel therapeutic strategies for neuropathic pain.

3. Objectives

In male mice with neuropathic pain caused by the chronic constriction of sciatic nerve (CCI), the aims of this thesis are to assess:

1. The effects of treatment with two slow-releasing H_2S donors on the allodynia and hyperalgesia provoked by sciatic nerve injury, their impact on the anxiety- and depressive-like behaviors associated with neuropathic pain and the underlying mechanisms in CNS and PNS.

2. The interaction between H_2S and the opioid system in the treatment of neuropathic pain. The role of H_2S on the antinociceptive actions and expression of MOR and DOR, and on the oxidative and apoptotic responses caused by CCI in the MS and/or DRG of animals with neuropathic pain.

3. The relationship between H_2S and CB2R systems in modulating neuropathic pain and the accompanying emotional disorders. The impact of H_2S on the expression of CB2R and the inflammatory, neurotrophic, and oxidative responses caused by CCI in the PFC, vHIP and PAG were also evaluated.

4. Manuscripts

4.1. **Bai X,** Batallé G, Pol O.

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Article

The Anxiolytic and Antidepressant Effects of Diallyl Disulfide and GYY4137 in Animals with Chronic Neuropathic Pain

Xue Bai [1,2], Gerard Batallé [1,2] and Olga Pol [1,2,*]

[1] Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau, Hospital de la Santa Creu i Sant Pau, 08041 Barcelona, Spain; xue.bai@e-campus.uab.cat (X.B.); gerard.batalle@e-campus.uab.cat (G.B.)
[2] Grup de Neurofarmacologia Molecular, Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
* Correspondence: opol@santpau.es; Tel.: +34-619-757-054

Abstract: When neuropathic pain is maintained long term, it can also lead to the development of emotional disorders that are even more intense than pain perception and difficult to treat. Hydrogen sulfide (H_2S) donors relieve chronic pain, but their effects on the associated mood disorders are not completely elucidated. We evaluated if treatment with DADS (diallyl disulfide) or GYY4137 (morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex), two slow-releasing H_2S donors, inhibits the anxiety- and depressive-like behaviors that concur with chronic neuropathic pain generated by sciatic nerve injury in mice. The modulatory role of these drugs in the inflammatory, apoptotic, and oxidative processes implicated in the development of the affective disorders was assessed. Our results revealed the anxiolytic, antidepressant, and antinociceptive properties of DADS and GYY4137 during neuropathic pain by inhibiting microglial activation and the up-regulation of phosphoinositide 3-kinase/phosphorylated protein kinase B and BAX in the amygdala (AMG) and/or periaqueductal gray matter (PAG). Both treatments also normalized and/or activated the endogenous antioxidant system, but only DADS blocked ERK 1/2 phosphorylation. Both H_2S donors decreased allodynia and hyperalgesia in a dose-dependent manner by activating the Kv7 potassium channels and heme oxygenase 1 signaling pathways. This study provides evidence of the anxiolytic and antidepressant properties of DADS and GYY4137 during neuropathic pain and reveals their analgesic actions, suggesting that these therapeutic properties may result from the inhibition of the inflammatory, apoptotic, and oxidative responses in the AMG and/or PAG. These findings support the use of these treatments for the management of affective disorders accompanying chronic neuropathic pain.

Keywords: anxiety; apoptosis; depression; hydrogen sulfide; neuropathic pain; oxidative stress

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1. Introduction

Several studies have demonstrated that the prevalence of neuropathic pain in the general population is around 6.9–10% [1]. It is also well known that, when neuropathic pain is maintained long term, in addition to the symptoms of the pain itself, it is very common to develop emotional disorders that are even more intense than pain perception, making their treatment a challenge [2]. Current treatments reduce pain symptoms but have limited efficacy in reducing the mood disorders that co-occur with neuropathic pain; consequently, new therapies are urgently needed.

Hydrogen sulfide (H_2S) is a gaseous neurotransmitter that regulates numerous physiological and pathophysiological processes [3]. It is highly implicated in modulating the cellular redox state and protects cells from oxidative stress [4,5]. Previous works reported the anxiolytic and/or antidepressant effects of fast exogenous H_2S donors such as sodium hydrosulfide (NaHS) [6] and sodium sulfide (Na_2S) [7] and the improvement of the anxiety- and/or depressive-related behaviors accompanying diabetes [8–10]. In addition, the antidepressant effects of H_2S donors that could release H_2S in a more controlled manner, such

as diallyl disulfide (DADS), a garlic component, in animals with depressive-like behaviors induced by chronic mild stress (CMS) have been also demonstrated [11]. GYY4137 (morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex), a synthetic H_2S donor that also slowly releases H_2S over a long time period [12], has been also recognized as a promising therapeutic agent in cardiovascular diseases, inflammatory processes, diabetes, and cancer [13]. Nevertheless, the potential effects of DADS and GYY4137 in modulating the emotional disorders associated with chronic pain have not been established.

Microglial activation and the increased synthesis of phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) induced by nerve injury in the spinal cord and several brain regions provoke central sensitization, thus contributing to the development of anxiety- and/or depressive-like behaviors associated with chronic pain both in animals [14,15] and patients [16]. Microglial activation has been exhibited in the amygdala (AMG) of mice with anxiety-like behaviors, and its blockage inhibited this emotional behavior [17].

Recent studies also reveal the direct involvement of oxidative stress in anxiety- and depressive-like behaviors [18,19]. Oxidative stress, displayed with high levels of reactive oxygen species (ROS) and/or low levels of antioxidant enzymes, such as heme oxygenase 1 (HO-1) and quinone oxidoreductase 1 (NQO1) in the spinal cord, hippocampus, and prefrontal cortex, is also implicated in the maintenance of neuropathic pain triggered by the chronic constriction of the sciatic nerve (CCI) [15,20]. In consequence, the inhibition of the PI3K/p-Akt signaling pathways attenuated neuropathic pain [21,22] and treatment with antioxidants, such as oxindoles and Nrf2 and/or HO-1 inducers, exerted anxiolytic and antidepressant effects by enhancing and/or avoiding the depletion of antioxidant enzymes induced by neve injury or peripheral inflammation [20,23,24]. Earlier studies reveal that NaHS blocked oxidative stress and increased the synthesis of superoxide dismutase (SOD) in the hippocampus of diabetic rats [8]. Similarly, isothiocyanates such as allyl-isothiocyanate (A-ITC) and phenyl-isothiocyanate (P-ITC) also avoided the enhanced expression of p-Akt/AKT or p-ERK $\frac{1}{2}$ and improved the protein levels of HO-1, NQO1, and glutathione S-transferase Mu 1 (GSTM1) in the hippocampus and prefrontal cortex of animals with neuropathic pain [25]. Nevertheless, the effects of DADS and GYY4137 in the nociceptive and oxidative responses provoked by peripheral nerve lesion in the central nervous system have not been assessed.

It is well known that oxidative stress leads to cell apoptosis as manifested with the up-regulation of Bcl2-associated X (BAX), an apoptosis-related protein, in the midbrain of rats with neuropathic pain [26,27]. In this study, the effects of DADS and GYY4137 in the apoptotic reactions generated by nerve injury will be also evaluated.

The AMG is an important brain area involved in the modulation of emotional disorders, such as anxiety and depression, as well as in the development of chronic pain including neuropathic pain [11,28–30]. In addition, several direct and indirect pathways between the periaqueductal gray area (PAG) and AMG participating in the modulation of neuropathic pain have also been demonstrated [11,31], thus supporting the participation of these brain areas in the control of neuropathic pain and associated affective disorders.

In this study, we evaluated the anxiolytic and antidepressant effects of DADS and GYY4137 in mice with neuropathic pain induced by CCI. The mechanisms implicated in their analgesic actions and their effects in microglial activation and expression of PI3K/p-Akt, BAX, and several antioxidant enzymes in the AMG and PAG were further investigated.

2. Materials and Methods

2.1. Animals

Experiments were carried out with male C57BL/6 mice (21–26 g; 5–6 weeks old), acquired from Envigo Laboratories (Barcelona, Spain), which were housed under standard light/dark (12/12-h), temperature (22 °C), and humidity (66%) conditions with free access to food and water. Experiments were performed after 7 days of acclimatization to the environmental conditions, conducted between 9:00 a.m. and 5:00 p.m., and in conformity

with the guidelines of the European Commission's directive (2010/63/EC) and the Spanish Law (RD 53/2013) regulating animal research, and approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona (ethical code: 9863). Maximal efforts were made to reduce the number and suffering of animals used.

2.2. Induction of Neuropathic Pain

Neuropathic pain was provoked by CCI. Sciatic nerve ligation was carried out under isoflurane anesthesia conditions (3% induction, 2.5% maintenance). The biceps femoris and the gluteus superficialis were separated by blunt dissection, and three ligatures right (4/0 silk) around the sciatic nerve were performed taking care to preserve epineural circulation as described by [32]. The same conditions were applied to control animals without nerve ligation (sham).

2.3. Mechanical Allodynia

Mechanical allodynia was evaluated by measuring the hind paw withdrawal response after the stimulation with the von Frey filament of different bending forces (0.008–3.5 g). Animals were placed in Plexiglas tubes (20 cm high × 9 cm diameter) with a wire grid bottom through which the filaments (North Coast Medical, Inc., San Jose, CA, USA) were applied by using the up–down paradigm [33]. A filament of 0.4 g was applied first, and the filament of 3.0 g was used as a cut-off. The strength of the next filament was increased or decreased depending on the animal's response. The threshold of the response was calculated using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that included curve fitting of the data.

2.4. Thermal Hyperalgesia

Thermal hyperalgesia was measured by assessing the paw withdrawal latency in response to radiant heat in the plantar test (Ugo Basile, Varese, Italy) [34]. Mice were placed in Plexiglas tubes (20 cm high × 9 cm diameter) placed on a glass surface. The heat source positioned under the plantar surface of the hind paws was activated with a light beam intensity until the paw withdrawal. We used a cut-off time of 12 s. Mean paw withdrawal latencies were determined from the average of three separate trials.

2.5. Cold Allodynia

Cold allodynia was evaluated using the cold plate apparatus (Ugo Basile, Italy). The number of elevations of each hind paw from mice exposed to the cold plate (4 ± 0.5 °C) during 5 min was recorded.

In all tests, animals were habituated to the environment for 1 h before the experiment. Both ipsilateral and contralateral paws were tested.

2.6. Anxiety Behavioral Tests

The anxiety-like behavior was assessed by utilizing the elevated plus maze (EPM) [35] and the open file (OF) tests [36]. The EPM has an X-shape with 4 arms each of 5 cm wide and 35 cm long, two open and two closed, with walls 15 cm high. The height from the labyrinth to the ground is 45 cm. The animal was placed in the center of the maze facing the open arms and its behavior was recorded by a digital camera for 5 min. The number of entries into the open and closed arms and the percentage of time stay in the open arms were calculated for each animal.

In the OF test, mice were placed in the center of the arena of a 44 × 44 cm box with a grey non-reflecting base and four walls, and their behavior was recorded by a digital camera for 5 min. Animals were allowed to move freely around the maze and to explore the environment. The number of entries in the central area, the time spent in it and the number of squares crossed was assessed.

2.7. Depressive Behavioral Tests

The evaluation of the depressive-like behaviors was performed by using the tail suspension test (TST) and the forced swimming test (FST) in which the duration of immobility of the animals was quantified according to the methods described by [37,38], respectively.

In the TST, mice isolated acoustically and visually were suspended by the tail from a horizontal wooden bar (35 cm above the floor) using adhesive tape (1 cm from the tip of the tail). The immobility time in seconds was recorded for 6 min. In the FST, each mouse was placed in a transparent Plexiglas cylinder (25 cm × 10 cm) containing water to a depth of 10 cm at 24 °C ± 0.1 °C. Each animal was subjected to forced swimming for 6 min, and the total duration of immobility was measured during the last 4 min, when mice show a sufficiently stable level of immobility. In both tests, mice were considered immobile when they remained completely quiet.

All of these experiments were performed by experimenters blinded to the experimental conditions.

2.8. Western Blot Analysis

Mice were euthanized by cervical dislocation at 30 days after surgery (CCI or SHAM). The contralateral AMG and the PAG were extracted and preserved at 80 °C until use. We analyzed the expression of PI3K, p-Akt/Akt, p-ERK1/2/ERK1/2, CD11b/c, BAX, HO-1, NQO1, SOD-1, and glutathione S-transferase Mu 1(GSTM1) by Western blot assay. The sonication of tissues was made in cold lysis buffer RIPA Buffer (Sigma-Aldrich). After solubilization for 1 h at 4 °C, crude homogenates were sonicated for 10 s and centrifuged at 700× *g* for 20 min at 4 °C. The supernatant (60 µg of total protein) was mixed with 4X Laemmli loading buffer and loaded onto a 4% stacking/12% separating sodium dodecyl sulfate polyacrylamide gels. Proteins were electrophoretically transferred onto a polyvinylidene fluoride membrane for 120 min and successfully blocked with phosphate-buffered saline (PBS) containing 5% nonfat dry milk, Tris-buffered saline with Tween 20 containing 5% bovine serum albumin (BSA), or 5% nonfat dry milk and PBS with Tween 20 containing 5% BSA for 75 min; they were then incubated with specific rabbit primary antibodies anti PI3K (1:150; Abcam, Cambridge, United Kingdom), phospho-Akt (1:150; Cell Signaling Technology, Danvers, MA, USA), total Akt (1:250; Cell Signaling Technology, Danvers, MA, USA), phospho-ERK 1/2 and total ERK 1/2 (1:250; Cell Signaling Technology, Danvers, MA, USA), BAX (1:250; Cell Signaling Technology, Danvers, MA, USA), HO-1 (1:250; Abcam, Cambridge, UK), NQO1 (1:200; Abcam, Cambridge, UK), SOD-1 (1:150; Novus Biologic, Littleton, CO, USA), and GSTM1 (1:150; Novus Biologic, Littleton, CO, USA) or β-actin (1:5000, Abcam Cambridge, UK) overnight at 4 °C. The blots were then incubated with anti-rabbit secondary polyclonal antibodies conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h at room temperature. Proteins were detected by using chemiluminescence reagents provided in the ECL kit (GE, Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometric analysis was carried out using Image-J program (National Institutes of Health, Bethesda, MD, USA).

2.9. Experimental Procedures

In a first set of experiments, we investigated if the repetitive administration of 150 µmols/kg of DADS from day 28 to 30 after surgery or 32 µmols/kg of GYY4137 from day 27 to 30 after surgery (two times per day) inhibited the anxiety- and depressive-like behaviors associated with chronic pain. The effects of these treatments in the mechanical allodynia, thermal hyperalgesia, and cold allodynia provoked by nerve injury were also assessed (n = 6–8 animals per group). The dose of DADS and GYY413 was selected in accordance with other studies [11,39].

In other groups of animals, we evaluated the antinociceptive effects produced by the acute administration of different doses of DADS (12.5–200 µmols/kg) and GYY4137 (2–64 µmols/kg) in CCI mice. The reversion of the analgesic effects made by the acute administration of high doses of DADS (200 µmols/kg) or GYY4137 (64 µmols/kg) with

8.0 µmol/kg of the selective Kv7 potassium channel blocker, XE-991, or 14.5 µmol/kg of the HO-1 inhibitor (tin protoporphyrin IX, SnPP) [40] were also evaluated (n = 6 animals per group). In all experiments, sham-operated mice were used as controls.

Sciatic nerve-injured animals treated with DADS, GYY4137, or vehicle (0.9% saline solution, SS) were euthanized by cervical dislocation, and the protein levels of PI3K, p-Akt, p-ERK 1/2, CD11b/c, BAX, HO-1, NQO1, SOD-1, and GSTM1 in the AMG and PAG were evaluated by Western blot. In these experiments, sham-operated mice treated with vehicle were used as controls (n = 3–4 samples per group).

2.10. Drugs

DADS and GYY4137 acquired from Sigma-Aldrich (St. Louis, MO, USA) were dissolved in SS and intraperitoneally administered in a final volume of 10 ml/kg, 1 h before testing, in accordance with our previous pilot studies and other works [11,41]. XE-911 and SnPP purchased in Tocris Bioscience (Ellisville, MO, USA) and Frontier Scientific (Livchem GmbH & Co., Frankfurt, Germany) were dissolved in dimethyl sulfoxide (1% in SS) and administered via intraperitoneal at 8 µmols/kg and 14.5 µmol/kg in a final volume of 10 mL/kg, 30 min before conducting the behavioral tests in accordance with previous studies [42,43]. All drugs were freshly prepared before use. For each group treated with a drug, the respective control group received the same volume of the corresponding vehicle.

2.11. Statistical Analyses

Data are expressed as the mean values ± standard error of the mean (SEM). We used the GraphPad software (version 8.0) for the statistical analysis. A one-way ANOVA followed by the Student–Newman–Keuls test was utilized for evaluating the effects of DADS and GYY4137 administered alone and combined with XE-911 or SnPP. The effects of DADS and GYY4137 in the expression of several proteins were also analyzed by using a one-way ANOVA and the post hoc Student–Newman–Keuls test. The ED_{50} of the drugs was calculated by linear regression analysis. A value of $p < 0.05$ was considered significant.

In the von Frey filaments and plantar tests, antinociception is expressed as the percentage of maximal possible effect, where the test latencies pre-drug (baseline) and post-drug administration are compared and calculated in accordance with the following equation:

$$\text{Maximal possible effect (\%)} = [(\text{drug-baseline})/(\text{cut-off-baseline})] \times 100 \quad (1)$$

In the cold plate, antinociception is expressed according to the following equation:

$$\text{Inhibition (\%)} = [(\text{number of paw elevations at baseline} - \text{number of paw elevations after drug})/\text{number of paw elevations at baseline})] \times 100. \quad (2)$$

3. Results

3.1. Anxiolytic and Antidepressant Effects of DADS and GYY4137 in Animals with Chronic Neuropathic Pain

The effects of the repetitive intraperitoneal administration of DADS at 150 µmols/kg, two times a day, during 3 consecutive days and those of GYY4137 administered at 32 µmols/kg, two times a day, during 4 days in the anxiety- and depressive-liked comportments associated with chronic neuropathic pain were evaluated. The results show that both DADS and GYY4137 normalized the diminished number of entries into the open arms ($p < 0.013$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with their respective sham-operated mice treated with vehicle; Figures 1A and 2A) and the percentage of time spend in its ($p < 0.008$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with their respective sham-operated mice treated with vehicle; Figures 1B and 2B). No changes in the number of entries into the closed arms were observed (Figures 1C and 2C). We further assessed the effects of DADS and GYY4137 in the OF test to verify their anxiolytic effects during neuropathic pain. The administration of DADS and GYY4137 both normalized the reduced number of entries into the central

area ($p < 0.004$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with sham-operated mice treated with vehicle; Figures 1D and 2D) and the shorter amount of time spent in the central area ($p < 0.029$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with sham-operated mice treated with vehicle; Figures 1E and 2E), but did not modify the number of squares crossed (Figures 1F and 2F).

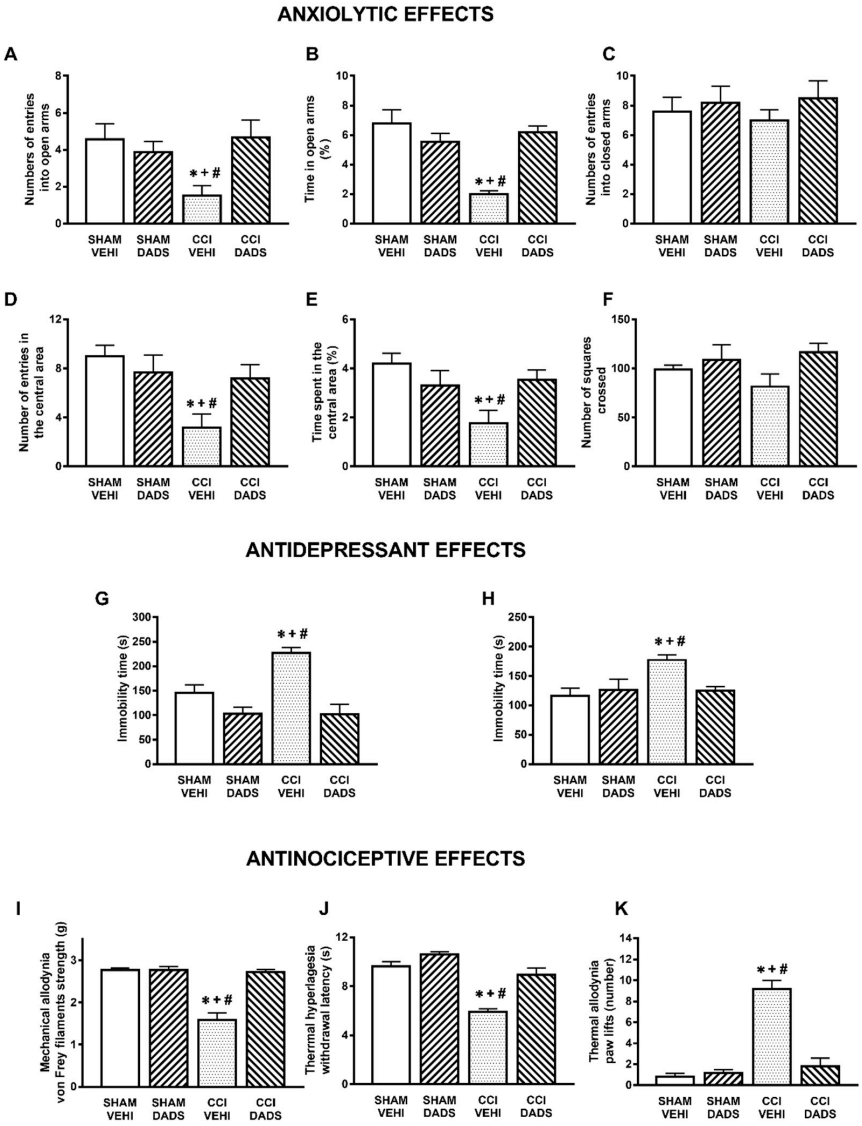

Figure 1. Treatment with DADS inhibited the anxiety- and depressive-like behaviors and the nociceptive responses induced by nerve injury. Effects of the repeated administration with DADS or vehicle at 150 μmol/kg for 3 days, 2 times for each day, on the anxiety-, depressive-, and nociceptive-like behaviors induced by nerve injury at 30 days after surgery. The effects of DADS or vehicle in sham-operated mice are also displayed. In the EPM test, the number of entries to the open arms (**A**), percentage of the time spent in the open arms (**B**), and the number of entries into the closed arms (**C**) are shown. In the OF test, the number of entries in the central area (**D**), the percentage of time spent in the central area (**E**), and the number of squares crossed (**F**) are presented. In the TST (**G**) and FST (**H**), the immobility times (s) are shown. The effects of DADS in the mechanical allodynia (**I**), thermal hyperalgesia (**J**), and thermal allodynia (**K**) in the ipsilateral paw of sham-operated or sciatic nerve-injured mice are also indicated. For each test: * denotes significant differences vs. sham-operated mice treated with vehicle, + vs. sham-operated mice treated with DADS, and # vs. CCI mice treated with DADS ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). Mean values ± SEM; n = 6–8 animals.

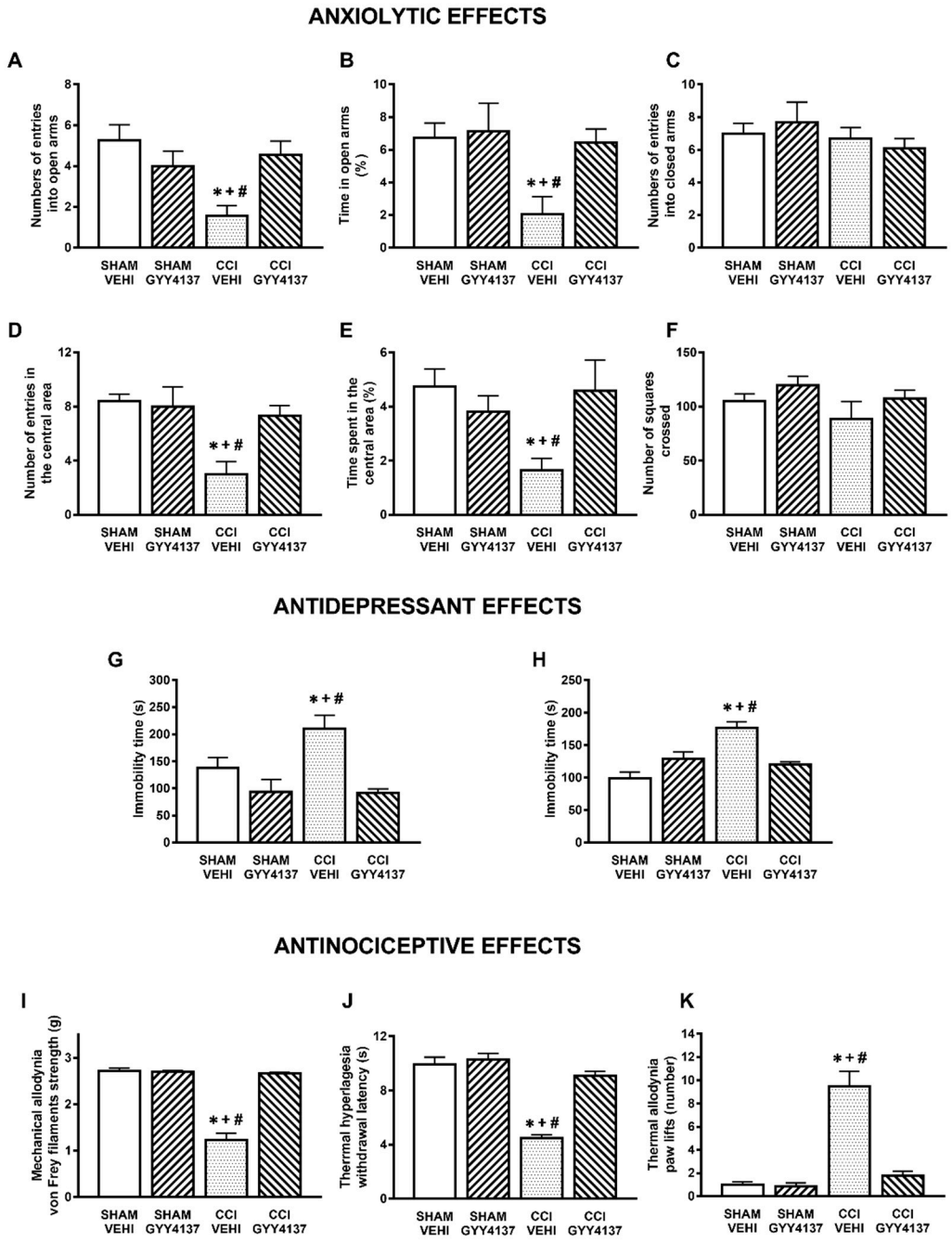

Figure 2. Treatment with GYY4137 inhibited the anxiety- and depressive-like behaviors and the nociceptive responses induced by nerve injury. Effects of the repeated administration with GYY4137 or vehicle at 32 μmol/kg for 4 days, at 2 times for each day, on the anxiety-, depressive-, and nociceptive-like behaviors induced by nerve injury at 30 days after surgery. The effects of GYY4137 or vehicle in sham-operated mice are also displayed. In the EPM test, the number of entries to the open arms (**A**), percentage of the time spent in the open arms (**B**), and the number of entries into the closed arms (**C**) are shown. In the OF test, the number of entries in the central area (**D**), the percentage of time spent in the central area (**E**), and the number of squares crossed (**F**) are presented. In the TST (**G**) and FST (**H**), the immobility times (s) are shown. The effects of GYY4137 in the mechanical allodynia (**I**), thermal hyperalgesia (**J**), and thermal allodynia (**K**) in the ipsilateral paw of sham-operated or sciatic nerve-injured mice are also indicated. For each test: * denotes significant differences vs. sham-operated mice treated with vehicle, + vs. sham-operated mice treated with GYY4137, and # vs. CCI mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). Mean values ± SEM; n = 6–8 animals.

Regarding the antidepressant effects, both treatments reduced the high immobility time observed in sciatic nerve-injured animals treated with vehicle in the TST ($p < 0.001$;

one-way ANOVA followed by the Student–Newman–Keuls test, as compared with sham-operated mice treated with vehicle; Figures 1G and 2G) and in the FST ($p < 0.021$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with sham-operated mice treated with vehicle; Figures 1H and 2H), thus revealing the antidepressant effects of these H_2S donors under neuropathic pain conditions.

Our data also demonstrated the completed reversion of the mechanical allodynia ($p < 0.001$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with sham-operated mice treated with vehicle) (Figures 1I and 2I), thermal hyperalgesia ($p < 0.001$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with sham-operated mice treated with vehicle) (Figures 1J and 2J) and cold allodynia ($p < 0.001$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with sham-operated mice treated with vehicle) (Figures 1K and 2K) provoked by nerve injury in animals treated with DADS or GYY4137 during 3 and 4 days, respectively. The repetitive administration of DADS or GYY4137 did not have any significant effect either in the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown).

3.2. The Acute Administration of DADS and GYY4137 Inhibited Mechanical Allodynia, Thermal Hyperalgesia, and Cold Allodynia Induced by Sciatic Nerve Injury in a Dose-Dependent Manner

The administration of DADS (12.5–200 μmol/kg) or GYY4137 (2–64 μmols/kg) inhibited mechanical allodynia (Figure 3A), thermal hyperalgesia (Figure 3B), and cold allodynia (Figure 3C) incited by sciatic nerve injury in a dose-dependent manner. As a consequence, the mechanical antiallodynic and thermal antihyperalgesic effects produced by high doses of DADS (100, 150 or 200 μmols/kg) were significantly higher than those produced by lower doses ($p < 0.001$; one-way ANOVA). Similarly, the inhibitory effects produced by 16, 32, or 64 μmols/kg of GYY4137 were significantly higher than those produced by lower doses of this drug ($p < 0.001$; one-way ANOVA). In all tests, the effects of DADS at 50, 100, or 200 μmols/kg and GYY4137 at 16, 32, or 64 μmols/kg were greater than those produced by vehicle ($p < 0.001$; one-way ANOVA). Our findings also revealed that DADS or GYY4137 intraperitoneally administered did not have any significant effect either in the ipsilateral paw of sham-operated mice or in the contralateral paw of sciatic nerve-injured or sham-operated animals (data not shown). Regarding the ED_{50}, GYY4137 was about 11.8, 7.2 and 9.9 times more effective than DADS in inhibiting mechanical allodynia, thermal hyperalgesia, and cold allodynia, respectively (Table 1).

3.3. Reversion of the Antinociceptive Effects of DADS and GYY4137 with the Administration of XE-991 or SnPP

The involvement of H_2S and HO-1 in the antinociceptive actions of DADS and GYY4137 during neuropathic pain was demonstrated by the reversion of their effects with the selective Kv7 potassium channel blocker, XE-991 (8.0 μmol/kg), and the HO-1 inhibitor, SnPP (14.5 μmol/kg). Our results showed that both XE-991 and SnPP reversed the mechanical anti-allodynic effects produced by 200 μmol/kg of DADS ($p < 0.001$, one-way ANOVA vs. saline + vehicle treated mice) or 64 μmol/kg of GYY4137 ($p < 0.001$, one-way ANOVA vs. saline + vehicle treated mice) (Table 2), in addition to the thermal antihyperalgesic and anti-allodynic effects produced by DADS ($p < 0.001$, one-way ANOVA vs. saline + vehicle treated mice) and GYY4137 ($p < 0.001$, one-way ANOVA vs. saline + vehicle treated mice) in animals with neuropathic pain. The administration of XE-991 and SnPP alone did not produce any significant effect in the ipsilateral and contralateral paws of CCI animals (data not shown).

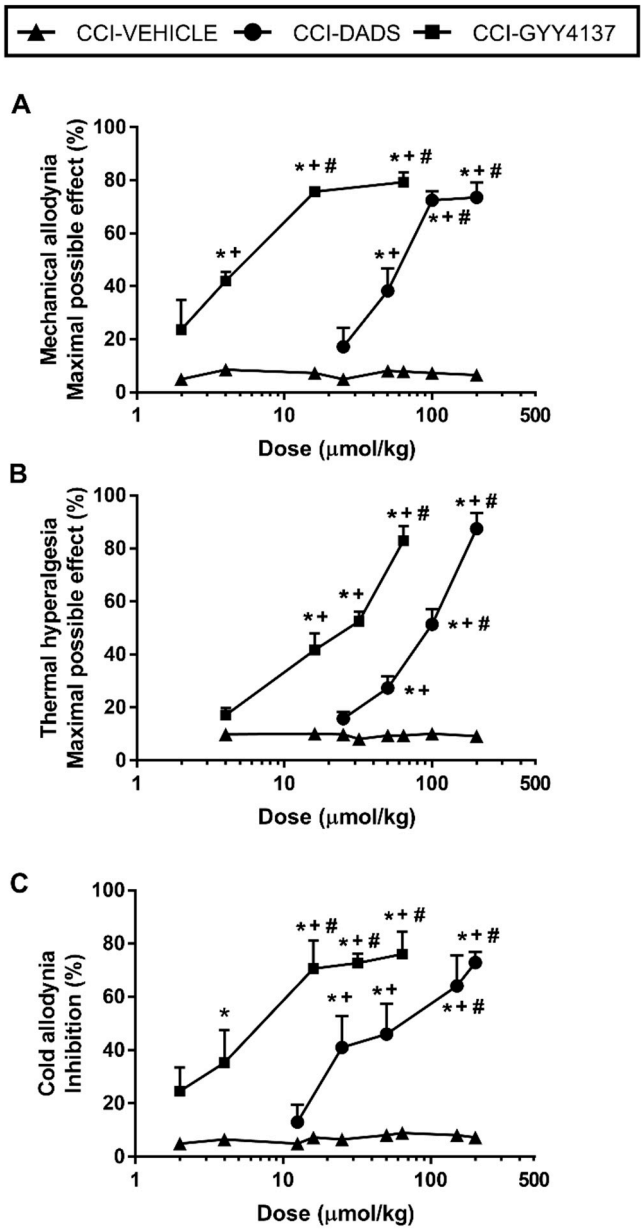

Figure 3. Effects of the acute administration of DADS and GYY4137 in the allodynia and hyperalgesia induced by sciatic nerve injury. Mechanical antiallodynic (**A**), thermal antihyperalgesic (**B**), and thermal antiallodynic effects (**C**) of different doses (logarithmic axis) of DADS and GYY4137 (μmols/kg) are shown. For each dose evaluated, * indicates significant differences vs. animals treated with vehicle, + indicates significant differences vs. the effect produced by the lowest dose of DADS or GYY4137, # vs. the effect produced by other doses of DADS or GYY4137 ($p < 0.05$; one-way ANOVA, followed by Student–Newman–Keuls test). Data are expressed as mean values of maximal possible effect (%) for mechanical allodynia and thermal hyperalgesia and as % inhibition for cold allodynia. Mean values ± SEM ($n = 6$ animals per dose).

Table 1. Comparison of the potencies (ED_{50}) of DADS and GYY4137 in the inhibition of the mechanical allodynia, thermal hyperalgesia, and thermal allodynia induced by sciatic nerve injury. Data are expressed as ED_{50} values (μmol/Kg) ± SEM (n = 6 animals per dose). For each test, the ratio of the ED_{50} values between drugs is also indicated.

Treatments	Mechanical Allodynia	Thermal Hyperalgesia	Thermal Allodynia
DADS	54.1 ± 8.1	186.5 ± 22.5	60.3 ± 6.4
GYY4137	4.6 ± 1.7	25.9 ± 6.0	6.1 ± 3.4
Ratio (DADS/GYY4137)	11.8	7.2	9.9

Table 2. Reversal of the effects produced by the acute intraperitoneal administration of 200 μmol/kg of DADS or 64 μmols/kg of GYY4137 with 8 μmols/kg of XE-911 or 14.5 μmol/kg of SnPP in the inhibition of the mechanical allodynia, thermal hyperalgesia and thermal allodynia induced by sciatic nerve injury in the ipsilateral paw. Data are expressed as mean ± SEM (n = 6 animals per treatment). In each test, * denotes significant differences vs. saline + vehicle treated animals ($p < 0.05$, one-way ANOVA, followed by the Student–Newman–Keuls test).

Treatments	Mechanical Allodynia	Thermal Hyperalgesia	Thermal Allodynia
Saline + vehicle	7.8 ± 1.1	9.4 ± 1.5	5.8 ± 1.3
DADS + vehicle	73.6 ± 5.7 *	81.1 ± 4.4 *	74.8 ± 5.0 *
DADS + XE-911	7.3 ± 4.9	5.5 ± 2.1	9.4 ± 5.6
DADS + SnPP	6.5 ± 4.3	4.5 ± 0.7	14.3 ± 6.4
GYY4137 + vehicle	79.3 ± 3.1 *	83.0 ± 5.6 *	76.0 ± 5.0 *
GYY4137 + XE-911	13.8 ± 7.0	7.2 ± 4.2	24.7 ± 10.1
GYY4137 + SnPP	6.9 ± 4.1	5.0 ± 3.2	12.0 ± 8.2
Saline + XE-911	10.2 ± 6.6	4.4 ± 2.8	15.2 ± 9.7
Saline + SnPP	10.0 ± 4.3	3.6 ± 1.9	12.1 ± 7.6

3.4. Effects of Treatment with DADS and GYY4137 in the Protein Levels of PI3K, p-Akt, p-ERK 1/2, CD11b/c, and BAX in the AMG and PAG of Mice with Neuropathic Pain

Sciatic nerve injury caused an up-regulation of PI3K ($p < 0.032$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figures 4A and 5A); p-Akt ($p < 0.018$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figures 4B and 5B), p-ERK 1/2 ($p < 0.006$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figures 4C and 5C) and BAX ($p < 0.031$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figures 4E and 5E) in the AMG and PAG. In both areas, the up regulation of PI3K and p-Akt were reversed with DADS and GYY4137 treatments, whereas p-ERK 1/2 activation was only inhibited with DADS. Moreover, while the up regulation of BAX in the AMG was normalized with DADS and GYY4137, only DADS reversed its up regulation in the PAG. Microglial activation induced by nerve injury in the AMG ($p < 0.003$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figure 4D), but not in the PAG (Figure 5D), was inhibited with both DADS and GYY4137.

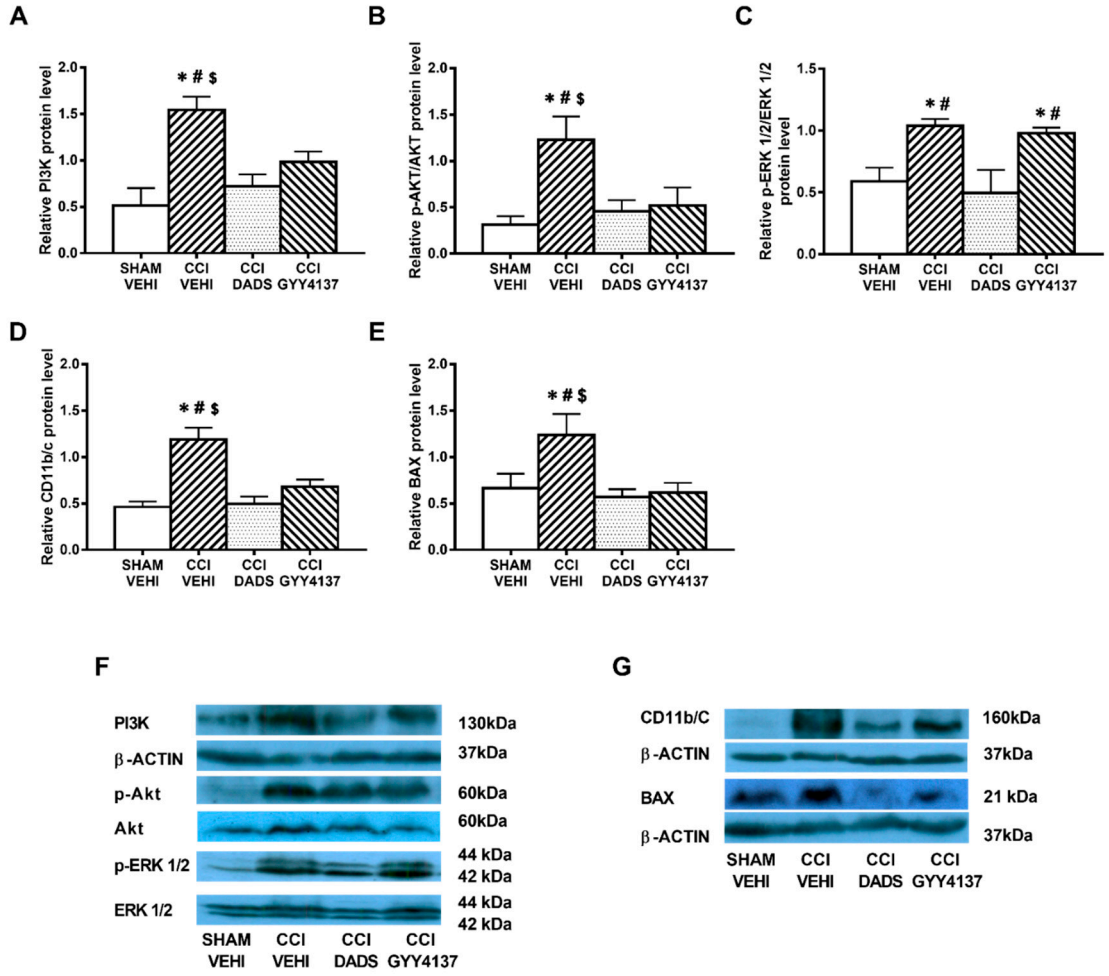

Figure 4. The effects of DADS and GYY4137 on the expression of PI3K, p-AKT, p-ERK 1/2, CD11b/c, and BAX in the AMG of animals with neuropathic pain. Treatment with DADS and GYY4137 normalized the up-regulation of PI3K (**A**), p-AKT (**B**), p-ERK 1/2 (**C**), CD11b/c (**D**), and BAX (**E**) in the AMG of animals with CCI-induced neuropathic pain. We used sham-operated mice treated with vehicle as controls. Non-phosphorylated proteins are expressed relative to β-actin protein levels while phosphorylated proteins are expressed relative to their corresponding total protein levels. Representative blots for PI3K, β-actin, p-Akt/Akt, and p-ERK 1/2/total ERK 1/2 (**F**) and for CD11b/c, BAX, and β-actin (**G**) are shown. In all panels, * represents significant differences vs. sham-operated mice treated with vehicle; # vs. sciatic nerve-injured mice treated with DADS; and $ vs. sciatic nerve-injured mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). Mean values ± SEM; n = 3–4 samples.

3.5. Effects of Treatment with DADS and GYY4137 on the Protein Levels of HO-1, NQO1, SOD-1, and GSTM1 in the AMG and PAG of Mice with Neuropathic Pain

In both brain areas, we further evaluated the effects of DADS and GYY4137 in expression of the antioxidant proteins HO-1, NQO1, SOD-1, and GSTM1. Decreased expression of HO-1 ($p < 0.003$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figure 6A) and increased levels of NQO1 ($p < 0.003$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figure 6B) and GSTM1 ($p < 0.018$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figure 6E) were demonstrated in the AMG of sciatic nerve-injured mice. Moreover, both DADS and GYY4137 treatments normalized the decreased expression of HO-1 (Figure 6A) and maintained the elevated levels of NQO1 (Figure 6B) and GSTM1 (Figure 6E) in the AMG. In the PAG, GYY4137 improved the protein levels of HO-1 ($p < 0.004$; one-way ANOVA vs. sham-operated and CCI vehicle treated mice) (Figure 7A) and NQO1 ($p < 0.003$; one-way ANOVA vs. sham-operated and CCI vehicle treated mice) (Figure 7B). Regarding GSTM1, both treatments improved its expression in the

PAG ($p < 0.009$; one-way ANOVA vs. sham-operated vehicle treated mice) (Figure 7E). No changes in the expression of SOD-1 were manifested either in the AMG (Figure 6D) or in the PAG (Figure 7D) of animals with sciatic nerve injury.

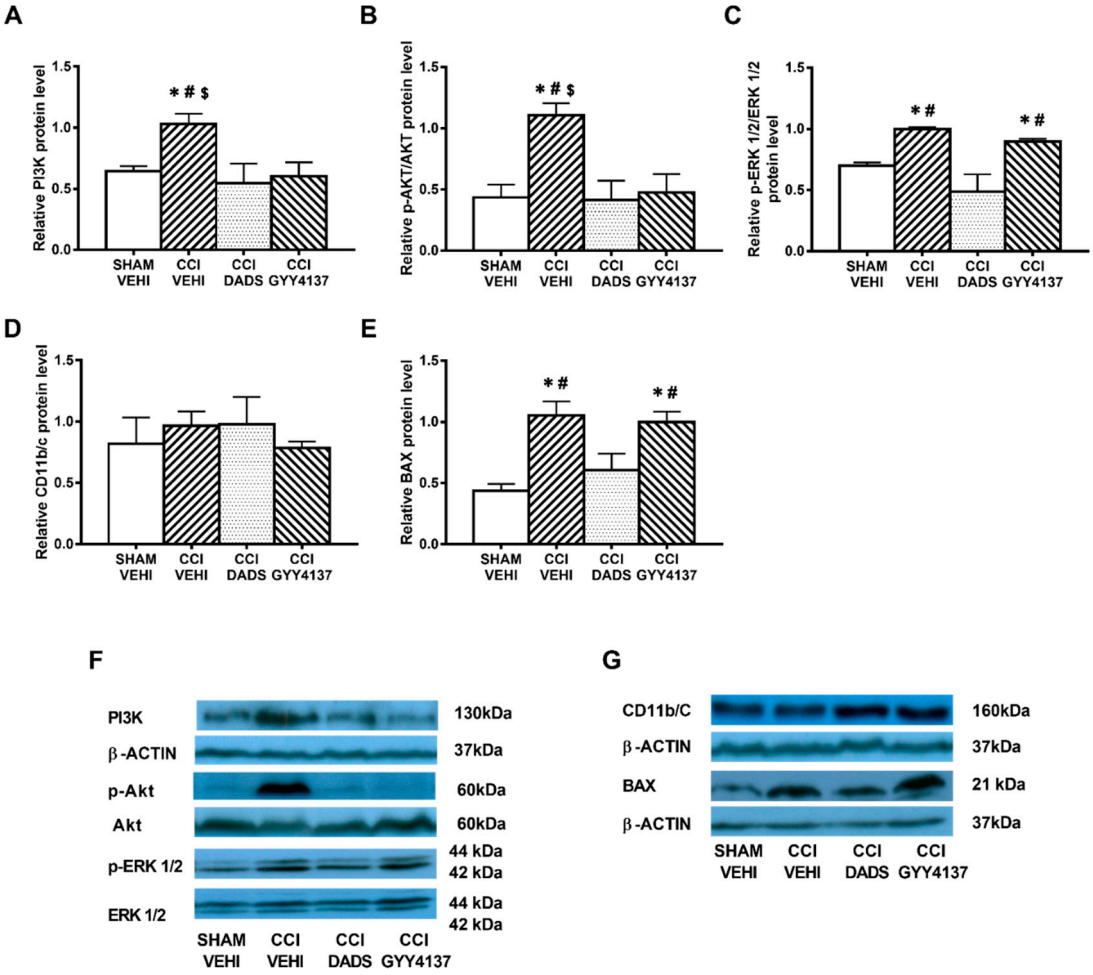

Figure 5. The effects of DADS and GYY4137 on the expression of PI3K, p-AKT, p-ERK 1/2, CD11b/c, and BAX in the PAG of animals with neuropathic pain. Treatment with DADS and/or GYY4137 normalized the up-regulation of PI3K (**A**), p-AKT (**B**), p-ERK 1/2 (**C**), and BAX (**E**) in the PAG of animals with CCI-induced neuropathic pain. No changes in the expression of CD11b/c were observed (**D**). Sham-operated mice treated with vehicle were used as controls. Non-phosphorylated proteins are expressed relative to β-actin protein levels while phosphorylated proteins are expressed relative to their corresponding total protein levels. Representative blots for PI3K, β-actin, p-Akt/Akt, and p-ERK 1/2/total ERK 1/2 (**F**) and for CD11b/c, BAX and β-actin (**G**) are displayed. In all panels, * represents significant differences vs. sham-operated mice treated with vehicle; # vs. sciatic nerve-injured mice treated with DADS; and $ vs. sciatic nerve-injured mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). Mean values ± SEM; n = 3–4 samples.

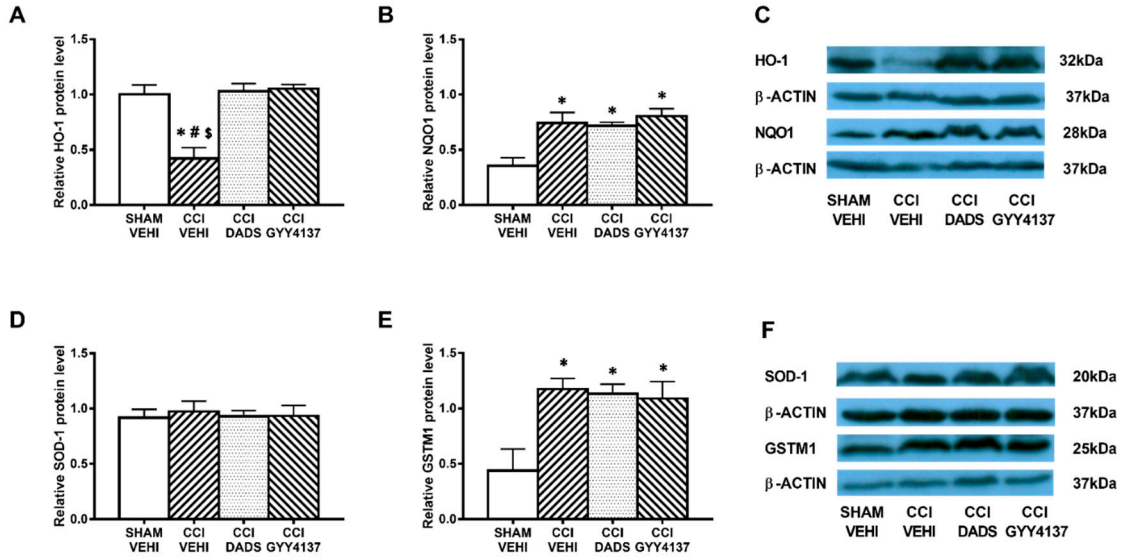

Figure 6. The effects of DADS and GYY4137 on the expression of HO-1, NQO1, SOD-1, and GSTM1 in the AMG of animals with neuropathic pain. Treatment with DADS and GYY4137 normalized and/or increased the protein levels of HO-1 (**A**), NQO1 (**B**), and GSTM1 (**E**) in the AMG of animals with CCI-induced neuropathic pain. No changes in the expression of SOD-1 (**D**) were detected. We used sham-operated mice treated with vehicle as controls. Proteins are expressed relative to β-actin protein levels. Representative blots for HO-1, NQO1, and β-actin (**C**) and for SOD-1, GSTM1, and β-actin (**F**) are shown. In all panels, * represents significant differences vs. sham-operated mice treated with vehicle; # vs. sciatic nerve-injured mice treated with DADS; and $ vs. sciatic nerve-injured mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). Mean values ± SEM; n = 3–4 samples.

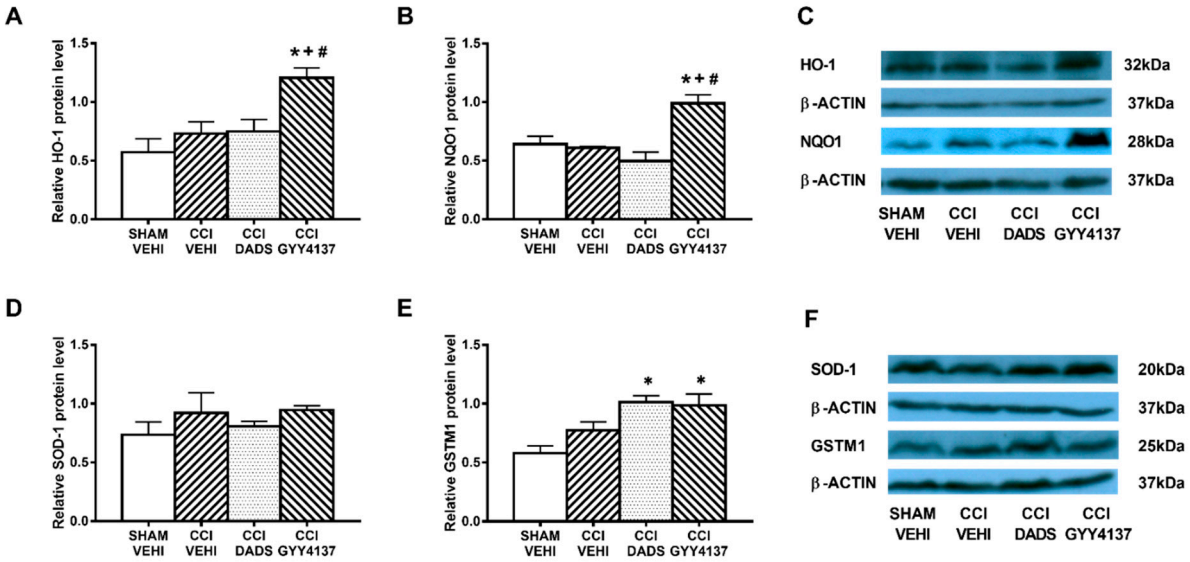

Figure 7. The effects of DADS and GYY4137 on the expression of HO-1, NQO1, SOD-1, and GSTM1 in the PAG of animals with neuropathic pain. Treatment with GYY4137 and/or DADS increased the protein levels of HO-1 (**A**), NQO1 (**B**), and GSTM1 (**E**) in the PAG of animals with CCI-induced neuropathic pain. No changes in the expression of SOD-1 (**D**) were observed. We used sham-operated mice treated with vehicle as controls. Proteins are expressed relative to β-actin protein levels. Representative blots for HO-1, NQO1, and β-actin (**C**) and for SOD-1, GSTM1, and β-actin (**F**) are displayed. In all panels, * represents significant differences vs. sham-operated mice treated with vehicle; + vs. sciatic nerve-injured mice treated with vehicle; and # vs. sciatic nerve-injured mice treated with DADS ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). Mean values ± SEM; n = 3–4 samples.

4. Discussion

This study demonstrated the anxiolytic and antidepressant effects of DADS and GYY4137 in mice with chronic neuropathic pain and that GYY4137 is more potent than DADS in inhibiting the allodynia and hyperalgesia provoked by sciatic nerve injury. These activities are mainly produced via inhibiting the PI3K/p-AKT and p-ERK 1/2 up-regulation, microglial activation, and apoptotic responses provoked by nerve injury as well as by modulating the endogenous antioxidant system in the AMG and/or PAG. Both treatments mediated their antinociceptive effects via activating the Kv7 potassium channels and the HO-1 signaling pathway.

It is well known that chronic pain concurs with several emotional disorders, which treatment has not completely resolved. This study demonstrates, for the first time, the inhibition of the anxiolytic and depressive-like behaviors induced by DADS and GYY4137 during neuropathic pain. These results are consistent with the anxiolytic and antidepressant actions of other H_2S donors, Na_2S, NaHS, and garlic, in different animal models of anxiety and depression as well as in diabetic rats with depressive-like behaviors [7,10,11]. Our results further confirmed the antidepressant properties of other slow H_2S releasers such as several isothiocyanates in animals with depressive-like behaviors but were in contrast to the lack of anxiolytic effects of these compounds during chronic osteoarthritic or neuropathic pain [25,43]. These dissimilar effects might be probably related with the different chemical structure of isothiocyanates vs. natural garlic derivates (DADS) or the synthetic H_2S donor (GYY4137). In relation to other known compounds that also have an impact on the emotional component such as gabapentinoids and several antidepressants [44–46], it is important to emphasize that although some of them can inhibit neuropathic pain and improve the associated anxiety-like behaviors, for example, gabapentin and pregabalin, they did not modify the depressive-like behaviors [47,48]. In contrast, different antidepressants, such as duloxetine, decreased pain hypersensitivity and depression-like behaviors in animals with CCI-induced neuropathic pain [49], but did not inhibit the anxiety-like behaviors [47]. In accordance with our results, several antidepressants for, instance, imipramine, milnacipran, and paroxetine, in addition to attenuating the nociceptive responses also have anxiolytic actions during neuropathic pain [50].

In summary, our data revealed, for the first time, the effectivity of DADS and GYY4137 in reducing emotional disorders (anxiety- and depressive-like behaviors) accompanying chronic neuropathic pain. Moreover, and in accordance with other studies [11,51], our findings reported the lack effects of DADS and GYY4137 in the locomotor activity of CCI-injured mice, thus showing the low side effects induced by both treatments during neuropathic pain.

Microglial activation and oxidative stress play an important role in the control of the anxiety and depressive-like behaviors [52]. In accordance, microglial activation has been shown in different brain regions of depressive patients suffering chronic pain [16] and in the hippocampus of animals with anxiety- and depressive-like behaviors associated with neuropathic pain [15,24]. Our data further demonstrate that nerve injury also activates microglia in the AMG, thus reinforcing the key role played by this brain area in the control of the affective components of neuropathic pain [30,53]. The normalization of the overexpression of CD11b/c induced by DADS and GYY4137 suggests the participation of microglia in their anxiolytic and antidepressant effects in CCI-injured mice.

Sciatic nerve ligation also produces important neuroplastic changes in the brain, which further contribute to the development of the anxiety- and depressive-like behaviors present in prolonged pain syndromes [9]. Our results reinforced these findings by showing increased p-ERK 1/2 levels in the AMG, thus supporting the correlation between ERK activation and the depressive-like behaviors observed in sciatic nerve-injured mice [53]. The inhibition of ERK activation induced by DADS might also contribute to its antidepressant and/or anxiolytic actions.

Depression and anxiety disorders are also related to an imbalance between ROS and antioxidant enzyme levels [54–56]. Therefore, animals with oxidative stress in the hip-

pocampus and prefrontal cortex [25] or in the peripheral blood granulocytes exhibited signs of anxiety- and depressive-like behaviors [57]. Interestingly, DADS and GYY4137 both avoided the decreased expression of HO-1 and maintained the elevated protein levels of NQO1 and GSTM1 in the AMG, revealing that the potent antioxidant actions of these compounds might also take part in the attenuation of the anxiety- and depressive-like behaviors induced by both compounds during neuropathic pain. Similarly, other authors revealed that garlic and different organosulfur compounds of garlic also potentiated the synthesis of antioxidant enzymes such as glutathione peroxidase in diabetic animals [58] and that the effectivity of GYY4137 against several neurological diseases is mainly accomplished via activating HO-1 synthesis [51,59,60].

Our data further revealed that DADS and GYY4137 inhibited the mechanical and cold allodynia, and thermal hyperalgesia induced by CCI in a dose-dependent manner. These results agree with the proven analgesic effects of GYY4137 in the neuropathy- induced by chemotherapeutic agents [39,41] and further reported the potential pain reliever actions of both DADS and GYY4137 treatments under neuropathic pain conditions generated by nerve injury. Regarding their effectiveness, our data showed that GYY4137 is 11.8 times more potent that DADS in inhibiting the mechanical allodynia and between 9.9 and 7.2 times more effective in decreasing cold allodynia and thermal hyperalgesia induced by CCI, respectively. Our findings reinforce the hypothesis that systemic administration of H_2S slow-release agents is particularly effective in relieving chronic pain [61,62] and reveal the greater efficacy of synthetic (GYY4137) versus natural H_2S donors (DADS). Moreover, we also demonstrate that the relief of neuropathic pain induced by DADS and GYY4137 was mediated via activation of the voltage gated Kv7 potassium channels as validated with the blockage of their antiallodynic and antihyperalgesic effects with the Kv7 potassium channel blocker, XE-991 [41].

In this study, we also demonstrated the neuroprotective properties of DADS and GYY4137 in animals with neuropathic pain with the reversion of the enhanced BAX levels induced by sciatic nerve-injury in the AMG and PAG. These results agree with the protective effects of DADS in neuronal cells against apoptosis both in vitro [63] and in vivo [64]. The neuroprotective effects of DADS and GYY4137 also participate in the painkiller actions of these compounds.

It is well known that PAG plays an important role in the descending modulation of pain [65] and the PI3K/p-Akt and ERK activation induced by nerve injury in this area supported the significant role played by these proteins during the maintenance of neuropathic pain [26]. The inhibition of these pathways with DADS and/or GYY4137 treatments revealed that their antinociceptive effects are mainly mediated via PAG regulation. In addition, the antioxidant effects of GYY4137 and DADS in this brain section proved by the upregulation of the expression of HO-1, NQO1, and GSTM1 might also contribute to the relief of chronic pain. Moreover, the reversal of the analgesic effects of DADS and GYY4137 with SnPP (an HO-1 inhibitor) suggest that the slow releasing H_2S donors alleviate neuropathic pain by activating the HO-1 signaling pathway. For the first time, we have demonstrated the participation of HO-1 in the antinociceptive effects of these treatments, and we postulate a positive interaction between H_2S and carbon monoxide systems under neuropathic pain conditions.

5. Conclusions

In summary, our results reveal the anxiolytic and antidepressant effects of GYY4137 and DADS during neuropathic pain as well as their analgesic properties by blocking the nociceptive, microglial, and apoptotic responses and activating the antioxidant system in the AMG and PAG. Thus, we suggest the potential use of these treatments, especially GYY4137, as desirable candidates for the management of affective disorders accompanying chronic neuropathic pain.

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Hydrogen Sulfide Increases the Analgesic Effects of μ- and δ-Opioid Receptors during Neuropathic Pain: Pathways Implicated

Xue Bai [1,2], Gerard Batallé [1,2], Gianfranco Balboni [3] and Olga Pol [1,2,*]

[1] Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau (IIB Sant Pau), 08041 Barcelona, Spain; xue.bai@e-campus.uab.cat (X.B.); gerard.batalle@e-campus.uab.cat (G.B.)
[2] Grup de Neurofarmacologia Molecular, Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
[3] Unit of Pharmaceutical, Pharmacological and Nutraceutical Sciences, Department of Life and Environmental Sciences, University of Cagliari, 09042 Cagliari, Italy; gbalboni@unica.it
* Correspondence: opol@santpau.es; Tel.: +34-619-757-054

Abstract: Recent studies have revealed that hydrogen sulfide (H_2S) increases the analgesic actions of the δ-opioid receptor (DOR) in inflammatory pain. However, the possible improvement of the analgesia of μ-opioid receptor (MOR) and DOR agonists during neuropathic pain, through pretreatment with two slow-releasing H_2S donors—DADS (diallyl disulfide) and GYY4137 (morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex)—is still unknown. In male C57BL/6J mice with neuropathic pain incited by chronic constriction of the sciatic nerve (CCI), we evaluated: (1) the influence of DADS (3.5 mg/kg) and GYY4137 (0.7 mg/kg) on the inhibition of the allodynia and hyperalgesia produced by the systemic or local administration of morphine (3 mg/kg or 65 μg) and UFP-512 (1 mg/kg or 12.5 μg); (2) the reversion of the antinociceptive actions of high doses of DADS (30 mg/kg) and GYY4137 (24 mg/kg) with MOR and DOR antagonists; and (3) the effects of H_2S donors on oxidative stress, apoptotic responses, and MOR and DOR expression in the medial septum (MS) and dorsal root ganglia (DRG). The results revealed that both DADS and GYY4137 improved the antiallodynic effects of morphine and UFP-512, possibly by up-regulating MOR and DOR expression in DRG. The administration of MOR and DOR antagonists blocked the analgesic properties of DADS and GYY4137, revealing the feasible participation of the endogenous opioid system in H_2S analgesic effects. Moreover, both H_2S donors inhibited oxidative stress and apoptosis generated by CCI in the MS and/or DRG. This study suggests the co-treatment of H_2S donors with MOR or DOR agonists as a potential therapy for neuropathic pain.

Keywords: analgesia; apoptosis; hydrogen sulfide; neuropathic pain; opioids; oxidative stress

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1. Introduction

Neuropathic pain has a high prevalence (6.9–10%), and is one of the most common clinical symptoms [1]. Neuropathic pain can be caused by a variety of etiologies, some of which are common, such as nerve injury, trauma, drugs, infections. Neuropathic pain can be also linked with several metabolic disorders—for example, diabetes and neurodegenerative diseases such as Parkinson and Alzheimer [2,3]. Neuropathic pain is characterized by different degrees of allodynia and hyperalgesia that severely affect the patient's quality of life. This type of pain is difficult to treat, given the variety and complexity of its manifestations, and the multiple adverse effects accompanying the current pharmacological treatments [4,5].

Oxidative stress, resulting from the increased production of reactive oxygen species (ROS) and the disruption of redox balance caused by damage [6], is an important pathophysiological process following nerve injury, and is deeply involved in the development

of neuropathic pain. Moreover, excessive production of ROS and oxidative stress causes abnormal mitochondrial structure, leading to mitochondrial dysfunction and apoptosis [7]. Several investigations have confirmed that mitochondrial dysfunction is an important cause of neurodegenerative diseases such as Alzheimer, Parkinson, and Huntington [8,9], and most of these diseases lead to motor neuron dysfunctions that in turn induce chronic pain [10–12].

Opioids are among the most-used analgesics in clinical practice, although μ-opioid receptor (MOR) agonists are relegated to third line clinically recommended treatments for neuropathic pain, due to their low efficacy and common undesirable side-effects, such as respiratory depression, constipation, addiction, and tolerance [13,14]. Consequently, several studies have demonstrated the low palliative efficacy of morphine in different preclinical models of neuropathic pain [15,16], and have revealed that the effectiveness of morphine in inhibiting neuropathy was much lower than it was for inhibiting inflammatory pain [16]. Interestingly, treatment with DOR agonists reduced chronic neuropathic [17,18] and inflammatory pain in rodents [19,20] with similar effectiveness, although its efficacy was moderate [16,18]. Thus, additional therapeutic strategies are needed to potentiate the efficacy of opioids for neuropathic pain.

Hydrogen sulfide (H_2S) is a gaseous neurotransmitter, widely distributed in the central (CNS) and peripheral (PNS) nervous systems, which modulates several physiological and pathological processes [21,22]. Recent preclinical studies have shown that treatment with two slow releasers of H_2S, a component of garlic, diallyl disulfide (DADS), and a synthetic H_2S donor, GYY4137 (morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex), relieved neuropathic [23] and osteoarthritic pain [24]. The analgesic actions of both compounds were mainly produced by inhibiting apoptotic responses, and activating the endogenous antioxidant system by triggering the synthesis of antioxidant enzymes—such as superoxide dismutase 1 (SOD-1), glutathione S-transferase Mu 1 (GSTM1), heme oxygenase 1 (HO-1), and quinone oxidoreductase 1 (NQO1)—in the amygdala (AMG) and periaqueductal gray matter (PAG) of mice with nerve-injury-induced neuropathy [23]. Nonetheless, DADS and GYY4137 actions in DRG—one of the initial parts of the ascending transmission pathways involved in the development and maintenance of neuropathic pain [25,26]—and in other brain areas involved in pain modulation, such as the medial septum (MS) [27,28], have not been previously evaluated.

In addition, a recent study proved that the administration of slow-releasing H_2S donors improved the antinociceptive effects of DOR agonists [D-Pen2, D-Pen5]-enkephalin, and H-Dmt-Tic-NH-CH(CH2-COOH)-Bid (UFP-512) during inflammatory pain, by enhancing the peripheral expression of the DOR [20]. Thus, the co-administration of H_2S donors with DOR agonists is a potential therapy for inflammatory pain. However, the possible potentiation of the analgesic properties of MOR and DOR agonists with their co-treatment with DADS and GYY4137 during neuropathic pain, and the possible mechanisms involved, are still unknown.

Therefore, in animals with neuropathic pain produced by the chronic constriction of the sciatic nerve (CCI), we evaluated: (1) the impact of the co-administration of DADS and GYY4137 with MOR (morphine) and DOR (UFP-512) agonists in the allodynic and hyperalgesic responses provoked by nerve injury; (2) the reversion of H_2S donor effects by MOR (naloxone) and DOR (naltrindole) antagonists; and (3) the influence of DADS and GYY4137 treatments on the oxidative stress, apoptosis, and protein levels of MOR and DOR in the MS and DRG.

2. Materials and Method

2.1. Animals

These studies were conducted with male C57BL/6 mice (21–26 g; 5–6 weeks old), purchased from Envigo Laboratories (Barcelona, Spain), which were accommodated under normal light/dark (12/12-h), temperature (22 °C), and humidity (66%) conditions, with free access to food and water. The experiments were conducted after 7 days of acclimatization

to the environment. The experiments were performed between 9:00 a.m. and 5:00 p.m. in compliance with the guidelines of European Commission Directive (2010/63/EC) and the Spanish Law (RD 53/2013) regulating animal research, and were approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona, Barcelona, Spain (ethical code 1319). The greatest efforts were made to minimize the number of animals employed, and their suffering.

2.2. Induction of Neuropathic Pain

The CCI performed under isoflurane anesthesia conditions (3% induction, 2.5% maintenance) was used as a model of neuropathic pain. After separation of the biceps femoris and the gluteus superficialis by blunt dissection, three ligatures right (4/0 silk) around the sciatic nerve were performed, taking care to maintain epineural circulation [29]; identical conditions were applied to control animals without nerve ligation (SHAM).

2.3. Mechanical Allodynia

Mechanical allodynia was evaluated by measuring hind paw withdrawal response after stimulation by von Frey filaments of different bending forces (0.4–3.5 g). Mice were put in Plexiglas tubes (20 cm high $\times$ 9 cm diameter) with a wire grid bottom, through which the filaments (North Coast Medical, Inc., San Jose, CA, USA) were applied by using the up–down paradigm [30]. Filaments of 0.4 and 3.5 g were used first and last, respectively. The strength of the following filament was increased or decreased depending on the animal's response. The threshold of the response was calculated using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that included curve fitting of the data.

2.4. Thermal Hyperalgesia

Thermal hyperalgesia was evaluated by measuring paw withdrawal latency in response to radiant heat in the plantar test (Ugo Basile, Varese, Italy) [31]. Mice were placed in Plexiglas tubes (20 cm high $\times$ 9 cm diameter) placed on a glass surface. The heat source positioned under the plantar surface of the hind paws was activated with a light beam intensity until the paw withdrawal. A cut-off time of 12 s was used to prevent paw damage. Mean paw-withdrawal latencies were determined from the average of three separate trials.

2.5. Cold Allodynia

A cold plate apparatus (Ugo Basile, Varese, Italy) was used for evaluating cold allodynia. The number of elevations of each hind paw of animals exposed to the cold plate (4 ± 0.5 °C) for 5 min, was recorded.

In all tests, the animals were habituated to the environment for 1 h before the experiment. Both the ipsilateral and the contralateral hind paws were tested, and the experiments were performed by experimenters blinded to the experimental conditions.

2.6. Western Blot Analysis

The animals were euthanized by cervical dislocation at 30 days after surgery (CCI or SHAM). The MS and ipsilateral DRG were extracted immediately, rapidly frozen in liquid nitrogen, and preserved at -80 °C until assay. The sonication of tissues was made in cold lysis RIPA Buffer (Sigma–Aldrich, St Louis, MO, USA), and after their solubilization for 1 h at 4 °C, the crude homogenates were sonicated for 10 s and centrifuged (700 g) for 20 min at 4 °C. The supernatant (60 µg of total protein) was mixed with 4X Laemmli loading buffer and loaded onto 4% stacking/12% separating sodium dodecyl sulfate polyacrylamide gels. Proteins were electrophoretically transferred onto a polyvinylidene fluoride membrane for 120 min, and blocked with phosphate-buffered saline (PBS; P-5493; Sigma–Aldrich, St. Louis, MO, USA) containing non-fat dry milk (5%), Tris-buffered saline with Tween 20 containing bovine serum albumin (5%) (BSA; Sigma–Aldrich, St. Louis, MO, USA), or non-fat dry milk (5%) and PBS with Tween 20 containing BSA (5%), for 75 min. Then, they were incubated overnight at 4 °C with specific rabbit primary antibodies: anti HO-1 (1:150;

Enzo Life Sciences, New York, NY, USA); NQO1 (1:200; Sigma–Aldrich, St. Louis, MO, USA); SOD-1 (1:150; Novus Biologic, Littleton, CO, USA); GSTM1 (1:150; Novus Biologic, Littleton, CO, USA); 4-HNE (1:200; Abcam, Cambridge, UK); BAX (1:250; Cell Signaling Technology, Danvers, MA, USA); MOR (1: 300; Abcam, Cambridge, UK); DOR (1:300; Abcam, Cambridge, UK); or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5000, Merck, Billerica, MA, USA). The blots were then incubated with anti-rabbit secondary polyclonal antibodies conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h at room temperature. The proteins were detected by utilizing chemiluminescence reagents provided in an ECL kit (GE, Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometric analysis was carried out using the Image-J program (National Institutes of Health, Bethesda, MD, USA).

2.7. Experimental Procedures

To investigate the effects of H_2S in the analgesic actions of MOR and DOR agonists, the inhibition of mechanical and cold allodynia, and thermal hyperalgesia produced by low doses of DADS (3.5 mg/kg) or GYY4137 (0.7 mg/kg) intraperitoneally injected in combination with the i.p. or subplantar (s.p.) injection of low doses of morphine (3 mg/kg, i.p.; 65 µg, s.p.) or UFP-512 (1 mg/kg, i.p.; 12.5 µg, s.p.), were evaluated. The local UFP-512 dose was extracted from the response curve executed in this study (Figure S1), and the intraperitoneally and/or subplantarly injected doses of UFP-512, morphine, DADS, and GYY4137 were selected in accordance with previous studies [18,23,29,32]. DADS and GYY4137 were injected 30 min before morphine or UFP-512 injections, and tests were conducted 30 min later (n = 6 animals for group).

In other experiments, we assessed the reversion, by the i.p. or s.p. administration of the MOR antagonist (naloxone, 3 mg/kg; 20 µg) or the DOR antagonist (naltrindole, 3 mg/kg; 50 µg), of the effects produced by high doses of DADS (30 mg/kg) or GYY4137 (24 mg/kg). DADS and GYY4137 were injected 15 min before naloxone or naltrindole administration, and tests were conducted 60 min later (n = 6 animals per group). The doses of DADS, GYY4137, naloxone, and naltrindole were selected in accordance with previous studies [18,23,29,32]. In all experiments, saline (SS, 0.4% NaCl) plus SS-treated animals were used as controls.

Finally, nerve-injured mice injected with DADS or GYY4137 were euthanized by cervical dislocation, and the HO-1, NQO1, SOD-1, GSTM1, 4-HNE, BAX, MOR, and DOR protein levels in the MS and DRG were assessed by Western blot. Sham-operated animals injected with SS were utilized as controls (n = 3 samples).

2.8. Drugs

DADS and GYY4137, purchased from Sigma–Aldrich (St. Louis, MO, USA), were dissolved in SS, and intraperitoneally injected in a final volume of 10 mL/kg at 1 h, before testing in compliance with other works [23,33].

Morphine hydrochloride purchased from Alcaiber S.A. (Madrid, Spain), UFP-512 synthesized by [34], and naloxone and naltrindole obtained from Sigma–Aldrich (St. Louis, MO, USA), were dissolved in SS and intraperitoneally or subplantarly injected in a final volume of 10 mL/kg or 30 µL, in agreement with previous studies [18,23,29,32].

All drugs were prepared just before use. For each group injected with a drug, the corresponding control group received the identical volume of SS.

2.9. Statistical Analyses

Data are expressed as the mean values ± standard error of the mean (SEM). GraphPad software (version 8.0) was used to perform the statistical analysis. In each behavior test, the evaluation of the effects of different doses of UFP-512 or SS was performed by using a one-way analysis of variance (ANOVA) followed by the Tukey test. A one-way ANOVA followed by the Tukey test was also applied to evaluate the effects of DADS and GYY4137 injected alone or mixed with morphine, UFP-512, naloxone or naltrindole.

The effects of the H_2S donors on the expression of several proteins were also analyzed by using a one-way ANOVA and the post hoc Tukey test.

A value of $p < 0.05$ was considered significant.

3. Results

3.1. The Effects of the Co-Administration of DADS or GYY4137 with Morphine or UFP-512 on the Allodynia and Hyperalgesia Caused by Nerve Injury

In mice with neuropathic pain induced by CCI, we assessed the mechanical and cold antiallodynic effects, as well as the antihyperalgesic effects induced by the i.p. administration of a low dose of DADS (3.5 mg/kg) or GYY4137 (0.7 mg/kg) combined with low doses of morphine (3 mg/kg or 65 µg) or UFP-512 (1 mg/kg or 12.5 µg), intraperitoneally or subplantarly injected. The effects of each of these treatments administered alone were also assessed.

Our results confirmed that CCI reduced the threshold of ipsilateral hind paw withdrawal from von Frey filaments stimulation ($p < 0.001$, one-way ANOVA followed by the Tukey test vs. sham-operated mice treated with SS plus SS; Figure 1A,B), as well as the withdrawal threshold of the ipsilateral hind paw in response to a thermal stimulus ($p < 0.001$, one-way ANOVA followed by the Tukey test vs. sham-operated mice treated with SS plus SS; Figure 1C,D), and increased the number of ipsilateral hind paw lifts caused by cold stimulus at day 30 after surgery ($p < 0.001$, one-way ANOVA followed by Tukey test; vs. sham-operated mice treated with SS plus SS; Figure 1E,F).

Moreover, treatment with DADS or GYY4137 did not alter but slightly inhibited the mechanical allodynia (Figure 1A,B), thermal hyperalgesia (Figure 1C,D), and cold allodynia (Figure 1E,F) provoked by nerve injury ($p < 0.001$; one-way ANOVA vs. CCI- SS plus SS treated mice). Co-administration of both H_2S donors with morphine increased the mechanical antiallodynic (Figure 1A,B), thermal antihyperalgesic (Figure 1 C,D), and cold antiallodynic (Figure 1E,F) effects produced by the i.p. and s.p. injection of morphine in the ipsilateral paw of nerve-injured mice ($p < 0.001$, one-way ANOVA followed by Tukey test; vs. their respective control group treated with SS plus SS or morphine, and with mice treated with DADS or GYY4137 plus SS). Nevertheless, while the co-administration of both H_2S donors with morphine completely blocked the mechanical and cold allodynia, thermal hyperalgesia was only significantly reduced ($p < 0.001$; one-way ANOVA vs. sham-operated mice treated with SS plus SS), thus revealing that the co-administration of H_2S plus morphine, systemically or locally administered, was more effective in inhibiting allodynia than hyperalgesia.

Our results also demonstrated that treatment with 3.5 mg/kg of DADS or 0.7 mg/kg of GYY4137 significantly enhanced the mechanical antiallodynic (Figure 2A,B), thermal antihyperalgesic ($p < 0.001$, one-way ANOVA; Figure 2C,D), and cold antiallodynic effects ($p < 0.001$, one-way ANOVA; Figure 2E,F) produced by the i.p. (1 mg/kg) or s.p. (12.5 µg) administration of UFP-512 ($p < 0.001$, one-way ANOVA, followed by Tukey test; vs. their respective control group treated with SS plus SS or UFP-512, and with mice treated with DADS or GYY4137 plus SS).

As with the morphine, whereas mechanical and cold allodynia were completely blocked with the co-administration of both H_2S donors with UFP-512, thermal hyperalgesia was not totally reduced ($p < 0.001$; one-way ANOVA vs. sham-operated mice treated with SS plus SS), thus revealing that the co-administration of H_2S donors plus UFP-512, systemically or locally administered, was more effective in inhibiting mechanical and cold allodynia than thermal hyperalgesia provoked by nerve injury.

Lastly, DADS and GYY4137 administered alone, or combined with the i.p. and s.p. injection of morphine or UFP-512, did not have any effect in the contralateral paws of sciatic nerve-injured animals, nor in the contralateral and ipsilateral paws of sham-operated treated animals (data not displayed).

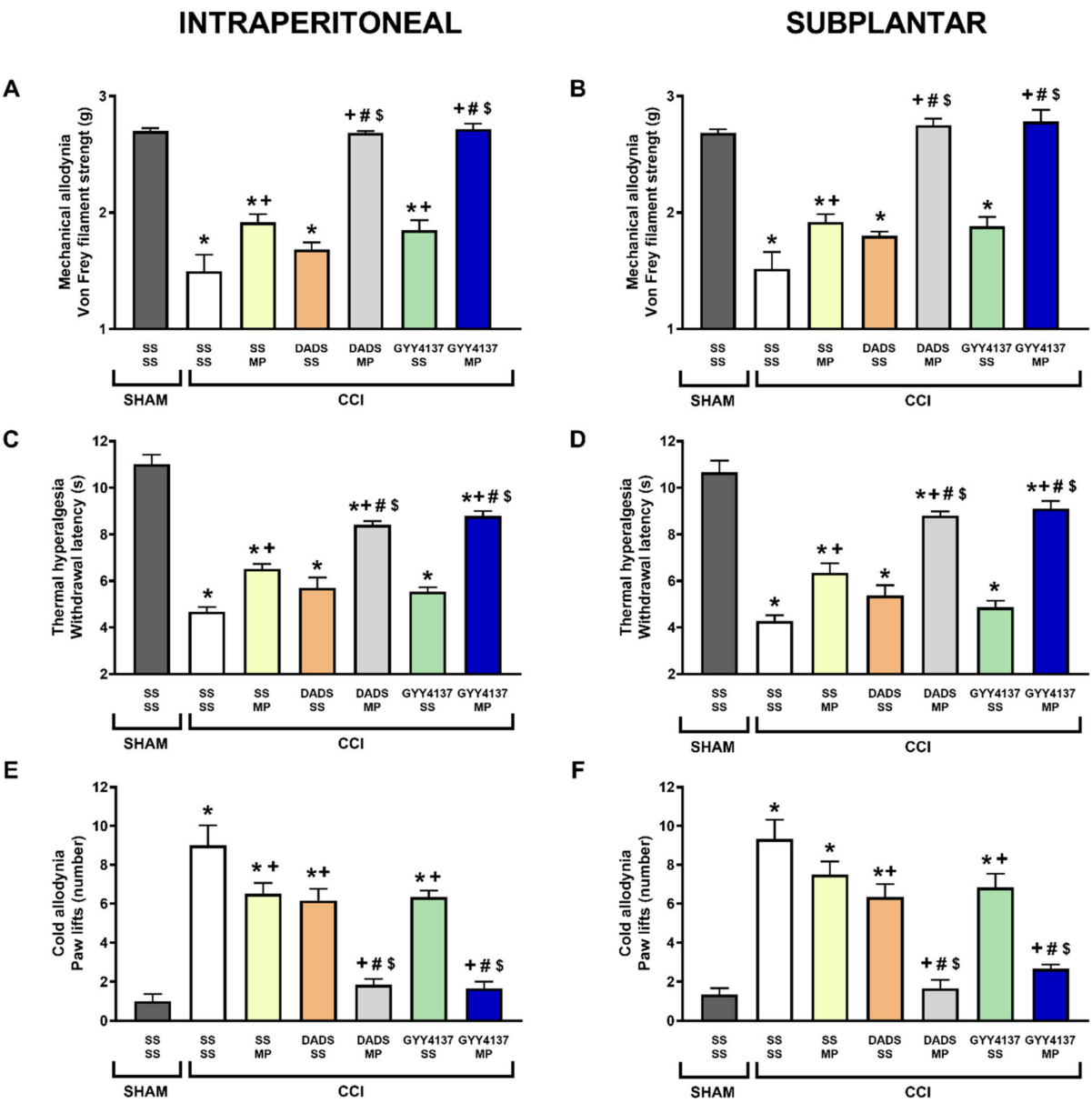

Figure 1. Effects of the co-administration of DADS or GYY4137 with morphine on the mechanical allodynia, thermal hyperalgesia, and cold allodynia induced by nerve injury in mice. Effects are represented of the i.p. administration of 3.5 mg/kg of DADS or 0.7 mg/kg of GYY4137 combined with 3 mg/kg or 65 μg of morphine (MP), intraperitoneally or subplantarly injected, on the mechanical allodynia (**A**,**B**), thermal hyperalgesia (**C**,**D**), and cold allodynia (**E**,**F**) provoked by sciatic nerve injury (CCI) in the ipsilateral paws. The effects of these treatments administered alone are also represented. Sham-operated (SHAM) animals treated with SS plus SS are also represented. For each test, * represents significant differences vs. SHAM-animals treated with SS plus SS, + vs. CCI-mice treated with SS plus SS, # vs. CCI-mice treated with SS plus MP and $ vs. CCI-mice treated with DADS or GYY4137 plus SS ($p < 0.05$; one-way ANOVA, followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 6$ animals for treatment).

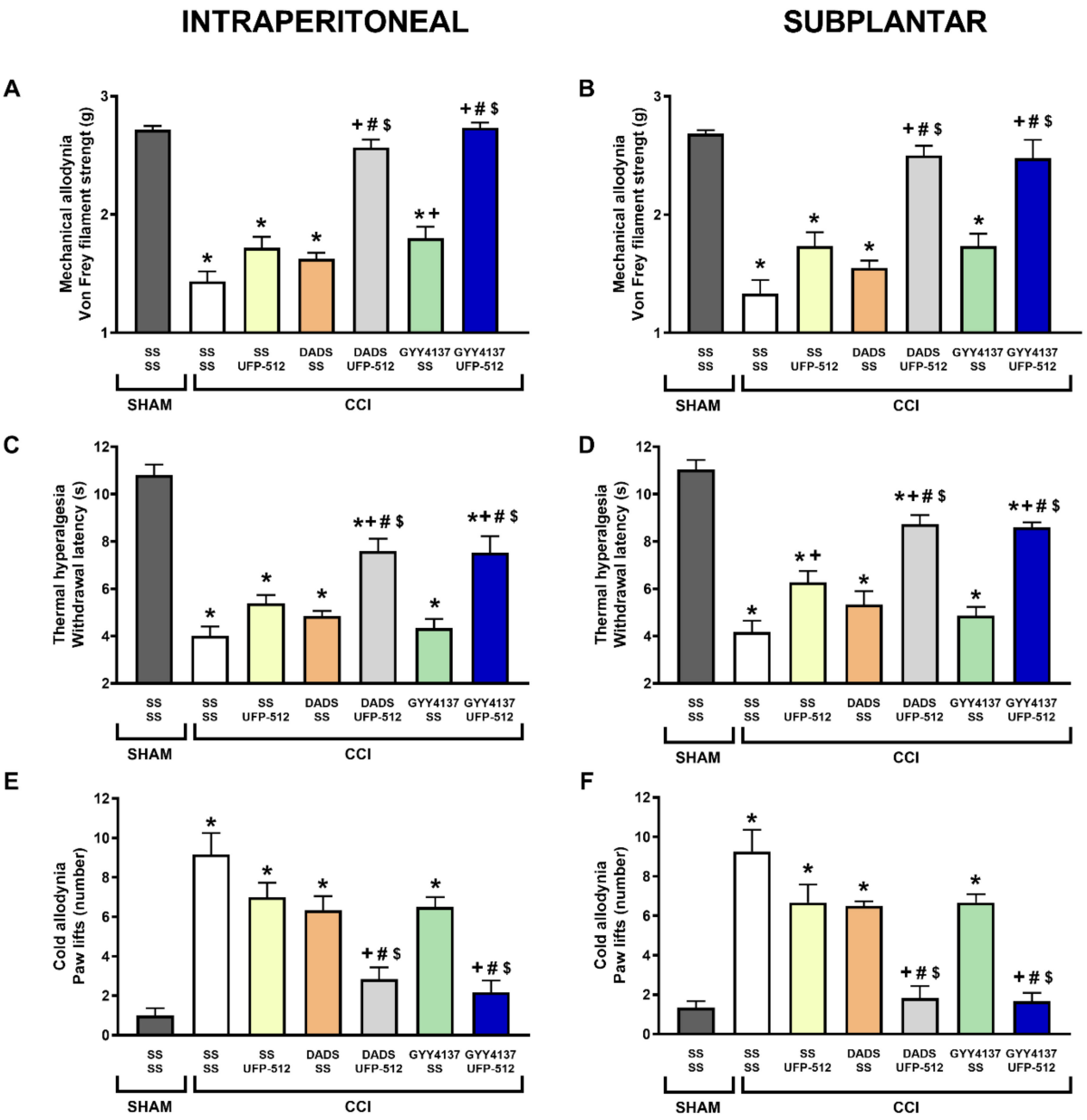

Figure 2. Effects of the co-administration of DADS or GYY4137 with UFP-512 on the mechanical allodynia, thermal hyperalgesia, and cold allodynia induced by nerve injury in mice. Effects are represented of the i.p. injection of DADS (3.5 mg/kg) or GYY4137 (0.7 mg/kg) co-administered with 1 mg/kg or 12.5 μg of UFP-512, intraperitoneally or subplantarly injected, on the mechanical allodynia (**A**,**B**), thermal hyperalgesia (**C**,**D**), and cold allodynia (**E**,**F**) provoked by sciatic nerve injury (CCI) in the ipsilateral paws. The effects of these treatments administered alone are also represented. Sham-operated (SHAM) animals treated with SS plus SS are also represented. For each test, * represents significant differences vs. SHAM-animals treated with SS plus SS, + vs. CCI-mice treated with SS plus SS, # vs. CCI-mice treated with SS plus UFP-512 and $ vs. CCI-mice treated with DADS or GYY4137 plus SS ($p < 0.05$; one-way ANOVA, followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 6$ animals for treatment).

3.2. Reversal of the Antinociceptive Effects of DADS and GYY4137 with MOR and DOR Antagonists

To investigate the possible involvement of the endogenous opioid system in the analgesic actions of DADS and GYY4137 during neuropathic pain, we assessed the reversion of the antinociceptive actions produced by a high dose of DADS (30 mg/kg) or GYY4137

(24 mg/kg) with the administration of the MOR antagonist, naloxone, injected at 3 mg/kg, i.p. or 20 µg, s.p. The effects induced by each of these treatments administered individually were also evaluated.

Our results demonstrated that systemic and local treatment with naloxone reversed the inhibition of the mechanical allodynia ($p < 0.001$, one-way ANOVA; Figure 3A,B), thermal hyperalgesia ($p < 0.001$, one-way ANOVA; Figure 3C,D) and cold allodynia ($p < 0.001$, one-way ANOVA; Figure 3E,F) induced by both H_2S donors. In all tests, i.p. or s.p. treatment with naloxone did not alter the nociceptive responses induced by CCI.

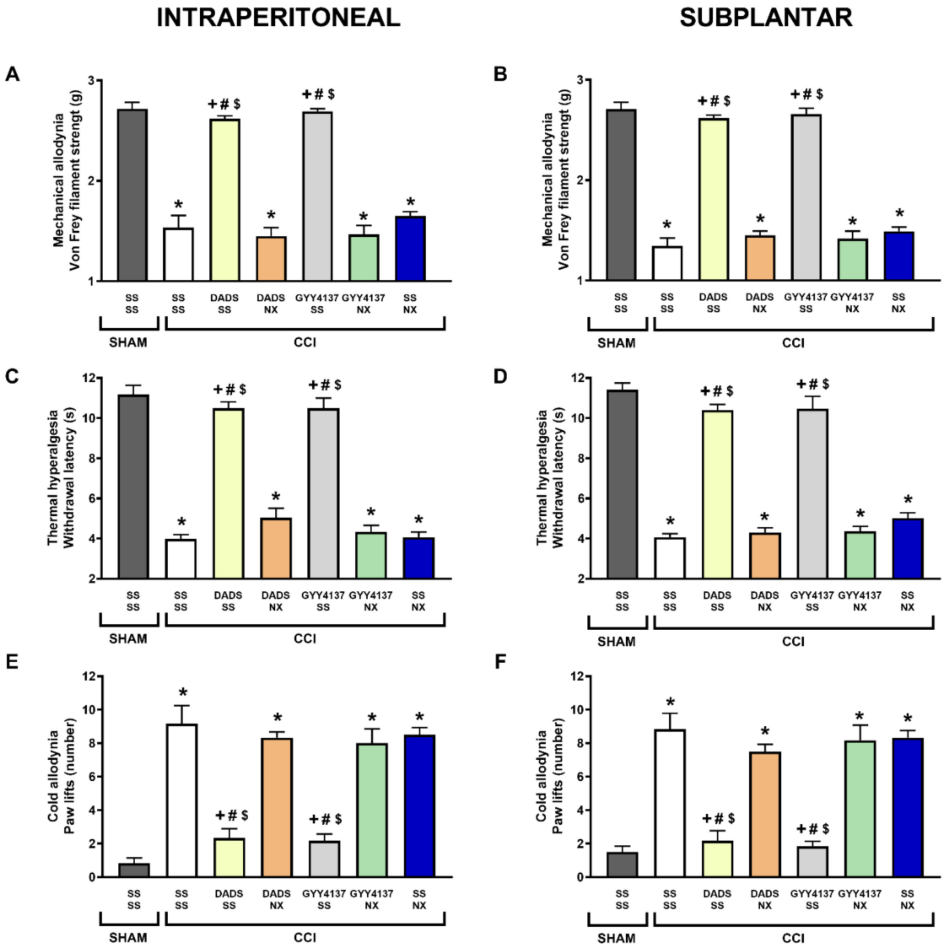

Figure 3. Reversal of the antiallodynic and antihyperalgesic effects of DADS and GYY4137 by naloxone during neuropathic pain. The inhibitory actions produced by i.p. injection of DADS (30 mg/kg) or GYY4137 (24 mg/kg) alone, and combined with naloxone (NX) intraperitoneally (3 mg/kg) (**A**,**C**,**E**) or subplantarly injected (20 µg) (**B**,**D**,**F**) in mice with CCI-provoked neuropathic pain, are represented. Sham-operated (SHAM) animals treated with SS plus SS are also represented. For each test, * represents significant differences vs. SHAM-animals treated with SS plus SS, + vs. CCI-mice treated with SS plus SS, # vs. CCI-mice treated with DADS or GYY4137 plus NX, and $ vs. CCI-mice treated with SS plus NX ($p < 0.05$; one-way ANOVA, followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 6$ animals for treatment).

Similar results were obtained with the co-administration of DADS (30 mg/kg) or GYY4137 (24 mg/kg) with the DOR antagonist, naltrindole. Indeed, the i.p. (3 mg/kg) or s.p. (50 µg) injection of naltrindole reversed the inhibition of the mechanical allodynia ($p < 0.001$, one-way ANOVA; Figure 4A,B), thermal hyperalgesia ($p < 0.001$, one-way ANOVA; Figure 4C,D), and cold allodynia ($p < 0.001$, one-way ANOVA; Figure 4E,F) produced by high doses of DADS and GYY4137. In all tests, i.p. or s.p. treatment with naltrindole did not alter the nociceptive responses induced by CCI.

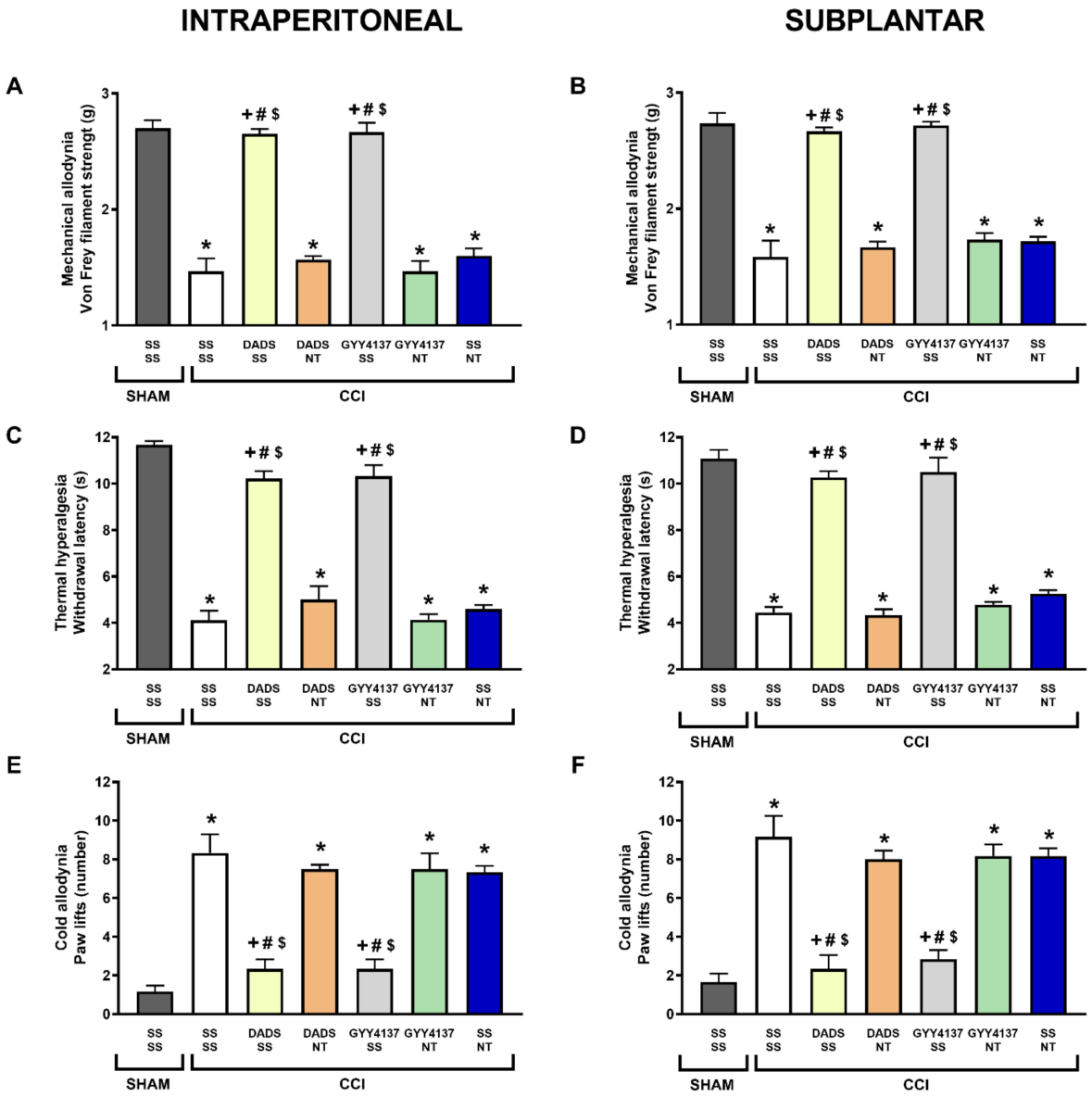

Figure 4. Reversal of the antiallodynic and antihyperalgesic effects of DADS and GYY4137 by naltrindole during neuropathic pain. The inhibitory actions induced by the i.p. injection of DADS (30 mg/kg) or GYY4137 (24 mg/kg) alone, and combined with naltrindole (NT) intraperitoneally (3 mg/kg) (**A**,**C**,**E**) or subplantarly injected (50 μg) (**B**,**D**,**F**) in mice with CCI-provoked neuropathic pain, are represented. Sham-operated (SHAM) animals treated with SS plus SS are also represented. For each test, * represents significant differences vs. SHAM-animals treated with SS plus SS, + vs. CCI-mice treated with SS plus SS, # vs. CCI-mice treated with DADS or GYY4137 plus NT, and $ vs. CCI-mice treated with SS plus NT ($p < 0.05$; one-way ANOVA, followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 6$ animals for treatment).

Finally, the administration of SS, DADS, or GYY4137 alone, and combined with naloxone or naltrindole, did not alter the responses obtained in the contralateral paws of nerve-injured animals, nor in the contralateral and ipsilateral paws of sham-operated mice (data not displayed).

3.3. Effects of DADS and GYY4137 on the Expression of Antioxidant Enzymes in the MS and DRG of Nerve-Injured Mice

To assess the effects of treatment with DADS and GYY4137 in the central and peripheral antioxidant system, the protein levels of HO-1, NQO1, SOD-1, and GSTM1 in the MS and

DRG were assessed. In the MS, sciatic nerve injury decreased the protein levels of SOD-1 ($p < 0.0104$; one-way ANOVA vs. sham-operated animals treated with SS) (Figure 5D), and both treatments, DADS and GYY4137, reversed its down-regulation. Moreover, both H_2S donors increased the HO-1 ($p < 0.0068$; one-way ANOVA vs. sham-operated and nerve-injured animals administered with SS) (Figure 5A) and GSTM1 levels ($p < 0.0018$; one-way ANOVA vs. sham-operated and nerve-injured mice administered with SS) (Figure 5E). No alterations in NQO1 levels were observed in any group (Figure 5B).

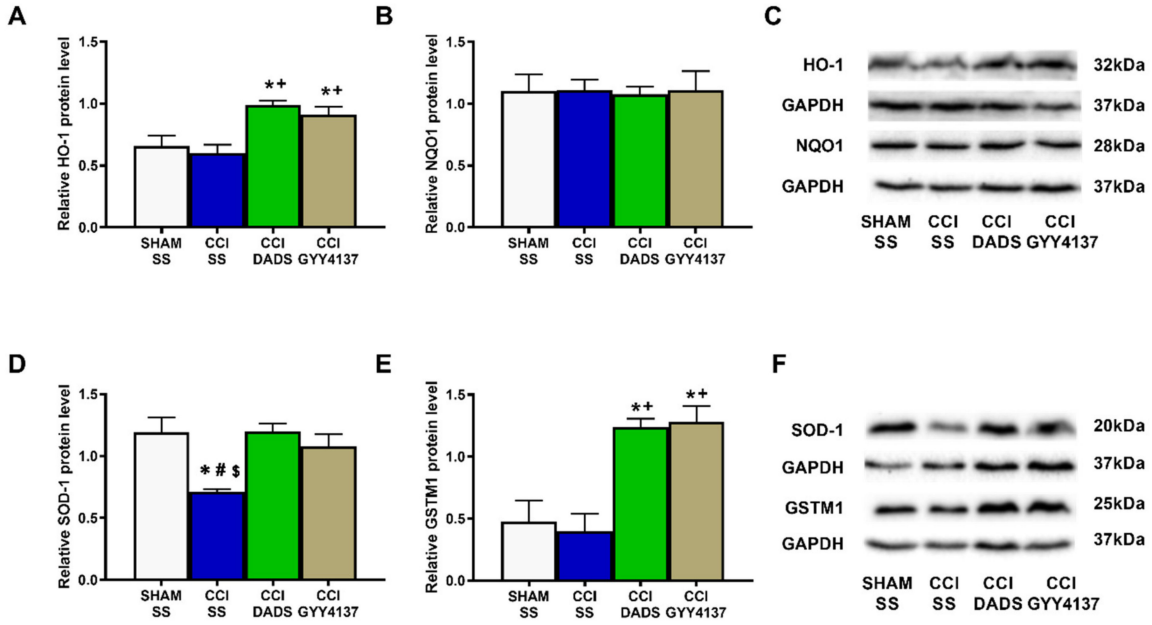

Figure 5. The influence of DADS and GYY4137 treatments on the expression of HO-1, NQO1, SOD-1, and GSTM1 in the MS of nerve-injured mice. Both treatments improved the HO-1 (**A**) and GSTM1 (**E**) levels, and normalized the downregulation of SOD-1 (**D**) induced by CCI. No alterations in the expression of NQO1 (**B**) were identified. Sham-operated (SHAM) mice treated with SS were used as control groups. All proteins are expressed relative to GAPDH levels. Blots for HO-1 and NQO1 (**C**), as well as for SOD-1 and GSTM1 (**F**), are displayed. In all pictures, * denotes significant differences vs. SHAM-animals treated with SS; + vs. CCI-mice treated with SS; # vs. CCI-mice treated with DADS; and $ vs. CCI-mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 3$ samples).

In the DRG, whereas no changes in the expression of HO-1 were manifested (Figure 6A), both DADS and GYY4137 increased the expression of NQO1 ($p < 0.0005$; one-way ANOVA vs. sham-operated and nerve-injured animals injected with SS; Figure 6B), SOD-1 ($p < 0.0092$; one-way ANOVA vs. sham-operated and nerve-injured mice injected with SS; Figure 6D), and GSTM1 ($p < 0.0179$; one-way ANOVA vs. sham-operated and nerve-injured mice injected with SS; Figure 6E).

3.4. Effects of Treatment with DADS and GYY4137 on the Protein Levels of 4-HNE and BAX in the MS and DRG of Nerve-Injured Mice

In the MS, nerve injury up-regulated the expression of 4-HNE ($p < 0.0115$; one-way ANOVA vs. sham-operated SS-injected animals; Figure 7A) and BAX ($p < 0.020$; one-way ANOVA vs. sham-operated SS-injected animals; Figure 7B), and both H_2S donors stabilized them. No changes in the 4-HNE or BAX levels were noted in the DRG of nerve-injured animals injected with SS, DADS, or GYY4137 (Figure 7D,E).

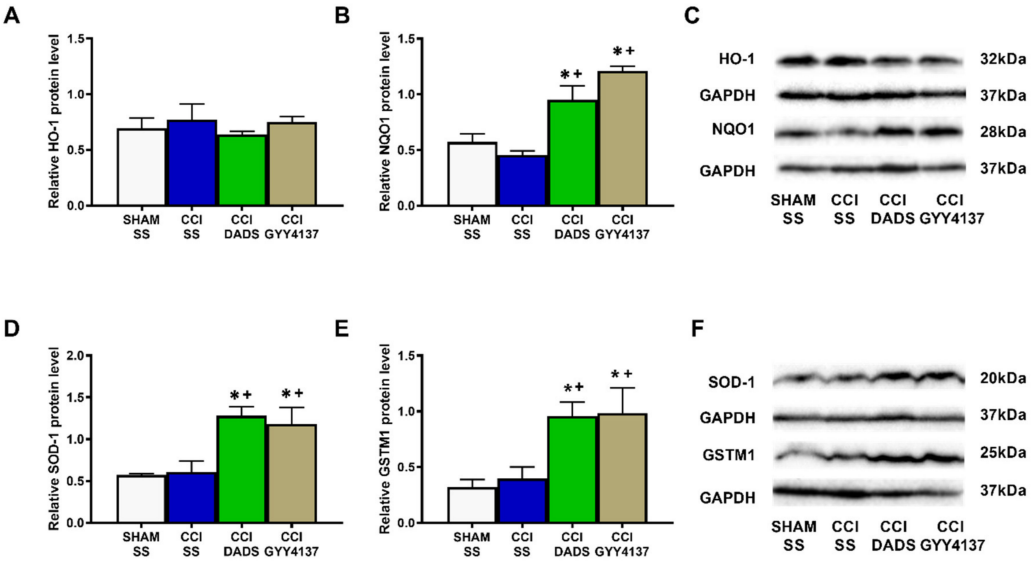

Figure 6. The influence of DADS and GYY4137 treatments on the HO-1, NQO1, SOD-1, and GSTM1 expression in the DRG of animals with neuropathic pain. Both treatments increased the expression of NQO1 (**B**), SOD-1 (**D**), and GSTM1 (**E**) in the DRG of mice with nerve injury-provoked neuropathic pain. No variations in HO-1 levels were identified (**A**). We used sham-operated (SHAM) mice treated with SS as a control group. All proteins are expressed in relation to GAPDH levels. Blots for HO-1 and NQO1 (**C**), as well as for SOD-1 and GSTM1 (**F**), are displayed. In all pictures, * symbolizes significant differences vs. SHAM-animals treated with SS and + vs. CCI-mice injected with SS ($p < 0.05$; one-way ANOVA followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 3$ samples).

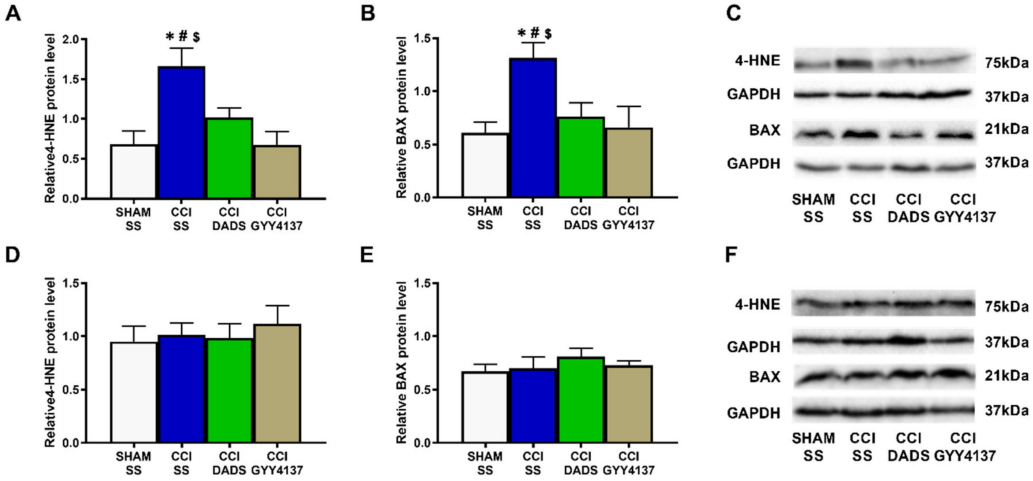

Figure 7. The influence of DADS and GYY4137 treatments on the expression of 4-HNE and BAX in the MS and DRG of mice with neuropathic pain. In the MS, both treatments regularized the overexpression of 4-HNE (**A**) and BAX (**B**). No changes in the expression of 4-HNE (**D**) or BAX (**E**) were detected in the DRG. Sham-operated (SHAM) mice treated with SS were used as control groups. 4-HNE and BAX are expressed relative to GAPDH levels. Blots for 4-HNE and BAX in the MS (**C**) and DRG (**F**) are presented. In all pictures, * symbolizes significant changes vs. SHAM-animals treated with SS; # vs. CCI-mice injected with DADS; and $ vs. CCI-mice injected with GYY4137 ($p < 0.05$; one-way ANOVA followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 3$ samples).

3.5. Actions of DADS and GYY4137 Treatments on the Protein Levels of MOR and DOR in the MS and DRG of Nerve-Injured Animals

The plausible mechanism implicated in the increased analgesic actions of morphine and UFP-512, induced by their co-treatment with H_2S donors, was evaluated by studying the expression of MOR and DOR in the MS and DRG of sciatic nerve-injured mice treated with DADS or GYY4137. Our data showed that no alterations in the MOR (Figure 8A) or DOR (Figure 8B) levels were revealed in the MS of nerve-injured animals injected with SS, DADS, or GYY4137. In contrast, both H_2S donors enhanced the protein levels of MOR ($p < 0.0093$; one-way ANOVA vs. sham-operated and nerve-injured animals injected with SS; Figure 8D), and normalized the decreased levels of DOR provoked by injury ($p < 0.0001$; one-way ANOVA vs. sham-operated animals injected with SS; Figure 8E) in the DRG of animals with neuropathic pain.

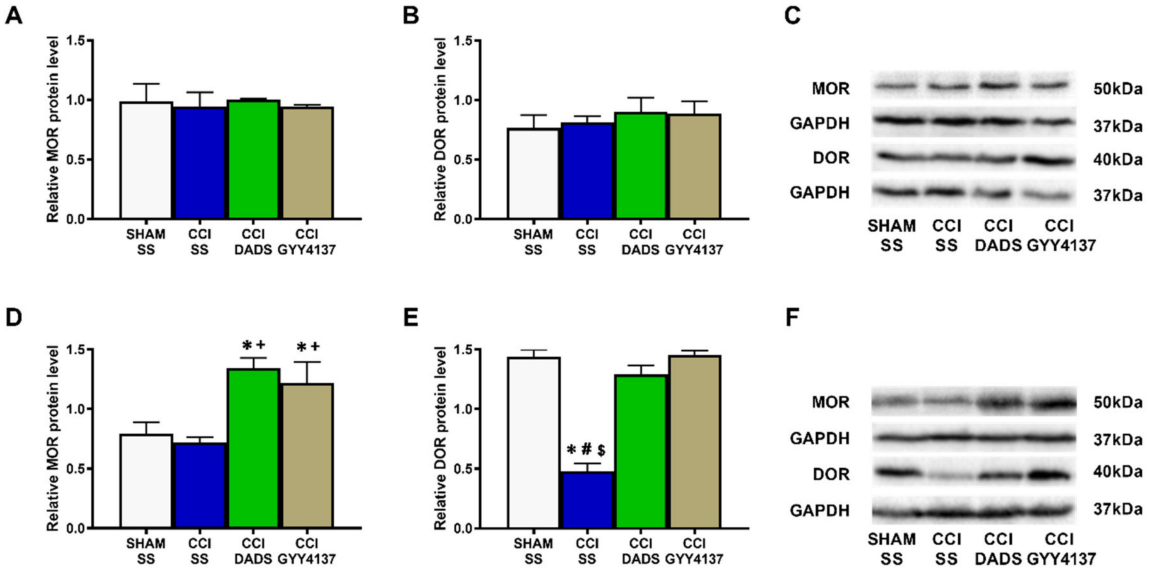

Figure 8. The influence of DADS and GYY4137 treatments on the expression of MOR and DOR in the MS and DRG of mice with neuropathic pain. Both treatments did not alter the protein levels of MOR (**A**) and DOR (**B**) in the MS, increased the expression of MOR (**D**), and avoided the down-regulation of DOR (**E**) in the DRG. Sham-operated (SHAM) mice treated with SS were used as control groups. MOR and DOR levels are expressed in relation to GAPDH. Blots for MOR and DOR in the MS (**C**) and DRG (**F**) are presented. In all pictures, * denotes significant changes vs. SHAM-animals injected with SS; + vs. CCI-mice injected with SS; # vs. CCI-mice injected with DADS; and $ vs. CCI-mice injected with GYY4137 ($p < 0.05$; one-way ANOVA followed by Tukey test). Each column represents the mean, and vertical bars indicate SEM ($n = 3$ samples).

4. Discussions

In this study, we have shown that treatment with DADS or GYY4137 enhances the antinociceptive effects of MOR and DOR agonists, and increases their expression in the DRG of mice with neuropathic pain. Our data also revealed that the antiallodynic and antihyperalgesic effects of DADS and GYY4137 are reversed with specific MOR and DOR antagonists. Furthermore, both H_2S donors modulate the oxidative and/or apoptotic reactions triggered by nerve injury in the MS and DRG of mice.

Chronic pain therapy is complex, basically due to the limited efficacy of conventional pharmacological treatments, especially with opioids [13], so increasing their efficacy is a potential strategy to improve the management of patients with neuropathic pain. Our study reveals that the combined administration of low doses of H_2S donors with MOR or DOR agonists significantly improves the systemic and local antiallodynic and antihyperalgesic actions of morphine and UFP-512 in neuropathic pain. Our results further reveal that, while the co-treatment of DADS or GYY4137 with morphine or UFP-512 fully prevents

CCI-induced mechanical and cold allodynia, these drug combinations do not completely reverse the thermal hyperalgesia provoked by nerve injury, thus revealing the greater effectiveness of these drug combinations in inhibiting allodynia than hyperalgesia caused by nerve-injury. Furthermore, because all tested combinations completely blocked mechanical and cold allodynia, and considering the few adverse effects induced by the local administration of opioids [14], the combination of local opioids with systemic H_2S donors offers a safer and more effective strategy for the treatment of neuropathic pain. These data agree with the significant improvement of the local painkiller actions of DOR agonists in mice with inflammatory pain pre-treated with H_2S donors [20]. Interestingly, other studies have demonstrated that the co-treatment of a carbon monoxide (CO)-slow-releasing compound, tricarbonyldichlororuthenium(II)dimer (CORM-2), or the HO-1-inducer, cobalt protoporphyrin IX (CoPP), increases the analgesic effects of systemically or locally administered morphine, but not those produced by DOR agonists during neuropathic pain [29]. These results underline the opposed actions induced by both gaseous neurotransmitters on the activation of DOR in the same pain model. Furthermore, our data indicate that both DADS and GYY4137 augment the peripheral expression of MOR and avoid the decreased levels of DOR observed in the DRG of nerve-injured mice, thus explaining the enhanced analgesic actions of morphine and UFP-512 in nerve-injured mice pre-treated with H_2S donors. These results are consistent with the increased expression of DOR-induced H_2S in the paws of mice with peripheral inflammation [20], and with the improved expression of MOR in the DRG of CCI-animals administered with CORM-2 or CoPP [29]. The lack of effects of CO/HO-1 activators in the expression of DOR in the DRG might explain the differing effects induced by H_2S and CO on the painkilling actions of DOR agonists under neuropathic pain conditions. Our findings might be of great relevance in clinical practice, by allowing the use of opioids combined with H_2S donors for the management of neuropathy. Moreover, and considering the lowered adverse effects of DOR agonists, its combination with DADS or GYY4137 should be considered as one interesting option to treat neuropathic pain.

Several studies have revealed that CO activates the synthesis of endogenous opioid peptides in inflammatory pain [35]. In this regard, our data demonstrate the reversion of the analgesic actions of both H_2S donors with the systemic or local administration of MOR and DOR antagonists, naloxone and naltrindole. These results suggest the participation of the endogenous opioid system in the antiallodynic and antihyperalgesic effects induced by slow-releasing H_2S donors during neuropathic pain. Our findings are consistent with the reversion of the antinociceptive actions of inhaled H_2S with the administration of MOR antagonists in rats with diabetic neuropathy [36], and with the blockade of the analgesia induced by Na2S, another H_2S releaser, with pre-treatment with selective antisense oligodeoxynucleotide probes against DOR and MOR [37]. This suggests that β-endorphins and enkephalins may be involved in the analgesic actions of H_2S donors during nerve injury-induced neuropathic pain in mice. These findings indicate that the potentiation of the analgesic effects of morphine and UFP-512 produced by H_2S might be exerted not only by enhancing the expression of MOR and DOR, but also by activating the endogenous opioid system in the CNS and PNS.

Oxidative stress participates in the progression of sensitization and cell apoptosis during neuropathic pain [38,39]. Indeed, elevated levels of 4-HNE, an endogenous α,β-unsaturated aldehyde generated during oxidative stress [40], were found in animals with carrageenan-induced inflammation [41] and with spinal cord injury-induced neuropathic pain [42]. Other studies have further revealed that the injection of 4-HNE incites mechanical allodynia [43], and that an accumulation of 4-HNE may disrupt many cell signaling pathways, including the regulation of apoptosis [40,44]. Moreover, increased levels of 4-HNE and/or BAX have been detected in the AMG and PAG of nerve-injured animals, and in the AMG of animals with osteoarthritis pain [23,24]. In accordance with these data, we observed high levels of 4-HNE and BAX in the MS of sciatic nerve-injured mice, which were normalized with DADS and GYY4137 treatments, therefore revealing the antioxidant

and antiapoptotic effects of both H_2S donors in the MS of mice with nerve injury-induced neuropathic pain. Both treatments further normalized the down-regulation of SOD-1 in the MS, and activated the expression of HO-1 and GSTM1 in this brain area, as well as the expression of NQO1, SOD-1, and GSTM1 in the DRG of nerve-injured mice. These results agree with the up-regulation of HO-1, NQO1, and GSTM1 provoked by GYY4137 in the AMG and PAG of nerve-injured animals [23], thus supporting the antioxidant properties of these H_2S donors in the CNS and PNS of animals with neuropathic pain. Moreover, and considering the analgesic properties of several antioxidant compounds [45,46], we hypothesize that the antioxidant and antiapoptotic actions of DADS and GYY4137 may also contribute to potentiating the analgesic activity of MOR and DOR agonists during neuropathic pain.

Finally, it is well recognized that gut microbiota have a great impact in human health [47,48]. Recent studies have shown that neuropathic pain induced by nerve injury [49,50] or by long-term use of morphine [51] can lead to gut dysbiosis, by altering the composition of the microbiota [52], thereby impairing intestinal immune function, promoting neurodegenerative diseases such as Alzheimer and Parkinson [48,53,54], and aggravating the pain associated with neurodegenerative disease [55]. Other studies have also demonstrated that fecal microbiota transplantation and treatments with prebiotics and probiotics are potential therapeutic approaches for Alzheimer [56,57] and Parkinson [58,59], by modifying gut microbiota. Moreover, these treatments also relieve neuropathic pain by inhibiting inflammatory signals or modulating pro-inflammatory and anti-inflammatory T cells [60–62]. Furthermore, it is worth noting that the reconstitution of intestinal microbiota biofilm [63,64] is another important pathophysiological feature of H_2S. Therefore, we postulate that the reversion of gut dysbiosis exerted by H_2S might also contribute to their analgesic effects. Furthermore, the co-administration of low doses of H_2S donors and opioids might also avoid the dysbiosis [51] and bacterial translocation [65,66] induced by high doses of opioids, thus maintaining the homeostasis of gut microbiota. Nevertheless, further studies are needed to demonstrate this hypothesis.

5. Conclusions

In summary, our results demonstrate an improvement in the analgesic properties of MOR and DOR agonists after their co-administration with slow-releasing H_2S donors in animals with neuropathic pain, and suggest that these effects could be explained by the peripheral up-regulation of MOR and DOR, and the activation of the endogenous opioid system induced by DADS and GYY4137. In addition, H_2S-induced antioxidant and antiapoptotic effects in the CNS and PNS could also contribute to potentiating the analgesic effects of opioids during neuropathic pain. This study proposes the co-treatment of H_2S donors with MOR or DOR agonists as a potential therapy for neuropathic pain.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/antiox11071321/s1, Figure S1: The inhibitory effects induced by the subplantar administration of UFP-512 on the mechanical allodynia, thermal hyperalgesia and cold allodynia induced by nerve-injury in mice.

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Hydrogen Sulfide Interacting with Cannabinoid 2 Receptors during Sciatic Nerve Injury-Induced Neuropathic Pain

Xue Bai [1,2], Gerard Batallé [1,2], Ignacio Martínez-Martel [1,2] and Olga Pol [1,2,*]

[1] Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau (IIB Sant Pau), 08041 Barcelona, Spain
[2] Grup de Neurofarmacologia Molecular, Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Barcelona, Spain
* Correspondence: opol@santpau.es; Tel.: +34-619-757-054

Abstract: Hydrogen sulfide (H_2S) donors make opioids more effective in inhibiting nociception during inflammatory and neuropathic pain. We examined whether the analgesic, anxiolytic and/or antidepressant actions of the cannabinoid 2 receptor (CB2R) agonist, JWH-133, might be improved by pretreatment with H_2S donors, DADS and GYY4137 in mice with sciatic nerve injury-provoked neuropathy (CCI). The reversion of the antinociceptive effects of these treatments with the CB2R antagonist, AM630, and the regulatory actions of H_2S in the phosphorylation of NF-κB inhibitor alpha (IKBα) and in the brain-derived neurotrophic factor (BDNF), CB2R, Nrf2 and heme oxygenase 1 (HO-1) levels in prefrontal cortex (PFC), ventral hippocampus (vHIP) and periaqueductal gray matter (PAG), were examined. Data showed that the analgesic effects of JWH-133, systemically and locally administered, were improved by the DADS or GYY4137 pretreatment. The co-treatment of GYY4137 with JWH-133 also stopped anxiodepressive-like activities that concur with neuropathy. Our data likewise showed that both H_2S donors normalized the inflammatory (p-IKBα), neurotrophic (BDNF) variations caused by CCI, increased the expression of CB2R and activated the Nrf2/HO-1 antioxidant pathway in PFC, v-HIP and/or PAG of animals with neuropathic pain. In addition, the blockade of the analgesia produced by high doses of DADS and GYY4137 with AM630 indicated the contribution of the endocannabinoid system in the effects of H_2S during neuropathic pain, thus supporting the positive interaction between H_2S and CB2R. Therefore, this study demonstrates the potential use of CB2R agonists combined with H_2S donors as a possible treatment for peripheral nerve injury-caused neuropathic pain and the associated emotional disturbances.

Keywords: analgesia; anxiety; BDNF; cannabinoids; cannabinoid 2 receptors; depression; hydrogen sulfide; neuropathic pain; oxidative stress

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1. Introduction

Neuropathic pain is a chronic disease with clinical features such as allodynia, hyperalgesia, paresthesia and spontaneous pain, among others. Patients with peripheral nerve injury causing neuropathic pain experience high rates of comorbidities, such as depression and anxiety, which can persist for a long time and have a negative impact on patients' lives [1,2].

Most pharmacological treatments used for the management of peripheral neuropathic pain, including the use of opioids, pregabalin and gabapentin, are ineffective and have serious side effects that limited their therapeutic use [3–5]. Therefore, there is a need to identify new therapies for the management of neuropathic pain and its accompanying affective disorders, with few side effects.

The therapeutic potential of the cannabinoid system in different neurological diseases has been widely demonstrated [6]. There are two types of cannabinoid receptors: type 1 (CB1R) and 2 (CB2R) [7,8]. CB1Rs are mostly found in the central nervous system (CNS) [9],

and CB2Rs are found in the microglia and immune cells, although they were also detected in the spinal cord and dorsal root ganglia [10]. More recent research further revealed that CB2Rs were also distributed in the hippocampus (HIP), cortex and cerebellum [11].

Interestingly, since CB2R agonists do not cause psychiatric disorders like CB1R agonists, their therapeutic role in pain has been extensively investigated [12–14]. Various studies showed that selective agonists of CB2R protected neurons from apoptosis [15] and reduced neuroinflammation after spinal cord injury [16]. Moreover, although some findings reported that CB2R agonists could induce anxiety-like behaviors and that the overexpression of CB2R produced depressive-like behaviors in mice [17,18], the antidepressant and anxiolytic effects of CB2R agonists in animals suffering diabetic neuropathy, inflammatory or osteoarthritis pain have also been proven [12,13,19]. Nevertheless, the possible effects of CB2R agonists in mood disorders associated with the chronic constriction of sciatic nerve (CCI)-provoked neuropathic pain have not been fully investigated.

Previous research demonstrated that the repetitive administration of two hydrogen sulfide (H_2S) donors, DADS, diallyl disulfide and/or GYY4137, morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate dichloromethane complex, alleviated neuropathic pain provoked by CCI [20], as well as of neuropathy caused by chemotherapy and osteoarthritic pain besides their accompanying anxiety- and depressive-like behaviors [21,22]. On the other hand, these two H_2S donors augmented the analgesic effects of opioids and those produced by carbon monoxide (CO) donors or a heme oxygenase 1 (HO-1) inducer in mice with neuropathic and joint pain [23,24]. However, whether treatment with DADS and/or GYY4137 could potentiate the analgesic actions of CB2R agonists, its effects on the emotional disorders and/or regulate the brain levels of CB2R in animals with CCI-induced neuropathic pain remains unknown, being the main objectives of this study.

Oxidative stress and central sensitization are inextricably linked to the development of neuropathic pain and its accompanying mood disorders [25,26]. These associated emotional disorders are also linked with neuroinflammation and high levels of proinflammatory targets such as the nuclear factor κB (NF-κB), of which the activation induces the phosphorylation of kappa-B inhibitor (p-IKBα) in the CNS and PNS [27,28]. Moreover, exogenous melatonin and irisin alleviated neuropathic pain-associated affective disorders and ethanol-induced behavioral deficits by suppressing the NF-κB activation pathway [29,30].

Brain-derived neurotrophic factor (BDNF) is important for controlling the neuroplasticity and neurogenesis in the CNS (cortex, HIP and subventricular zone) [31] and peripheral nervous system (PNS), for example, in the dorsal root ganglia [32]. Thus, previous studies have shown that stress, injury or inflammation could induce the upregulation of BDNF in the PNS [33], and the down- and upregulation in specific brain areas, such as the HIP and periaqueductal gray matter (PAG), respectively [34,35]. Nonetheless, the effects of H_2S in the brain expression of BDNF of animals with affective-like behaviors linked with chronic pain are not fully known.

Hence, in male mice with anxiodepressive-like behaviors accompanying CCI-incited neuropathic pain, we examined: (1) the result of the combined administration of DADS and GYY4137 with JWH-133 in modulating the nociceptive responses; (2) the reversion of the analgesics properties of JWH-133, DADS and GYY4137 with AM630 (a CB2R antagonist); (3) the anxiolytic and/or antidepressant actions of JWH-133 alone and co-administered with GYY4137; (4) the effects of H_2S in the expression of p-IKBα, BDNF, CB2R, NRF2 and HO-1 in the PFC, ventral HIP (vHIP) and PAG, areas deeply involved in the control of nociception and emotions [36–39].

2. Materials and Method

2.1. Animals

We used male C57BL/6 mice from Envigo Laboratories (Barcelona, Spain) to carry out the experiments. The mice (age, 5–6 weeks; weight, 21–26 g) were kept under standard conditions of light/dark (12/12 h), temperature (22 ± 1 °C), and relative humidity

(55 ± 10%), with free access to water and food. The experiments were performed between 9:00 a.m. and 5:00 p.m., after a week of acclimatization and according to the ethical guidelines of the European Commission's directive (2010/63/EC) and the Spanish Law (RD 53/2013), and were approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona (ethical code 9863). Maximal efforts to reduce the number and suffering of animals were accomplished.

2.2. Neuropathic Pain Induction

CCI was utilized for inducing neuropathic pain. Under isoflurane anesthesia conditions (3% induction, 2.5% maintenance), after the biceps femoris and the gluteus superficialis were separated by blunt dissection, three ligatures (4/0 silk) across the sciatic nerve were performed. The same process, excluding nerve ligation, was employed in the control mice (sham).

2.3. Mechanical Allodynia

Mechanical allodynia was assessed by evaluating the hind paw withdrawal reaction to the von Frey filament stimulation, from 0.4 to 3.5 g (North Coast Medical, Inc., San Jose, CA, USA). The mice were positioned in Plexiglas tubes (20 cm high × 9 cm diameter) with a wire grid bottom. Using the up–down paradigm [40], a filament of 0.4 g was applied first, and depending on the animal's reaction, the strength of the next filament was increased or reduced. The threshold of the response was estimated applying an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that incorporated curve-fitting of the data.

2.4. Thermal Hyperalgesia

Thermal hyperalgesia was evaluated by measuring the paw withdrawal latency in response to a radiant heat using the plantar test (Ugo Basile, Varese, Italy) [41]. The mice were arranged in Plexiglas tubes (20 cm high × 9 cm diameter) and positioned on a glass surface. The heat source sited on the plantar surface of the hind paws was activated with a light beam intensity until the paw withdrawal. The paw withdrawal latencies were obtained from the mean of the three separate trials.

2.5. Cold Allodynia

Cold allodynia was determined by recording the amount of elevation of each hind paw of the mice exposed to the cold plate (4 ± 0.5 °C) (Ugo Basile, Italy) for 5 min.

In all paradigms, both the ipsilateral and contralateral paws were assessed.

2.6. Anxiety-like Behaviors

We used the elevated plus maze (EPM), an X-shaped structure with 2 open and 2 closed arms, to evaluate the anxiety-like behaviors. All arms were 5 cm wide and 35 cm long, and the closed ones had 15 cm high walls. At the beginning of the test, the mouse was placed in the central area of the maze, always looking at the same open arm, and its behavior was recorded with a digital camera for 5 min. Both the number of entries into the open and closed arms were recorded, as well as the percentage of time spent in the open arms.

2.7. Depressive-like Behaviors

The tail suspension test (TST) and the forced swimming test (FST) were used to evaluate depressive-like behaviors.

In the TST, an adhesive tape, attached 1 cm from the end of the tail, was used to suspend the animals from a firm structure placed 35 cm from the ground. The animals were recorded for 8 min and their immobility time during the last 6 min was quantified.

In the FST, the mice were placed individually inside a plexiglass tube (25 cm high × 10 cm diameter) containing water at 24 ± 1 °C, up to a height of 10 cm. The mice were recorded for 6 min and their immobility time was measured for the last 4 min.

All these tests were performed by researchers blinded to the experimental conditions and the animals, between 9 and 10 weeks old, were familiarized with the testing room for 1 h before the start of the experiment.

2.8. Western Blot

The animals were euthanized by a cervical dislocation at 28 days after surgery (CCI or sham). The brain, obtained by performing a craniotomy, was dissected using a brain matrix. The PFC, HIP and PAG areas were identified following the Paxinos and Franklin's stereotaxic coordinates [42]. Additionally, vHIP was separated from the dorsal HIP using the methodology described by [43]. The specific areas were extracted by cutting with a clean scalpel or a punch, pushing the metal gently, but firmly, into the tissue. Rocking it back and forth allowed for making the cut and harvesting the region of interest, which was then placed into labeled pre-chilled Eppendorf tubes and stored at –80 °C until further use. The samples from two animals were pooled into one experimental sample to obtain sufficient protein levels to perform Western blot analysis. The expression of p-IKBα, BDNF, CB2R, NRF2 and HO-1 were evaluated. The tissues were sonicated in a cold lysis buffer, the RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA). After 1 h of dissolution (4 °C), the crude homogenate was sonicated (10 s) and centrifuged at 700× *g* (20 min) at 4 °C. After that, 60 µg of the total protein combined with 4x Laemmli loading buffer was loaded onto a 4% stacking/12% separating sodium dodecyl sulfate polyacrylamide gel. The electrophoretic transfer of the proteins onto polyvinylidene fluoride membranes for 120 min was successfully performed. The membranes were blocked with phosphate-buffered saline (PBS; Sigma-Aldrich, MO, USA) containing 5% nonfat dry milk, Tris-buffered saline with Tween 20 containing 5% bovine serum albumin (BSA; Sigma-Aldrich, MO, USA) or 5% nonfat dry milk and PBS with Tween 20 containing 5% BSA (75 min). The membranes were incubated with rabbit primary antibodies anti-p-IKBα (1:150), BDNF (1:150) and NRF2 (1:150) from Abcam (Cambridge, UK); IKBα (1:150) from Cell Signaling Technology (Danvers, MA, USA); HO-1 (1:100) from Enzo Life Sciences (New York, NY, USA); CB2R (1:150) from Cayman Chemical Company (Ann Arbor, MI, USA); and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000) from Merck (Billerica, MA, USA) as a loading control, at 4 °C overnight. The blots were incubated at room temperature for 1 h with secondary polyclonal antibodies conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The ECL kit (GE, Healthcare, Little Chalfont, Buckinghamshire, UK) was used to detect the proteins, and the Image-J program (National Institutes of Health, Bethesda, MD, USA) for densitometric analysis.

2.9. Experiments

Our objective is to find a treatment that inhibits not only the allodynia and hyperalgesia provoked by nerve injury, but also the anxiodepressive-like behaviors accompanying neuropathic pain. Therefore, considering that nerve injury leads to sensorial hypersensitivity from the onset of injury, whereas the associated anxiodepressive-like behaviors are only obvious after several weeks (4–6 weeks) of injury [44], all these experiments were performed at 28 days after CCI.

Firstly, baseline responses were established using the following test sequence: von Frey filaments, plantar and cold plate tests. After baseline measurements, neuropathic pain was induced by CCI, and the animals were tested again at day 28 after surgery using the same sequence as previously.

To examine the impact of H_2S in the pain reliever actions of JWH-133, a CB2R agonist [6] in neuropathic pain, the antinociceptive effects of the systemic co-administration of small doses of DADS (3.5 mg/kg) or GYY4137 (0.7 mg/kg) with JWH-133, 2 mg/kg, injected intraperitoneally and 5 µg subplantarly, were assessed (n = 8 animals/group).

The impact of the intraperitoneal co-injection of 0.7 mg/kg GYY4137 with 2 mg/kg JWH-133 in the anxiodepressive-like behaviors accompanying neuropathic pain were also determined in parallel within different groups of animals (n = 8 animals per group). These

experiments were performed at day 28 after CCI induction. The doses of JWH-133 were chosen from the dose-response curves of this study and those of the DADS and GYY4137 from preceding works [23]. In all cases, the animals were treated with DADS and GYY4137 15 min prior to JWH-133 injection, and the tests were accomplished after 45 min.

The reversal of the painkilling effects produced by 50 mg/kg or 150 µg JWH-133, 30 mg/kg DADS or 24 mg/kg GYY4137 with 3 mg/kg or 90 µg AM630, administered intraperitoneally or subplantarly, were also tested. The JWH-133 and H_2S donors were administered 15 min prior to AM630, and the tests were performed 45 min afterward (n = 8 mice/group). The doses of DADS, GYY4137 and AM630 were chosen in accordance with previous findings [23].

Lastly, using the samples of animals that have undergone functional tests, the effects of DADS or GYY4137 on the protein levels of p-IKBα, BDNF, CB2R, NRF2 and HO-1 in the PFC, v-HIP and PAG were evaluated. The controls were sham-operated mice administered with vehicle. In these experiments, 4 samples/group were evaluated based on the sample size analysis performed with the data obtained in a pilot experiment, accepting a risk of $\alpha = 0.05$ and $\beta = 0.2$ in a two-tailed test.

2.10. Drugs

Both GYY4137 and DADS from Sigma-Aldrich (St. Louis, MO, USA) were dissolved in 0.9% saline, and given intraperitoneally (10 mL/kg) 1 h before testing [21]. JWH-133 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in a saline solution with 1% Tween 80 (Sigma-Aldrich, St. Louis, MO, USA), and AM630 (LabClinics (Barcelona, Spain) dissolved in a mixed solution containing 90% saline, 5% DMSO and 5% Tween 80, were both injected in a volume of 10 mL/kg (intraperitoneal) or 30 µL (subplantar) [45,46].

All drugs were freshly prepared, and for each group treated with a drug, the respective control group received the same volume of the corresponding vehicle.

2.11. Statistical Analyses

We used the Prism 8.0 (Graphpad, La Jolla, CA, USA) and the SPSS (version 28, IBM, Madrid, Spain) programs for the statistical analysis. The normal distribution of the data was determined using the Kolmogorov–Smirnov test, and the sample size was calculated using GRANMO program. A two-way analysis of variance (ANOVA) was used to evaluate the antinociceptive actions of JWH-133 and the anxiolytic and antidepressant effects of JWH-133 alone and with GYY4137. A one-way ANOVA followed by a Tukey test was employed to see the effects of DADS and GYY4137 alone or with JWH-133 or AM630, as well as the impact of H_2S donors on protein levels. The results are presented as the mean values ± standard error of the mean (SEM). A p value < 0.05 was considered significant.

3. Results

3.1. The Antinociceptive Effects of JWH-133 during Neuropathic Pain

Our results showed that JWH-133, given intraperitoneally (1–50 mg/kg) and subplantarly (5–150 µg) dose-dependently inhibited the allodynia and hyperalgesia generated by CCI (Figure 1).

For all tests, the two-way ANOVA demonstrated the significant effects of the dose and group, and their interaction ($p < 0.001$).

Nerve injury reduced the threshold of paw withdrawal to a mechanical stimulus in comparison to sham-operated mice treated with the vehicle ($p < 0.001$, one-way ANOVA) (Figure 1A,D). Mechanical allodynia was progressively reduced with the administration of 1, 2 and 3 mg/kg or 5 and 10 µg of JWH-133, and was totally inhibited with 5, 10, 20 and 50 mg/kg or 30, 50 and 150 µg of this drug, given intraperitoneally or subplantarly ($p < 0.001$; one-way ANOVA vs. CCI vehicle-treated mice).

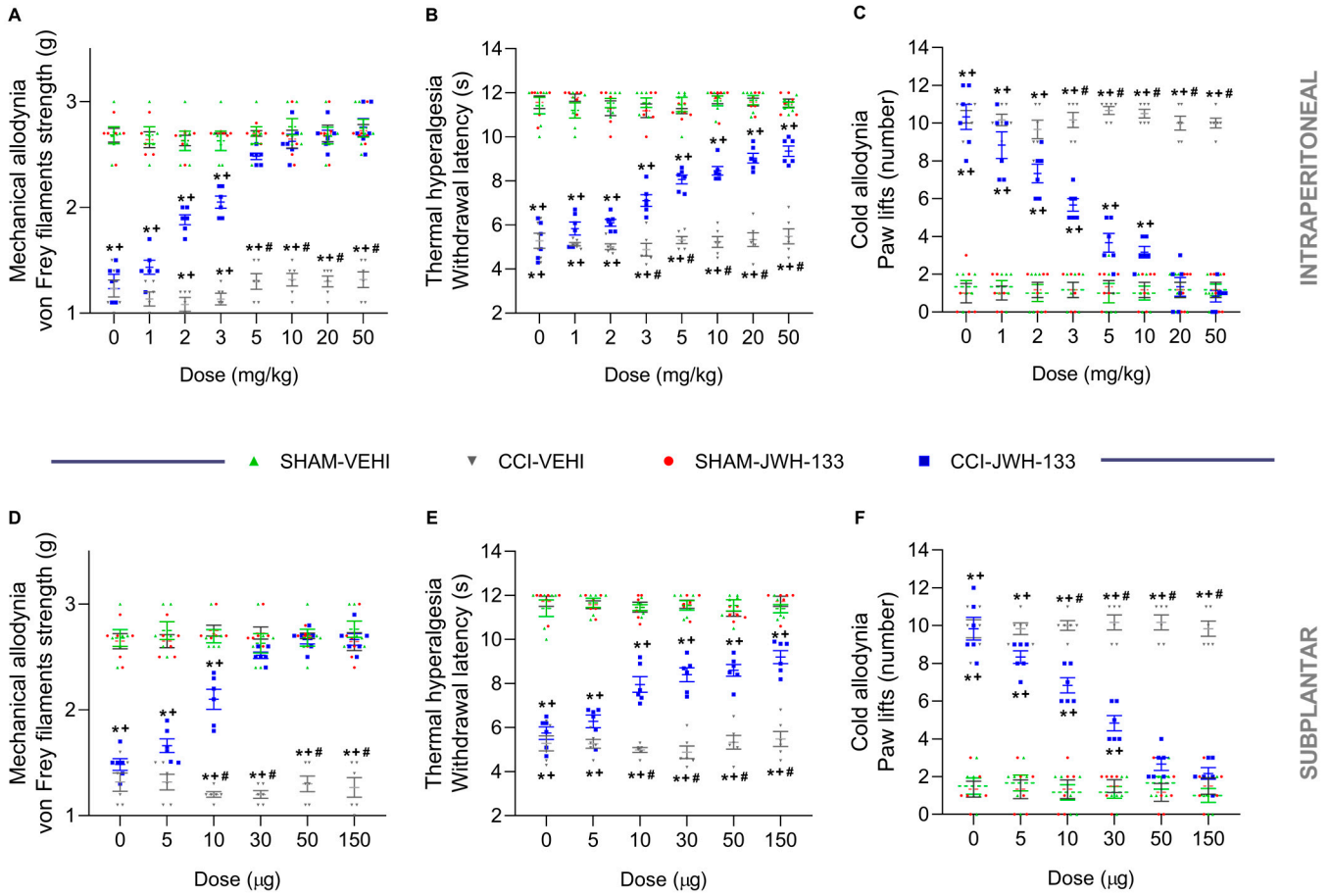

Figure 1. Effects of the intraperitoneal and subplantar administration of JWH-133 on the allodynia and hyperalgesia generated by CCI. Effects of several doses of JWH-133 intraperitoneally (**A**–**C**) and subplantarly (**D**–**F**) administered on the mechanical allodynia (**A**,**D**), thermal hyperalgesia (**B**,**E**) and cold allodynia (**C**,**F**) caused by CCI in the ipsilateral paws. The effects of JWH-133 or vehicle (VEHI) in the ipsilateral paws of sham-operated animals and those of the vehicle in the ipsilateral paws of CCI mice were also represented. For each assay, the symbols denote significant changes versus, * SHAM–VEHI, + SHAM–JWH-133 and # CCI–JWH-133 ($p < 0.05$; one-way ANOVA followed by Tukey test). Results are presented as the mean values ± SEM; $n = 8$ animals/group.

Nerve injury also significantly decreased the threshold for evoking paw withdrawal to a thermal stimulus ($p < 0.001$; one-way ANOVA) (Figure 1B,E). Thermal hyperalgesia was gradually reduced with the administration of 1 to 50 mg/kg or 5 to 150 µg of JWH-133 ($p < 0.001$; one-way ANOVA vs. CCI vehicle-treated mice).

The increased paw lift number provoked by the cold thermal stimulation was detected in CCI mice administered with the vehicle ($p < 0.001$; one-way ANOVA vs. sham-operated mice) (Figure 1C,F). Thermal allodynia was also gradually decreased with the administration of 1, 2, 3, 5 and 10 mg/kg or 5, 10 and 30 µg of JWH-133 and was entirely inhibited with 20 and 50 mg/kg or 50 and 150 µg of this drug injected intraperitoneally or subplantarly ($p < 0.001$; one-way ANOVA vs. CCI vehicle-treated mice).

JWH-133 or vehicle had no effect on the ipsilateral paws of sham-operated mice (Figure 1), nor the contralateral paws of sham-operated or CCI mice.

3.2. The Analgesic Effects of JWH-133 in Animals with Neuropathic Pain Co-Treated with DADS or GYY4137

The antiallodynic and antihyperalgesic activities produced by the systemic injection of 3.5 mg/kg or 0.7 mg/kg of DADS or GYY4137 mixed with JWH-133, intraperitoneally (2 mg/kg) or subplantarly (5 µg), were evaluated (Figure 2).

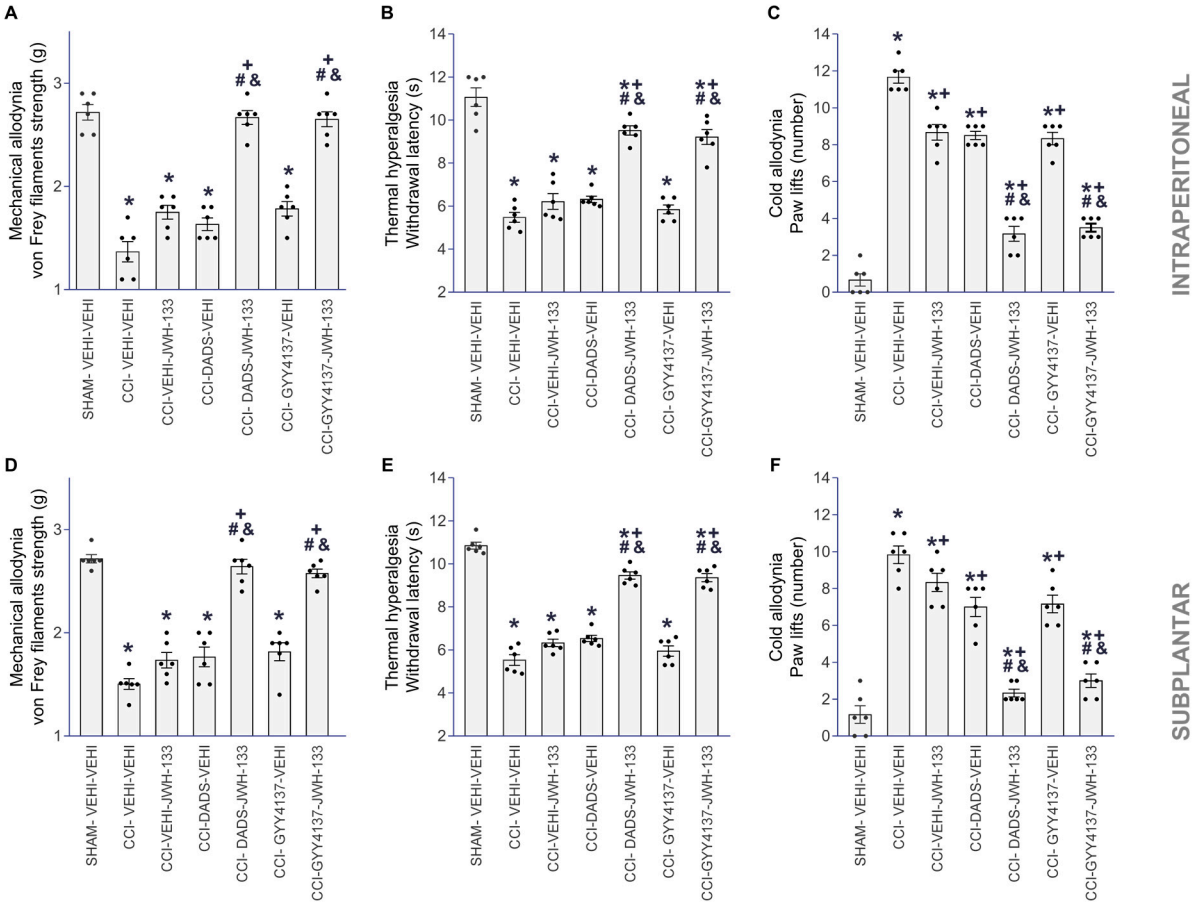

Figure 2. The analgesic actions of JWH-133 co-administered with DADS or GYY4137 in CCI mice. Effects of the co-administration of 3.5 mg/kg of DADS or 0.7 mg/kg of GYY4137 with 2 mg/kg or 5 µg of JWH-133, injected intraperitoneally or subplantarly, on the mechanical allodynia (**A**,**D**), thermal hyperalgesia (**B**,**E**) and cold allodynia (**C**,**F**) caused by CCI in the ipsilateral paws are displayed. The actions of these drugs injected alone are also presented. For each assay, the symbols denote significant changes versus * SHAM–VEHI–VEHI, + CCI–VEHI–VEHI, # CCI–VEHI–JWH-133 and & CCI–DADS–VEHI or CCI–GYY4137–VEHI ($p < 0.05$; a one-way ANOVA, followed by Tukey test). VEHI (vehicle). The results are presented as the mean values ± SEM; $n = 8$ animals/group.

The administration of DADS or GYY4137 significantly improved the mechanical (Figure 2A,D) and cold antiallodynic effects (Figure 2C,F), in addition to the thermal antihyperalgesic effects (Figure 2B,E) made by JWH-133, systemically or locally injected, as related with the effects produced by the vehicle, JWH-133, DADS or GYY4137 given alone (one-way ANOVA, $p < 0.001$).

DADS or GYY4137, administered separately and combined with JWH-133, intraperitoneally or subplantarly, had no impact on the contralateral paws of CCI- or sham-operated mice, nor on the ipsilateral paws of sham-operated animals.

3.3. Reversion of the Antinociceptive Actions of JWH-133, DADS and GYY4137 with AM630

The intraperitoneal (3 mg/kg) and subplantar (90 µg) administration of the CB2R antagonist AM630 reversed the mechanical (Figure 3A,D) and cold antiallodynic actions (Figure 3C,F), as well as thermal antihyperalgesic activities (Figure 3B,E) produced by JWH-133 injected intraperitoneally (50 mg/kg) or subplantarly (150 µg), respectively (one-way ANOVA, $p < 0.001$).

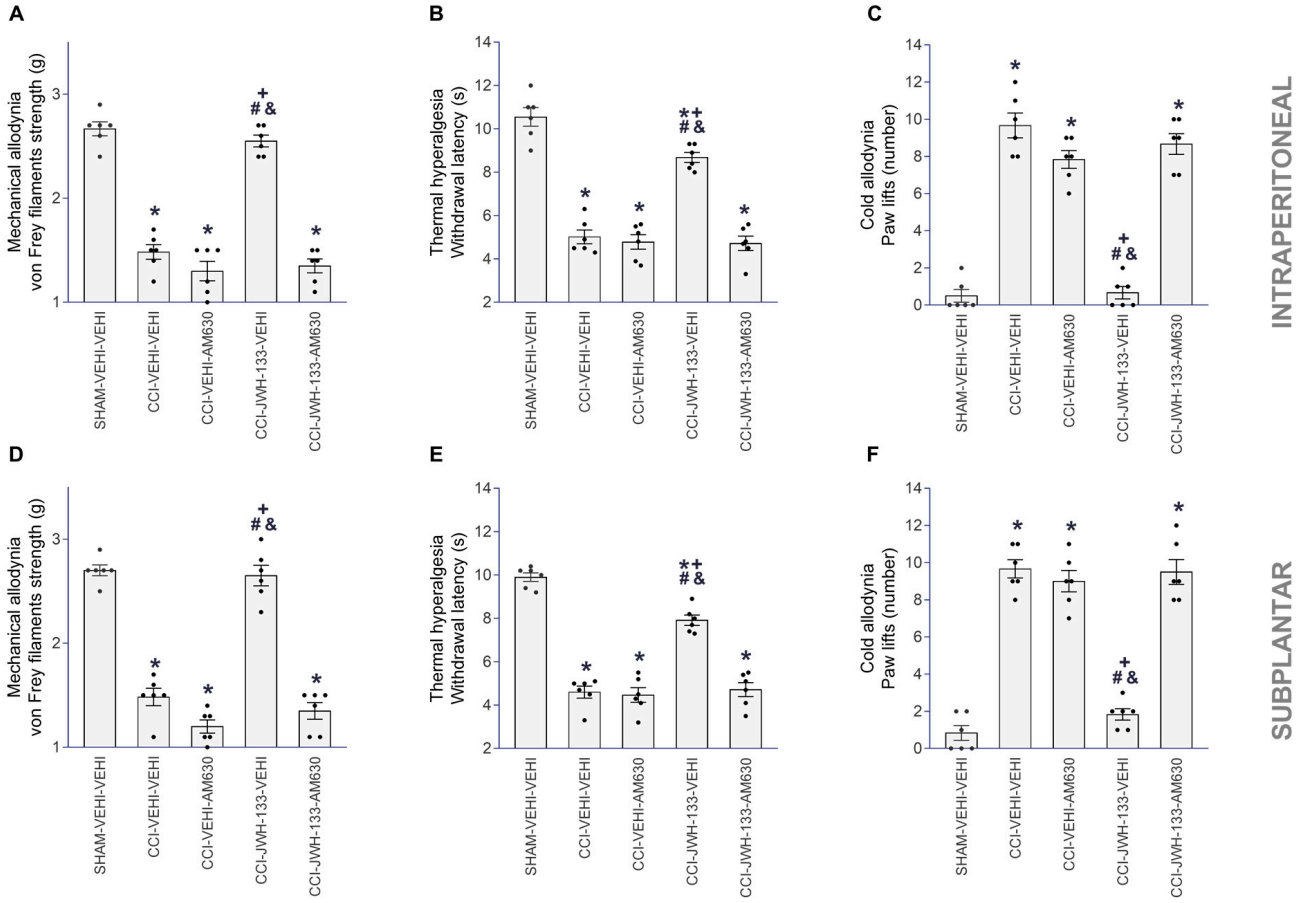

Figure 3. Reversal of the antiallodynic and antihyperalgesic effects of JWH-133 with AM630 in CCI mice. Effects of AM630 injected intraperitoneally (3 mg/kg) or subplantarly (90 µg) on the inhibition of the mechanical allodynia (**A**,**D**), thermal hyperalgesia (**B**,**E**) and cold allodynia (**C**,**F**) produced by the intraperitoneal (50 mg/kg) or subplantar (150 µg) administration of JWH-133 in the ipsilateral paws of CCI mice. For each assay and administration route, the symbols denote significant changes versus * SHAM–VEHI–VEHI, + CCI–VEHI–VEHI, # CCI–VEHI–AM630 and & CCI–JWH-133–AM630 ($p < 0.05$; one-way ANOVA, followed by Tukey test). VEHI (vehicle). The results are presented as the mean values ± SEM; $n = 8$ animals/treatment.

To examine the feasible contributions of the endocannabinoid system in the painkilling properties of H_2S donors, we assessed if AM630 could also reverse the painkiller effects produced by high doses of DADS or GYY4137 in animals experiencing neuropathic pain. Our results showed that the mechanical antiallodynic (Figure 4A,D), thermal antihyperalgesic (Figure 4B,E), and cold antiallodynic effects (Figure 4C,F) of 30 mg/kg DADS and 24 mg/kg GYY4137 were blocked with 3 mg/kg (intraperitoneal) or 90 µg (subplantar) of AM630 (one-way ANOVA, $p < 0.001$).

Furthermore, the treatment with VEHI, DADS, GYY4137, JWH-133 or AM630, alone or combined, given intraperitoneally or subplantarly, did not have any impact on the contralateral paws of the CCI animals nor in the contralateral and ipsilateral paws of sham-operated mice.

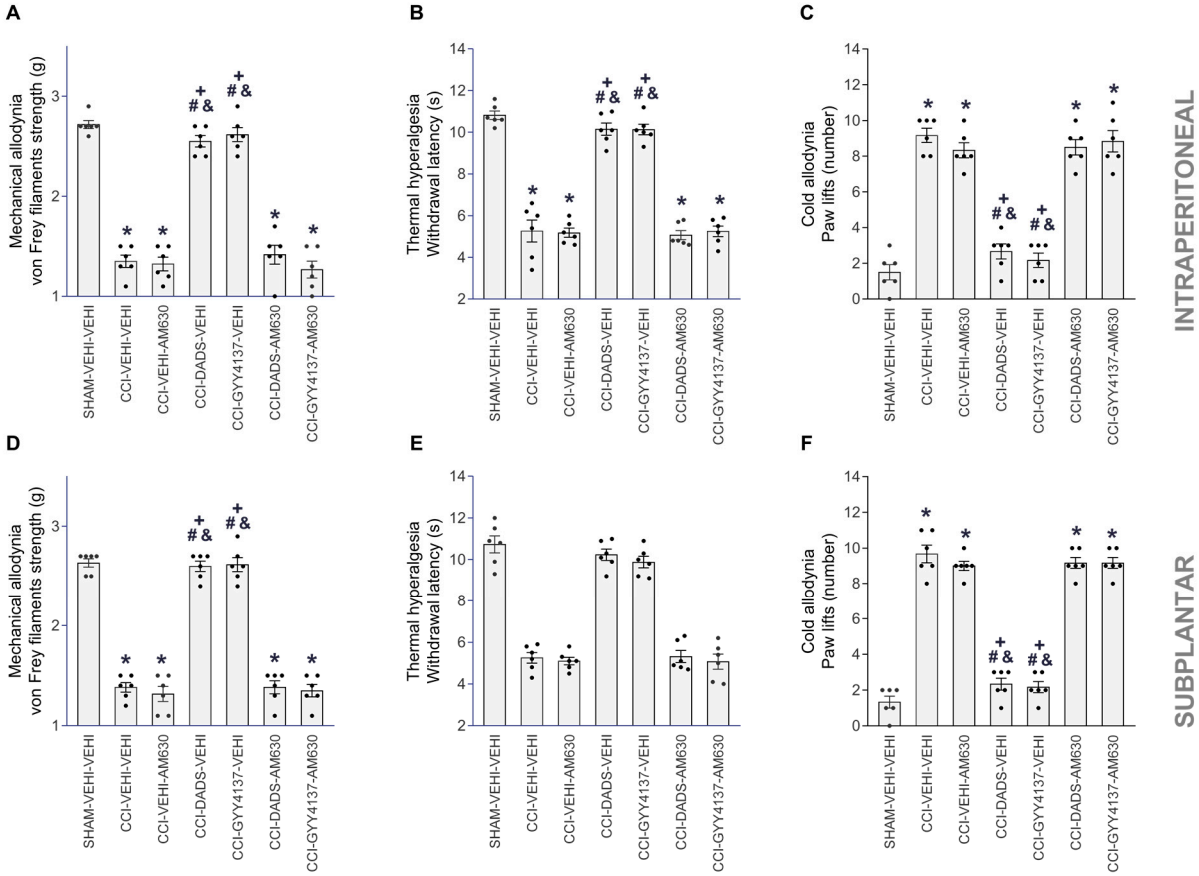

Figure 4. AM630 reversed the antiallodynic and antihyperalgesic effects of DADS and GYY4137 during neuropathic pain. The intraperitoneal (3 mg/kg) and subplantar (90 µg) administration of AM630 reversed the inhibition induced by 30 mg/kg of DADS or 24 mg/kg of GYY4137 in the mechanical allodynia (**A**,**D**), thermal hyperalgesia (**B**,**E**) and cold allodynia (**C**,**F**), provoked by CCI. For each assay and administration route, the symbols denote significant changes versus * SHAM–VEHI–VEHI, + CCI–VEHI–VEHI, # CCI–VEHI–AM630 and & CCI–DADS–AM630 or CCI–GYY4137–AM630 ($p < 0.05$; one-way ANOVA, followed by Tukey test). VEHI (vehicle). The results are presented as the mean values ± SEM; $n = 8$ animals/treatment.

3.4. Effects of JWH-133 Alone or Combined with GYY4137 on the Neuropathic-Pain-Associated Anxiodepressive-liked Behaviors

Considering the high antinociceptive effectiveness of the combined systemic treatment of GYY4137 and JWH-133, we assessed the possible anxiolytic and/or antidepressant actions produced by this combination in animals with neuropathic pain.

In the EPM test, the two-way ANOVA showed significant effects of surgery and treatment, as well as their interaction ($p < 0.001$) regarding the number of entries and the time that the animals remained in the open arms, but not in the number of times that they entered the closed arms. Therefore, the results showed that 2 mg/kg of JWH-133 alone and combined with 0.7 mg/kg of GYY4137 both regularized the reduced quantity of entrances into the open arms (one-way ANOVA, $p < 0.001$; Figure 5A) and the short time spent in them by the CCI animals treated with vehicle (one-way ANOVA, $p < 0.0031$; Figure 5B). The single treatment with GYY4137 did not change the number of entries and the time spent in the open arms of CCI mice. No differences in the number of closed arm entries were identified between the groups (Figure 5C).

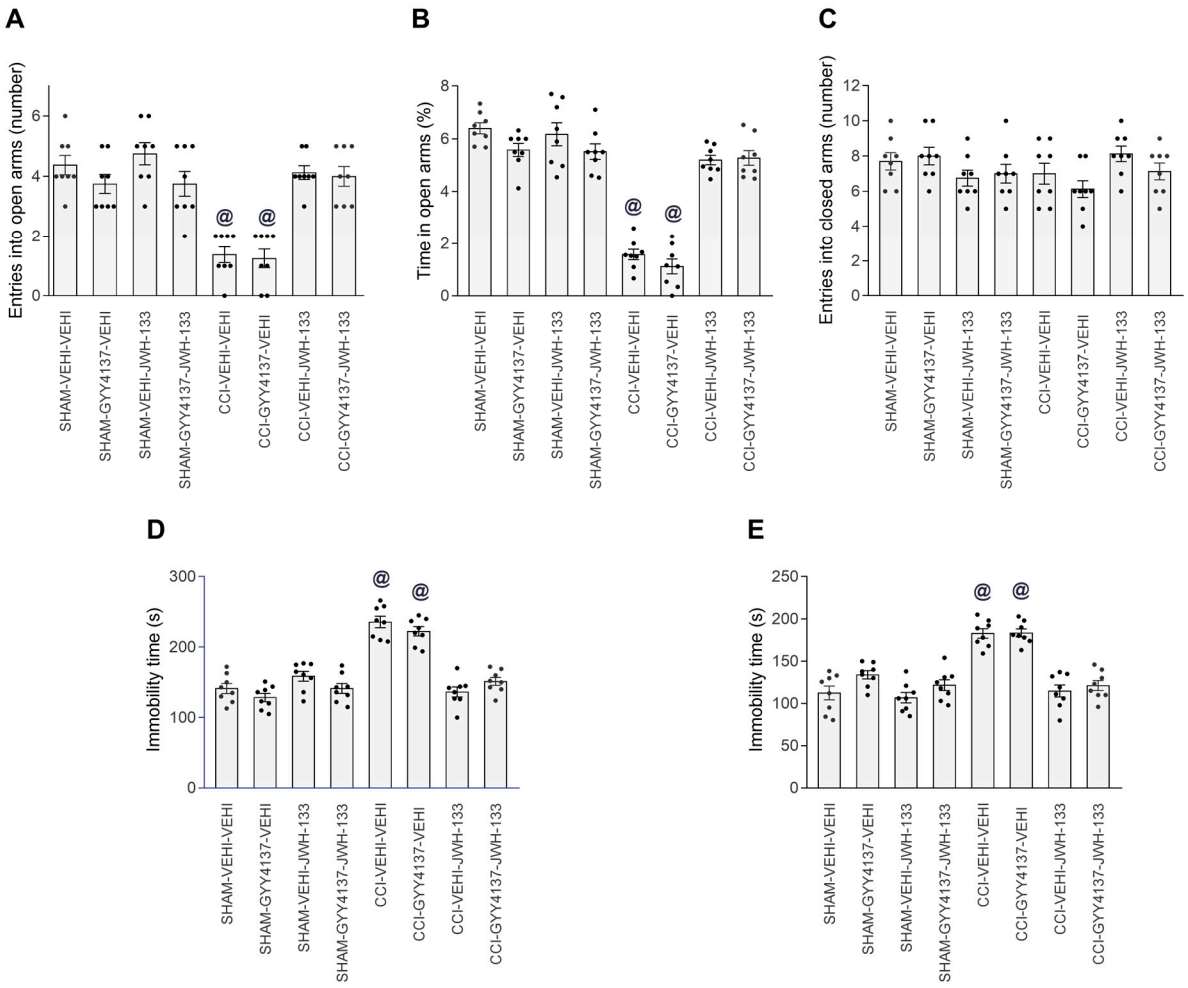

Figure 5. Combined treatment of GYY4137 with JWH-133 inhibited the anxiety- and depressive-like behaviors linked with neuropathic pain. Effects of the intraperitoneal administration of GYY4137 (0.7 mg/kg) and JWH-133 (2 mg/kg), alone and combined, on the anxiety- and depressive-like behaviors associated with CCI-provoked neuropathic pain. The effects of GYY4137, JWH-133, GYY4137 plus JWH-133 or the vehicle (VEHI) in sham-operated mice are also displayed. In the EPM test, the number of entrances into the open arms (**A**), proportion of time spent in the open arms (**B**) and the quantity of entrances into the closed arms (**C**) are shown. In the TST (**D**) and FST (**E**), the immobility times (s) are displayed. For each assay, @ denotes the significant differences versus the rest of the groups ($p < 0.05$; one-way ANOVA, followed by Tukey test). The results are presented as the mean values ± SEM; $n = 8$ animals/group.

The two-way ANOVA also demonstrated significant effects of surgery, treatment, and their interaction ($p < 0.001$) in the TST and FST. Therefore, the elevated immobility time of CCI mice treated with the vehicle in the TST ($p < 0.001$; one-way ANOVA; Figure 5D) and FST ($p < 0.001$; one-way ANOVA; Figure 5E) was decreased by JWH-133 alone or in combination with GYY4137. In these tests, the non-effects of GYY4137 alone were observed in CCI mice.

In addition, the non-effects of treatment with GYY4137, JWH-133 or GYY4137 plus JWH-133 were observed in the EPM, TST or FST of sham-operated mice (Figure 5).

These results revealed the anxiolytic and antidepressant actions produced by a CB2R agonist administered alone and combined with a H_2S donor in animals with affective disorders associated with neuropathic pain.

3.5. Effects of DADS and GYY4137 on the p-IKBα, BDNF, CB2R, NRF2 and HO-1 Levels in the PFC, vHIP and PAG of Mice with Neuropathic Pain

We analyzed the impact of DADS and GYY4137 treatments on the expression of p-IKBα, BDNF, CB2R, NRF2 and HO-1 in the PFC (Figure 6), vHIP (Figure 7) and PAG (Figure 8) of CCI animals. Sciatic nerve injury enhanced the expression of p-IKBα in PFC (Figure 6B; $p < 0.001$; one-way ANOVA) and PAG (Figure 8B; $p < 0.001$; one-way ANOVA) and DADS, as well as GYY4137, reversed these effects. Both treatments further normalized the low and high expression of BDNF in the vHIP (Figure 7C; $p < 0.007$, one-way ANOVA) and PAG (Figure 8C; $p < 0.001$, one-way ANOVA). Moreover, DADS and GYY4137 retained elevated CB2R levels in the PFC of CCI mice (Figure 6D; $p < 0.007$, one-way ANOVA) and activated its expression in the vHIP of these animals (Figure 7D; $p < 0.001$, one-way ANOVA). Both H_2S donors increased the NRF2 expression in the vHIP (Figure 7F; $p < 0.001$, one-way ANOVA), avoided the downregulation of HO-1 in PFC (Figure 6G; $p < 0.001$, one-way ANOVA) and increased its expression in the vHIP (Figure 7G; $p < 0.001$, one-way ANOVA). No changes in the expression of p-IKBα in the vHIP (Figure 7B), BDNF in the PFC (Figure 6C), CB2R in the PAG (Figure 8D), NRF2 in the PFC (Figure 6F) or PAG (Figure 8F), nor HO-1 in the PAG (Figure 8G) were identified.

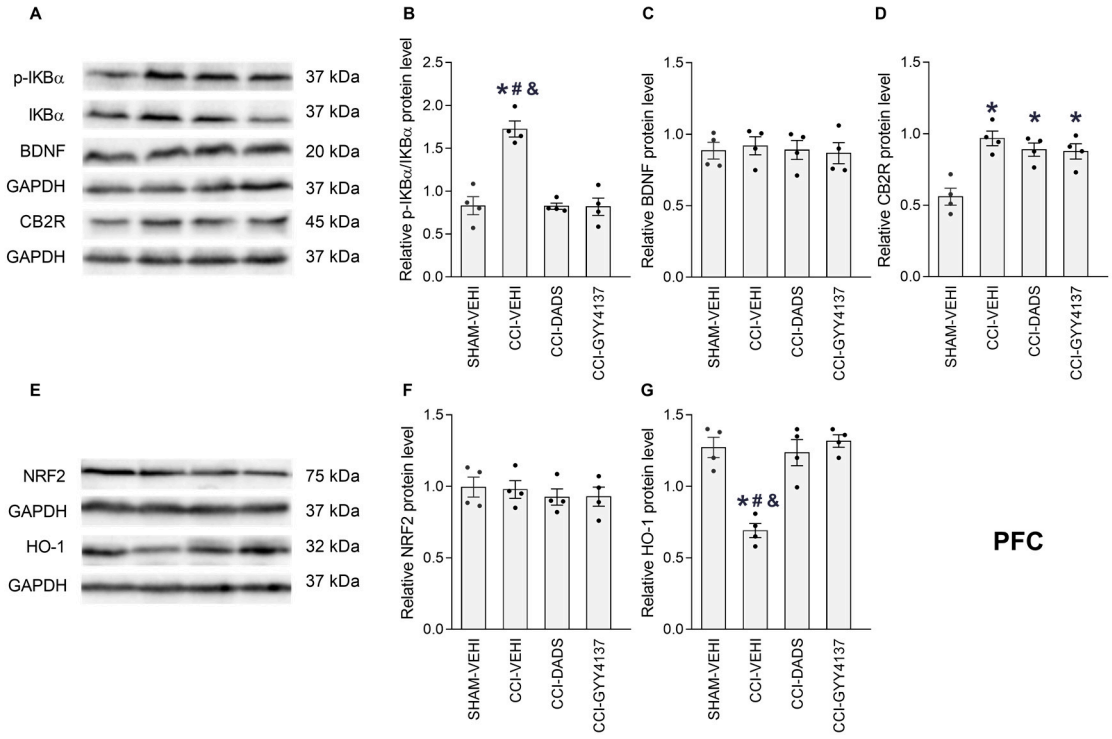

Figure 6. The effects of treatment with DADS and GYY4137 on the expression of p-IKBα, BDNF, CB2R, NRF2 and HO-1 in the PFC of mice with neuropathic pain. Both treatments reversed the up-regulation of p-IKBα (**B**) and the downregulation of HO-1 (**G**), and further maintained the high levels of CB2R (**D**) in CCI mice. No variations in the BDNF (**C**) or NRF2 expressions (**F**) were identified. Sham-operated mice treated with the vehicle (VEHI) were employed as the control group. All proteins are expressed relative to the GAPDH protein levels, except p-IKBα, which is relative to IKBα. Representative blots for p-IKBα, BDNF and CB2R (**A**), and for NRF2 and HO-1 (**E**) are shown. In all pictures, the symbols denote significant changes versus, * sham-operated mice treated with VEHI, # CCI mice treated whit DADS and & CCI mice treated GYY4137 ($p < 0.05$; one-way ANOVA, followed by Tukey test). The results are presented as the mean values ± SEM; $n = 4$ samples/group.

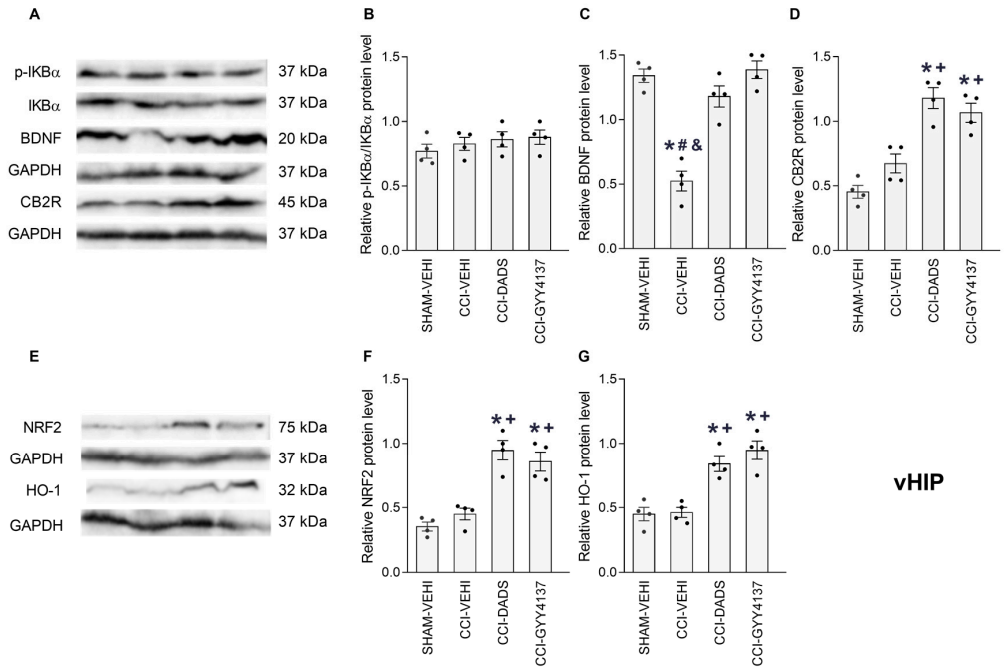

Figure 7. The effects of treatment with DADS and GYY4137 on the expression of p-IKBα, BDNF, CB2R, NRF2 and HO-1 in the vHIP of mice with neuropathic pain. Both treatments reversed the downregulation of BDNF (**C**), and increased the expression of CB2R (**D**), NRF2 (**F**) and HO-1 (**G**) in CCI mice. No variations in the p-IKBα expression (**B**) were identified. Sham-operated mice treated with the vehicle (VEHI) were employed as the control group. All proteins are expressed relative to the GAPDH protein levels, except p-IKBα, which is relative to IKBα. Representative blots for p-IKBα, BDNF and CB2R (**A**), and for NRF2 and HO-1 (**E**) are shown. In all pictures, the symbols denote significant changes versus, * sham-operated mice treated with VEHI, + CCI mice treated with VEHI, # CCI mice treated DADS and & CCI mice treated GYY4137 ($p < 0.05$; one-way ANOVA, followed by Tukey test). The results are presented as the mean values ± SEM; $n = 4$ samples/group.

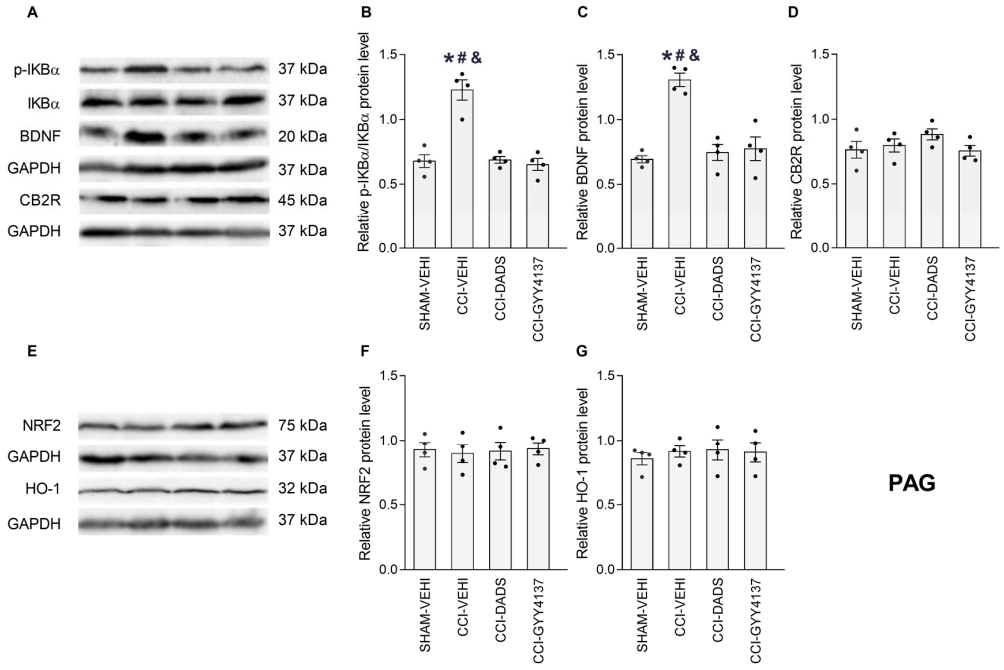

Figure 8. The effects of treatment with DADS and GYY4137 on the expression of p-IKBα, BDNF, CB2R, NRF2 and HO-1 in the PAG of mice with neuropathic pain. Both treatments reversed the

upregulation of p-IKBα (**B**) and BDNF (**C**) induced by CCI. No variations in the CB2R (**D**), NRF2 (**F**) and HO-1 (**G**) were identified. Sham-operated mice treated with the vehicle (VEHI) were employed as the control group. All proteins are expressed relative to the GAPDH protein levels, except p-IKBα, which is relative to IKBα. Representative blots for p-IKBα, BDNF and CB2R (**A**), and for NRF2 and HO-1 (**E**) are shown. In all pictures, the symbols denote significant changes versus, * sham-operated mice treated with VEH, # CCI mice treated whit DADS and & CCI mice treated GYY4137 ($p < 0.05$; one-way ANOVA, followed by Tukey test). The results are presented as the mean values ± SEM; $n = 4$ samples/group.

4. Discussion

Our results demonstrated that pretreatment with H_2S donors significantly improved the antinociceptive effects of JWH-133, a CB2R agonist, and preserved its anxiolytic and antidepressant actions in mice with mood disorders associated with neuropathic pain. The analgesic actions of JWH-133, DADS and GYY4137 were reversed by the local and systemic administration of the CB2R antagonist, AM630. Moreover, both H_2S donors normalized the inflammatory, neurotrophic and oxidative replies elicited by CCI, as well as modulated the expression of CB2R in the PFC, vHIP and/or PAG of animals with neuropathic pain.

Our results showed that JWH-133, given intraperitoneally or subplantarly, dose-dependently diminished the sciatic nerve injury-incited allodynia and hyperalgesia. In accordance, previous studies using JWH-133 and/or other CB2R agonists also demonstrated that their systemic and/or local administration decreased the allodynia and hyperalgesia in different models of neuropathic pain caused by nerve injury, brachial plexus avulsion or cisplatin injection [47–49]. Our data further revealed that the intraperitoneal and subplantar injection of AM630 abolished the analgesic actions of JWH-133, validating the specificity of the central and peripheral pain-relieving actions of this CB2R agonist under neuropathic pain conditions. Studies performed with CB2R-knockout mice supported these findings by demonstrating that the allodynia and hyperalgesia produced by nerve injury increased in these animals [50,51].

Different studies revealed a potentiation of the pain-relieving activity of CB2R agonists through the co-administration of Nrf2 transcription factor activators or HO-1 enzyme inducers in animals with inflammatory pain and neuropathy associated with diabetes [47]. Nevertheless, the effects of H_2S donors in the antinociceptive effects of CB2R, as well as in their plausible anxiolytic and/or antidepressant actions in animals with emotional disturbances accompanying neuropathic pain, have not been previously investigated. For the first time, this study showed that DADS and GYY4137, two slowly H_2S-releasing compounds, augmented the antinociceptive actions of JWH-133, administered systemically or locally, and further preserved the anxiolytic and antidepressant actions performed by this CB2R agonist in CCI mice. On the contrary, the co-administration of CB2R agonists with an HO-1-inducer compound decreased the pain-killing actions of CB2R in CCI mice [47]. Thus, reporting the different effects produced by the two gaseous neurotransmitters, H_2S and CO, in modulating the actions of CB2R agonists under sciatic nerve injury-induced neuropathic pain and highlighting the co-treatment of H_2S with CB2R as a good strategy for relieving CCI-generated neuropathic pain. Interestingly, the analgesia induced by high doses of DADS and GYY4137 administered systemically was reversed by AM630, given intraperitoneally and subplantarly, indicating that central and peripheral endocannabinoids might be involved in the antinociceptive actions of H_2S donors, as it occurs with the opioid system [23].

The endogenous cannabinoid system is also critical in the regulation of affective diseases [18]. Moreover, CB2Rs are found in several brain areas participating in the control of emotional behaviors, such as the amygdala, PFC and HIP, thus sustaining the potential use of CB2Rs in modulating affective disorders [6,18,52]. In this way, our data showed that the intraperitoneal administration of 2 mg/kg JWH-133 did not produce any effect on the EPM, TST or FST of sham-operated mice, but it was capable of reversing the reduced number of entrances and time spent in the EPM open arms, and also reduced the immobility time of CCI mice in the TST and FST. Thus, this showed the anxiolytic and antidepressant

properties of this CB2R agonist under neuropathic pain conditions. In accordance with our results, JWH-133 injected into CB2R-gene-knockout mice did not reverse the anxiogenic-like behaviors associated with neuropathic pain caused by the partial sciatic nerve ligation [14], and the administration of another CB2R agonist (GW405833) also reduced the depressive-like behaviors in rats with mononeuropathy [52].

In addition, this study revealed that the acute treatment with a low dose of GYY4137 did not impede the emotional disorders associated with neuropathic pain, the anxiety- nor depressive-liked behaviors, confirming that the repetitive administration of slow H_2S-releasing donors are needed to modulate the affective deficits under chronic pain conditions [21]. Interestingly, the combining treatment of GYY4137 with JWH-133 maintained the positive emotive actions of the CB2R agonist in CCI mice, suggesting that the effects produced by this combined therapy on mood disorders were mainly produced by JWH-133, while the enhanced analgesic actions of GYY4137 combined with JWH-133 in CCI mice seems to be the result of a positive interaction between both systems, H_2S and CB2R.

In this research, we furthermore analyzed the impact of DADS and GYY4137 on the p-IKBα, BDNF, CB2R, NRF2 and HO-1 protein levels in the PFC, vHIP and PAG of animals with nerve injury-provoked neuropathy. Many studies indicate that the induction and maintenance of neuropathic pain is linked to an inflammatory element [53,54]. Accordingly, elevated levels of p-IKBα were detected in the PFC and PAG of CCI mice that were inhibited by DADS and GYY4137, revealing the anti-inflammatory activity of these treatments, which can contribute to enhance the analgesic effects of CB2R agonists. It has been demonstrated that the excessive synthesis of inflammatory mediators induced by CCI produces a depletion of endogenous cannabinoids in the PFC and abnormal changes of endocannabinoid signaling during neuropathic pain [55]. Therefore, considering that the administration of ATB-352, a H_2S donor, inhibits the enzymes involved in the degradation of the endogenous cannabinoids in the gut [56], we speculated that the reduction of the inflammatory responses produced by DADS and GYY4137 in the PFC and PAG of CCI mice might inhibit the enzyme monoacylglycerol lipase (MGL), involved in the degradation of 2-arachidonoyl glycerol (2-AG), resulting in the normalization of the low levels of this endocannabinoid in the brain, and thus explaining the enhancement of the analgesic effects of JWH-133 produced by DADS and GYY4137 during neuropathic pain. In accordance, it has been demonstrated that the inhibition of MGL with JZL184 increased the 2-AG levels in the brain and reduced neuropathic pain induced by CCI [57,58]. Nonetheless, additional experiments are needed to confirm this theory.

BDNF is a neurotrophic factor with multiple roles in the body, including the regulation of neuronal survival, shaping neurons and synaptic plasticity [59]. It is also involved in the pathophysiology of pain, depression and anxiety [60,61]. BDNF is initially produced as a precursor (pro-BDNF), which is then broken down to produce mature BDNF (mBDNF). ProBDNF and mBDNF have different functions, while mBDNF promotes neuronal survival, differentiation and synaptic plasticity, pro-BDNF induces apoptosis and dendritic spine plasticity [62]. In this study, we evaluated the effects of DADS and GYY4137 on the expression of mBDNF in the PFC, vHIP and PAG of animals with neuropathic pain. Our results demonstrated that CCI increased the mBDNF expression in the PAG and decreased them in the vHIP. In agreement with this, Guo et al., 2006 [63], also demonstrated the high levels of mBDNF in the PAG and rostral ventromedial medulla of animals with peripheral injury, and demonstrated that the hypersensitization induced by central mBDNF is mediated by the activation of the TrkB-NMDAR-dependent excitatory transmission [64]. In this study, we also demonstrated that DADS and GYY4137 normalized the high expression of mBDNF in the PAG, suggesting that these actions might contribute to the modulation of nociception produced by these H_2S donors during neuropathic pain. Other authors indicated that peripheral proBDNF, but not mBDNF, contributes to pain hypersensitivity induced by peripheral inflammation [65], thus revealing the different effects induced by peripheral and/or central pro- and mBDNF in regulating pain.

It is also established that BDNF plays an important role in regulating anxiety and depression. Several studies reveal that pro- and mBDNF might develop different functions in controlling these emotional behaviors [66]. Indeed, the upregulation of proBDNF in the hippocampus of animals with anxiety- and depressive-like behaviors associated with peripheral inflammation [67], and the downregulation of mBDNF in the anterior cingulate cortex of mice with anxiodepressive-like behaviors accompanying neuropathic pain [68], support the different roles played by both forms of BDNF in regulating these affective disorders. Our results likewise demonstrated the downregulation of mBDNF in the vHIP of animals with anxiety- and depressive-like behaviors linked with CCI-induced neuropathic pain. Moreover, both H_2S donors regularized the low levels of BDNF in the v-HIP, contributing to the modulation of the affective disorders produced by them during neuropathic pain [69]. A limitation of this study is the non-evaluation of the proBDNF levels in the PFC, vHIP and PAG, which would have allowed us to know its role in the effects of DADS and GYY4137.

In agreement with another study that has shown increased spinal levels of CB2R during neuropathic pain [70], the high levels of this receptor were displayed in the PFC of CCI mice. Interestingly, both DADS and GYY4137 treatments preserved the upregulation of CB2R in PFC, and augmented its expression in the vHIP of CCI mice, thus explaining the improved painkiller actions and the conservation of the anxiolytic and/or the antidepressant actions of JWH-133 in CCI animals co-treated with GYY4137.

Oxidative stress is also important in the development and preservation of chronic pain [25,71]. In fact, the high levels of oxidative stress markers were observed in the PNS and CNS of animals with neuropathic pain [72,73]. In agreement with these data, low levels of HO-1 were detected in the PFC of CCI mice, and both H_2S donors normalized this downregulation and enhanced the expression of NRF2 and HO-1 in the vHIP. These findings agreed with the upregulation of HO-1 stimulated by GYY4137 in the medial septum of nerve-injured animals [23]. Our results highlight the antioxidant actions of slow H_2S releasers in the CNS of mice with neuropathic pain, suggesting that these properties might also contribute to potentiate the analgesic actions of CB2R agonists during neuropathic pain.

It is well recognized that nerve-injury-induced neuropathic pain altered the gastrointestinal microbiota [74–76] and that the gut microbiota influences neuropathic pain through the modulation of proinflammatory and anti-inflammatory T cells [77]. Moreover, alterations in the composition of the intestinal microbiota have been characterized in depressed patients [78], and studies on animals demonstrated that gut microbiota dysbiosis is associated with depressive- and anxiety-like behaviors [79,80]. In addition, probiotic intervention has been shown to reduce the anxiodepressive-like behaviors in stressed rats [81] and improve mood in depressive patients [82]. Therefore, and considering that the dysfunctions of intestinal microbiota led to reductions in endogenous H_2S [83] and the paucity of the precursors of the endocannabinoid system [84], while the administration of H_2S restored the intestinal microbiota biofilm and stabilized the microbiome–mucosa interface [85]. We postulated that the possible reversion of gut dysbiosis produced by DADS and GYY4137 treatments might normalize the endogenous H_2S levels and/or the endocannabinoid precursors, thus supporting the enhanced analgesic effects of JWH-133 and the preservation of its anxiolytic and antidepressant properties in CCI animals co-treated with DADS or GYY4137. Nevertheless, further experiments are needed to demonstrate this hypothesis.

5. Conclusions

This study demonstrated an enhancement in the antinociceptive effects of JWH-133 induced by H_2S donors, and revealed the anxiolytic and antidepressant effects of JWH-133 combined with GYY4137 in animals with mood disorders associated with neuropathic pain. The mechanism by which H_2S potentiates the analgesic effects of CB2R agonists might be related to the preservation of the high levels of CB2R, together with the inhibition of inflammation, oxidative stress and the BDNF overexpression produced by DADS and

GYY4137 in the PFC and/or PAG of animals with neuropathic pain. This study also revealed the participation of the endogenous cannabinoid system in the DADS and GYY4137 pain-reliever effects. Finally, based on our findings and considering the lack of tolerance or addiction liability of CB2R agonists [6] and the beneficial effects of H_2S on the gastrointestinal system [85], we propose the co-treatment of CB2R agonists plus H_2S donors as a potential effective therapy for peripheral nerve injury-caused neuropathic pain and the associated emotional disturbances, with few side effects.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Autonomous University of Barcelona (protocol code 9863 and date of approval 03-07-2018).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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5. Discussion

In a neuropathic pain model induced by CCI in male mice, our findings revealed that the slow-releasing H_2S donors, DADS and GYY4137, inhibited the allodynia and hyperalgesia, and that GYY4137 is more effectiveness than DADS. Moreover, the acute co-administration of these two H_2S donors with a MOR (morphine), DOR (UFP-512) or CB2R (JWH-133) agonist significantly enhanced the analgesic effects of opioids and cannabinoids during neuropathic pain. Furthermore, both H_2S donors administered alone or combined with JWH-133 suppressed the anxiety- and depressive-like behaviors associated with chronic neuropathic pain. We also found that the analgesic effects of H_2S are not only related with the activation of Kv7 potassium channels and the HO-1 signaling pathway, this gasotransmitter also activates the endogenous opioid and cannabinoid systems, and exhibits anti-inflammatory, anti-apoptotic, anti-nociceptive, and antioxidant actions in the CNS (AMG, PAG, MS, PFC, and/or vHIP) and/or PNS (DRG).

First, our study investigated the analgesic effects of slow-releasing H_2S donors, and opioids and cannabinoids administered alone. Our experimental data revealed that the systemic administration of two H_2S slow-releasing donors, DADS and GYY4137, inhibited the mechanical and cold allodynia, and thermal hyperalgesia caused by CCI at 30 days after injury. These findings support previous studies demonstrating the inhibitory effects of DADS and/or GYY4137 during neuropathic pain at 14 days after nerve injury and in neuropathic pain associated with chemotherapy (Qabazard et al., 2020; Wang et al., 2020). We further validated the analgesic effects induced by inhaled H_2S and other slow-releasing H_2S donors, for example, the isothiocyanates during nerve-injury provoked neuropathic pain (Kida et al., 2015; Lucarini et al., 2018). Moreover, comparing the analgesic effectiveness of DADS and GYY4137, our data showed that GYY4137 is 7.2, 9.9 and 11.8 times more potent than DADS in inhibiting of thermal hyperalgesia, cold allodynia, and mechanical allodynia, respectively. The possible reasons for these differences might be related to the distinct nature and chemical structures of these two donors (Szabo and Papapetropoulos, 2017) and the capacity of GYY4137 to liberate H_2S for longer time than DADS (Liang et al., 2015; Li et al., 2008).

Additionally, our results also demonstrated that the subplantar injection of UFP-512 suppressed neuropathic pain in a dose-dependent manner. This finding is consistent with the results obtained with the systemic administration of this DOR agonist in the same pain model (Polo et al., 2019). Likewise, the systemic and local administration of the CB2R agonist, JWH-133, also relieved the allodynia and hyperalgesia provoked by CCI in a dose-dependent manner. This in accordance with the dose-response analgesic curves described by Hervera et al. (2013) with another CB2R agonist, JWH-015, during neuropathic pain and with the attenuated allodynia and hyperalgesia observed in sciatic nerve-injured transgenic mice overexpressing CB2R (Racz et al., 2008). Our study further reveals that the systemic or local administration of the CB2R antagonist

AM630 reversed the analgesic effects of JWH-133, confirming the involvement of CB2R (rather than CB1R) in its the analgesic actions.

Following the analysis of the individual analgesic effects of these compounds, we investigated the effects produced by the administration of low doses of DADS or GYY4137 in combination with subanalgesic doses of MOR, DOR or CB2R agonists. Our results indicated that the co-treatment of these H_2S donors with the systemic or local administration of opioids or cannabinoids significantly enhanced their analgesic effects, showing that each of these combinations is more effective in inhibiting the allodynia and hyperalgesia that those produced by the single administration of each of them. In addition, considering the low doses of each drug used in the combination and the relatively minor side effects produced by the local administration of opioids (Stein, 2018, Pol, 2021), particularly, those produced by DOR agonists compared to MOR, the co-administration of H_2S donors with DOR, may emerge as a new safe and effective therapeutic strategy for neuropathic pain. On the other hand, and taking into account the few side effects produced by CB2R, compared to CB1R (Kibret et al., 2022), the combination of H_2S donors with CB2R agonists might also be a new safe alternative for treating the emotional disorders associated with chronic pain.

The treatment of the emotional disorders associated with chronic pain continues to be an important clinical problem due to the existing positive feedback between the emotional disruption and pain sensation. That is, negative emotional states can increase pain sensation (Bushell et al., 2013). Therefore, we analyzed the potential anxiolytic and antidepressant effects of the DADS and/or GYY4137 treatments in animals with anxiodepressive-like behaviors associated with neuropathic pain provoked by CCI. The experimental data showed that the low number of entries and short time stay in the open arms of the EPM test, together with the low number of visits and short time stay in the central area of the OF test observed in animals with neuropathic pain treated with vehicle, were completely normalized by the repetitive treatment with DADS and GYY4137, for 3 or 4 days, respectively. Additionally, the high immobility time observed in the TST and FST of mice with neuropathic pain, was also significantly decreased with both DADS and GYY4137 treatments. Thus, revealing the anxiolytic and antidepressant effects of these H_2S donors under these experimental conditions. Our results agree with the improvement of the anxiety-like and depression-like behaviors produced by garlic in diabetic rats (Rahmani et al., 2020) and with the amelioration of the anxiodepressive-like behaviors linked with osteoarthritis pain or chemotherapy induced by GYY4137 and/or DADS treatments (Batallé et al., 2022; Roch et al, 2022). Thus, revealing that these treatments show a more complete therapy for the emotional disorders accompanying neuropathic pain than some of the used first-line drugs for its treatment. That is, while gabapentinoids inhibit neuropathic pain and improve the accompanying anxiety-like behaviors, they do not avoid the associated depressive-like behaviors (La Porta et al., 2016; Alles and Smith, 2018). Moreover, even though the effectiveness of antidepressants for treating pain and depression, they do not reduce the anxiety-like behaviors, and produce significant undesirable effects (Grégoire et al., 2012; Hu et al., 2016). However,

DADS and GYY4137 not only inhibited neuropathic pain, but also suppressed the associated anxiety- and depressive-like behaviors.

Subsequently, we investigated the effects of the CB2R agonist, JWH-133, on the emotional disorders accompanying neuropathic pain. We found that acute administration of low doses of JWH-133 in nerve-injured mice exhibited anxiolytic and antidepressant actions and, as occurs with DADS and GYY4137, did not alter the locomotor activity, revealing the few side effects induced by this treatment in animals with neuropathic pain. Our results are consistent with the antidepressant effects of GW405833 (another CB2R agonist) during neuropathic pain (Hu et al., 2009). Moreover, and given that the common clinical approach for treating mood disorders normally involves the repetitive drug administration, and the long-term use of opioids leads to tolerance, while CB2R agonists do not induce this side effect during neuropathic pain (Hervera et al., 2012). To minimize the potential side effects and maximize the beneficial and safety effects of treatments, we chose to combine low doses of GYY4137 with JWH-133 to assess their effects on the anxiety- and depressive-like behaviors associated with neuropathic pain. The data showed that whereas the single administration of a low dose of GYY4137 did not have any effect on the anxiodepressive-like behaviors, its combined administration with JWH-133, beside increasing the analgesic effects of CB2R agonist, also maintained its efficacy in inhibiting the mood disorders, suggesting that this drug combination might be a novel approach for treating the emotional disorders associated with neuropathic pain.

In addition, we explored the possible mechanisms involved in the analgesic, anxiolytic, and antidepressant effects of DADS and GYY4137, as well as those involved in the increased analgesic actions of opioids and cannabinoids induced by H_2S. The fact that the Kv7 potassium channel blocker XE-991 completely reversed the antiallodynic and antihyperalgesic effects of both H_2S donors, revealed that the analgesic effects of DADS and GYY4137 are mainly produced by activating the Kv7 potassium channels. Consistent with our findings, other studies also showed the participation of these channels, in these and other slow-releasing H_2S donors, in mice with chemotherapy-induced neuropathic pain or with osteoarthritis pain (Di Cesare Mannelli et al., 2017; Batallé et al., 2020). Thus, indicating that, whether natural or synthetic, H_2S donors exert, at least in part, their pain-relieving actions through the activation of Kv7 potassium channels in different chronic pain models.

Our results further displayed that the HO-1 inhibitor (SnPP) completely abolished the analgesic effects of DADS and GYY1437, suggesting the involvement of antioxidant enzyme HO-1 in the analgesic actions of both H_2S donors during neuropathic pain. It's interesting to remark that the HO-1 enzyme, besides its antioxidant and anti-stress properties, it is also implicated in the generation of CO, a gasotransmitter with potent antinociceptive actions in chronic pain (Pol, 2021). Our results are accordingly with the interaction between H_2S and CO/HO-1 demonstrated in mice with osteoarthritis pain (Batallé et al., 2022) as well as in the gastrointestinal system of rodents (Głowacka et al., 2020. In summary, under neuropathic pain conditions, both the Kv7

potassium channels and the HO-1 enzyme are involved in the painkiller actions of both H_2S donors.

Oxidative stress and apoptosis are closely associated with the development of neuropathic pain (Cui et al., 2022). Then, the up regulation of the oxidative stress marker, 4-HNE (Martins et al., 2021), the induction of cellular apoptosis (Dalleau et al., 2013) and the imbalance of the endogenous antioxidant system caused by nerve injury, are implicated in pain sensitization and the accompanying depressive- and anxiety-like behaviors (Krolow et al., 2014, Balmus et al., 2016). In this study, we demonstrated the potent antioxidant and anti-apoptotic properties of H_2S donors by inhibiting the over expression of 4-HNE in the MS and the elevated levels of BAX in the AMG, PAG and MS of animals with neuropathic pain. The treatment with DADS and/or GYY4137 also increased and/or normalized the reduced levels of the Nrf2 transcription factor and its triggering antioxidant enzymes HO-1, NOQ1, SOD-1, and/or GSTM1 in diverse areas of the CNS of the sciatic nerve-injured mice, including AMG, PFC, vHIP, MS, and PAG, as well as of the PNS, such as DRG. As mentioned in the introduction, due to the different functions that have the brain areas analyzed in this study in modulating the nociceptive and emotional responses. Then, it seems that the antioxidant and anti-apoptotic effects of DADS and GYY4137 in the PAG, MS, DRG and/or PFC might contribute to their analgesic actions, while their antioxidative effects in the AMG, vHIP and/or PFC might be involved in the anxiolytic and antidepressant actions produced by both H_2S donors during neuropathic pain. These findings are in accordance with the antioxidant and anti-apoptotic properties of slow-releasing H_2S donors in the CNS and/or PNS of animals with neuropathic pain linked with chemotherapy and/or diabetes (Tang et al., 2015; Roch et al., 2022; Wang et al., 2023).

Microglial activation contributes to the development of chronic pain and the emotional deficits associated (Carrasquillo and Gereau, 2007; Loggia et al., 2015). Therefore, Díaz et al., (2019) demonstrated microglial activation in the HIP and an increase in the expression of p-IKBα in the SC of animals with anxiety- and depression-like behaviors linked with neuropathic pain. In the same way, this study revealed microglial activation in the AMG and an up-regulation of p-IKBα in the PFC and PAG of mice with neuropathic pain, that were inhibited with both DADS and GYY4137 treatments. Thus, suggesting that the anti-inflammatory actions produced both H_2S donors might contribute to the anxiolytic and antidepressant properties, as well as to the analgesia produced by them during neuropathic pain.

In the other hand, the PI3K/AKT and MAKP signaling pathways activated by microglia also play a relevant role in neuropathic pain (Ji et al., 2019; 2013; Liu et al., 2018). In our study, both H_2S donors inhibited the over expression of PI3K and p-AKT in the AMG and PAG of nerve-injured mice, revealing the central mechanism of that the H_2S-mediated antinociception. Additionally, DADS also suppress the ERK phosphorylation in the AMG and PAG, showing a link between the activation of this MAPK and neuropathic pain as well with the anxiety- and depressive-like behaviors associated (Carrasquillo and Gereau, 2007).

Peripheral nerve injury also provokes the release of BDNF, a neurotrophic factor closely related to pain sensitization and emotional disturbances accompanying chronic pain (Coull et al., 2005; Trang et al., 2012; Zhang et al., 2016). Our data demonstrated that nerve damage led to the up regulation of mBDNF in the PAG, supporting its pronociceptive role, and decreased its expression in the vHIP, consistently with the depressive-like behaviors related with diabetic neuropathy (Ge et al., 2019) or postoperative pain (Yang et al., 2020). Our data showed that both DADS and GYY4137 treatments reversed the dysregulation of mBDNF detected in the PAG and vHIP of nerve-injured animals, indicating that the regulatory expression of this neurotrophic factor induced by H_2S donors might be implicated their analgesic, anxiolytic and/or antidepressant effects.

To study the possible mechanisms involved in the enhanced analgesic actions of morphine and UFP-512 in animals pre-treated with slow-releasing H_2S donors, we analyzed the effects of DADS and GYY4137 on the expression of MOR and DOR in the CNS and PNS. Interestingly, DADS and GYY4137 increased the MOR levels and prevented the down regulation of DOR in the DRG, suggesting that these effects might explain the improvement of the pain reliever actions of morphine and UFP-512 induced by H_2S during neuropathic pain. This is in agreement with the investigations of Porta et al. (2021), reporting that DADS stimulated the expression of DOR in the PNS of animals with inflammatory pain. Moreover, the fact that the systemic and local administration of MOR and DOR antagonists, for example naloxone and naltrindole, effectively blocked the analgesia produced by both H_2S donors, indicates that this gas suppresses neuropathic pain, in part, by activating the endogenous opioid system. Therefore, the improvement of the analgesic actions of MOR and DOR agonists induced by H_2S might be supported by the up regulation of their receptors and the activation of the endogenous opioid system. This finding is consistent with the involvement of enkephalins in the analgesia produced by inhaled H_2S in rats with diabetic neuropathy (Li et al., 2020).

Regarding cannabinoids, we also found that the systemic and local administration of AM630, a selective CB2R antagonist, not only completely reversed the effects of JWH-133, but also the analgesic actions of DADS and GYY4137, indicating that these two H_2S donors can also activate the endogenous cannabinoid system to exhibit their pain-relieving properties. This fact can also contribute to the enhancement of the analgesic actions induced by low-doses of JWH-133 when is combined with DADS or GYY4137. In addition, the endocannabinoid system also plays an important role in mood disorders, especially CB2R (Kibret et al., 2022). Therefore, it has been described that CB2R agonists alleviated the anxiodepressive-like behaviors in mice with dopamine neuron loss (Liu et al., 2022). In accordance, our study demonstrated the anxiolytic and antidepressant actions of JWH-133 in mice with neuropathic pain and suggested that the increased or sustained high expression of CB2R in the PFC and vHIP, together with the activation of the endocannabinoid system induced by H_2S, might explain the potentiation of the JWH-133 analgesic effects and the maintenance of its anxiolytic and antidepressant properties in GYY4137 co-treated animals.

Finally, and considering that inflammation can induce a decrease in the endogenous cannabinoid levels in the PFC (Chen et al., 2023), we hypothesize that the reduction of the inflammatory responses induced by DADS and GYY4137, in the PFC and PAG, might inhibit MGL, an enzyme responsible for the degradation of 2-AG, that what it would lead to the restoration of the endogenous cannabinoid levels in the brain. Being this another possible explanation of the augmented analgesic effects produced by JWH-133 combined with DADS and GYY4137. In agreement with this theory, it has been shown that the inhibition of MGL increased the 2-AG levels in the brain and inhibited neuropathic pain (Kinsey et al., 2009; Ignatowska-Jankowska et al., 2015) (Figure 6).

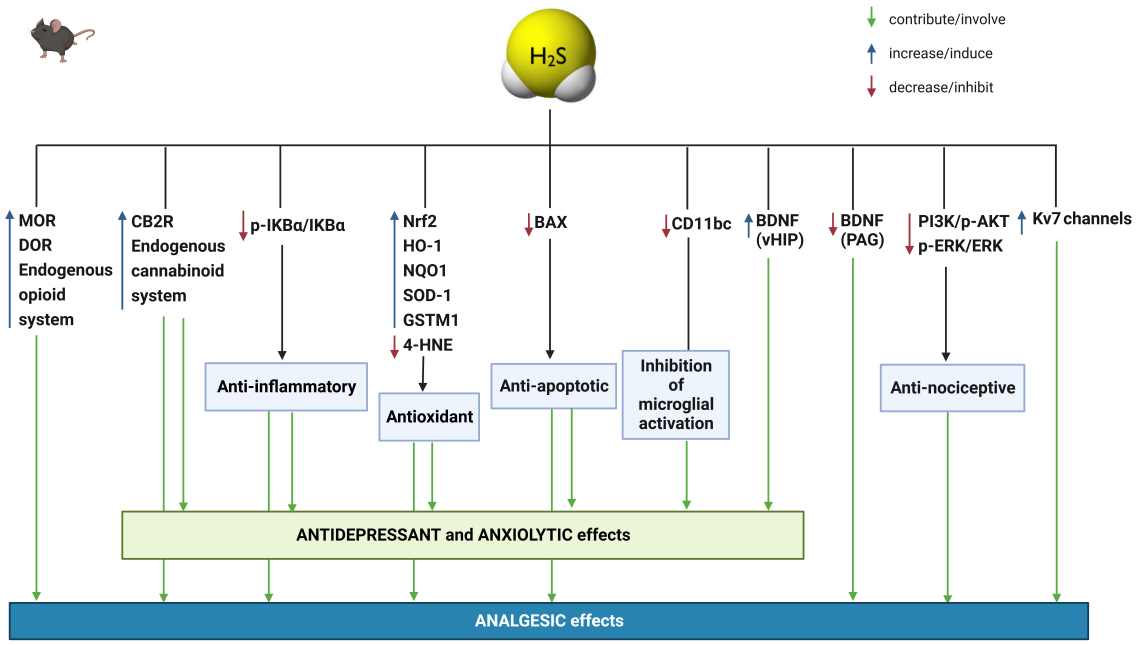

***Figure 6**. The summary of the effects produced by H_2S in neuropathic pain. Our results demonstrated that DADS and GYY4137 produced antidepressant, anxiolytic and analgesic effects by increasing the expression of various antioxidant enzymes (including HO-1), decreasing the levels of inflammatory, oxidative, and apoptotic mediators, modulating mBDNF expression in distinct brain regions, and inhibiting microglial activation. Moreover, the Kv7 potassium channels activation and the inhibition of PI3k/p-AKT and p-ERK 1/2 expression also contributed to the painkiller actions of H_2S donors. Finally, DADS and GYY4137 improved the analgesic effects of MOR, DOR and CB2R agonists by stimulating the endogenous opioid and cannabinoid system and enhancing the expression of their receptors.*

6. Conclusions

In male mice with neuropathic pain and emotional disorders associated, this study demonstrated:

1. The antinociceptive, anxiolytic and antidepressant effects produced by two slow-releasing H_2S donors, DADS and GYY4137, during neuropathic pain. The higher efficacy of GYY4137 vs DADS treatment in inhibiting pain. Thus, suggesting the potential use of these treatments, especially GYY4137, as a desirable candidate for the management of affective disorders accompanying neuropathic pain.

2. The effects of these H_2S donors are mainly produced by blocking the nociceptive, inflammatory, oxidative and/or apoptotic responses provoked by nerve injury, modulating the BDNF expression and activating the Nrf2 signaling pathway in different areas of the CNS and/or PNS. Moreover, the involvement of Kv7 potassium channels and the HO-1 enzyme in the analgesic actions of DADS and GYY4137 was also demonstrated.

3. The improvement of the analgesic properties of MOR and DOR agonists produced by their co-treatment with slow-releasing H_2S donors during neuropathic pain. These effects might be related to the peripheral up-regulation of MOR and DOR, and the activation of the endogenous opioid system induced by H_2S. These findings propose the combined treatment of H_2S donors with opioids, especially DOR agonists, as a potential therapy for neuropathic pain.

4. The enhancement of the antinociceptive actions and the maintenance of the anxiolytic and antidepressant effects of CB2R produced by H_2S in animals with mood disorders associated with neuropathic pain. The involvement of the endogenous cannabinoid system in the DADS and GYY4137 pain-reliever actions and the preservation of the elevated levels of CB2R, might explain these improved effects. These data propose the co-treatment of CB2R agonists plus H_2S donors as an effective treatment for peripheral nerve injury-caused neuropathic pain and the associated emotional disturbances, with few side effects.

In summary, treatment of H_2S donors alone and combined with CB2R agonists are two new options for the treatment of the emotional disorders accompanying neuropathic pain in mice.

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