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New strategies for the treatment of osteoarthritis pain and associated comorbidities

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List of Abbreviations

ACC: anterior cingulate cortex
A-ITC: allyl isothiocyanate
AKT: protein kinase B
AMG: amygdala
CAT: cysteine amino transferase
CBS: cystathionine-b-synthase
CNS: central nervous system
CO: carbon monoxide
CoPP: cobalt protoporphyrin IX
CORM-2: tricarbonyldichlororuthenium (II) dimer
COX-2: cyclooxygenase-2
CSE: cystathionine γ-lyase
DADS: diallyl disulfide
DMARD: disease-modifying antirheumatic drugs
DRG: dorsal root ganglia
GYY4137: morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex
GSTA1: glutathione S-transferase alpha 1
GSTM1: glutathione S-transferase mu 1
HIP: hippocampus
4-HNE: 4-hydroxy-2-nonenal
HO: heme oxygenase
HO-1: heme oxygenase-1
HO-2: heme oxygenase-2
H_2S: hydrogen sulfide
JNK: mitogen-activated protein kinase
IF-PFC: infralimbic prefrontal cortex
IL: interleukins
MIA: monosodium iodoacetate
mPFC: medial prefrontal cortex
3-MST: 3-mercaptopyruvate sulfurtransferase
NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells
NO: nitric oxide
NOS: nitric oxide synthase
NOS1: neuronal nitric oxide synthase
NOS2: inducible nitric oxide synthase
NQO1: NAD(P)H:quinone oxidoreductase-1
Nrf2: nuclear factor erythroid 2-related factor 2
NSAID: non-steroidal anti-inflammatory drugs
OA: osteoarthritis
PAG: periaqueductal gray matter
PFC: prefrontal cortex
PI3K: phosphatidylinositol 3-kinase
P-ITC: phenyl isothiocyanate

PNS: peripheral nervous system
ROS: reactive oxygen species
SOD-1: superoxide dismutase-1
TNFα: tumor necrosis factor α

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Abstract

Osteoarthritis (OA) pain and its associated comorbidities is an important clinical problem that has a negative impact on the quality of life of patients, and its treatment is not completely resolved. Therefore, it's important to investigate new ways to relieve OA pain and the accompanying emotional and cognitive deficits, with few side effects. In this study, we examined the effects of two gaseous neurotransmitters, hydrogen sulfide (H_2S) and carbon monoxide (CO), in modulating the allodynia and the grip strength deficits caused by OA. The potential interaction between both gasotransmitters in inhibiting these effects and the impact of H_2S in the emotional disturbances and memory deficits linked with OA pain were also evaluated. In C57BL/6 female mice, with OA pain induced by the intra-articular injection of monosodium iodoacetate, we demonstrated that: **1**) the repetitive administration of low doses of different slow-releasing H_2S donors, such as allyl isothiocyanate (A-ITC), phenyl isothiocyanate (P-ITC), morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex (GYY4137) and diallyl disulfide (DADS) inhibited the mechanical allodynia and grip strength deficits provoked by OA, **2**) while the administration of A-ITC and P-ITC only reversed the depressive-like behaviors associated with OA pain, treatment with GYY4137 and/or DADS inhibited both the anxiety- and depressive-like behaviors as well as the memory deficits linked with OA pain, **3**) the effects of A-ITC and P-ITC were mainly mediated by inhibiting the microglial activation, normalizing the up regulation of inducible nitric oxide synthase (NOS2) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT), and maintaining a high expression of antioxidant/detoxificant enzymes in the hippocampus, **4**) in the case of DADS and GYY4137, both treatments normalized the oxidative stress and apoptotic responses in the amygdala (AMG), the up-regulation of PI3K/p-AKT in the AMG, periaqueductal gray matter (PAG), infralimbic prefrontal cortex (IF-PFC) and/or anterior cingulate cortex and the NOS2 over expression in the AMG, PAG and IF-PFC of mice with OA pain. Thus revealing the antioxidant, anti-inflammatory, and anti-apoptotic actions of these slow-releasing H_2S donors in different brain areas involved in the modulation of pain, emotional and cognitive behaviors, **5**) the co-administration of low doses of DADS or GYY4137 with a CO releasing molecule or a heme oxygenase-1 enzyme inducer improved the antiallodynic and grip strength recovery effects produced by each of these compounds separately administered and **6**) the involvement of the nuclear factor erythroid 2-related factor 2 pathway in the pain-relieving actions of DADS and GYY4137 during OA. In summary, this study reveals a positive interaction between H_2S and CO on the modulation of OA pain and suggests that treatment with low doses of slow-releasing H_2S donors might be an interesting approach for treating, not only the mechanical allodynia and functional disabilities provoked by OA, but also the emotional and memorial deficits accompanying OA pain.

2.1) What is the osteoarthritis?

Osteoarthritis (OA) is a chronic multifactorial and degenerative disease and one of the most habitual forms of arthritis (Bortoluzzi et al., 2018; Hunter et al., 2020) characterized by producing a progressive degradation of articular cartilage, new bone formation, and synovial proliferation (Alcaraz et al., 2010). OA affects more than 100 million people worldwide, with a high incidence in women, and its etiology and treatment are not completely known (Hunter et al., 2020). The most notable features of OA include inflammation, progressive loss of articular cartilage and alterations of the subchondral bone causing intense pain and functional deficits that make it difficult to perform daily tasks (Eitner et al., 2017; Alcaraz et al., 2019). Furthermore, OA pain is frequently accompanied by different comorbidities, such as fear, attention difficulties, or alterations in working memory (Heuts et al., 2004; Innes and Sambamoorthi, 2018), as well as of several emotional disorders, for example anxiety- and depressive-like behaviors, which at the same time can exert a negative influence in pain perception (Vaeroy et al., 2005; Liu et al., 2023).

The pathogenesis of OA is influenced by some genetic and environmental factors which are associated with the activation of different morphological, biochemical, and biomechanical changes that contribute to the progression of articular injury (Xia et al., 2014). Furthermore, although the biochemical mechanisms involved in the development of OA are not totally resolved, inflammation and oxidative stress are related to this process (Goldring, 2000; Lepetsos and Papavassiliou, 2016) through the activation of the nociceptive (Conaghan et al., 2019b), inflammatory (Pelletier et al., 2001), and apoptotic pathways (Hwang and Kim, 2015), causing severe structural and functional damages in join cartilage (Figure 1).

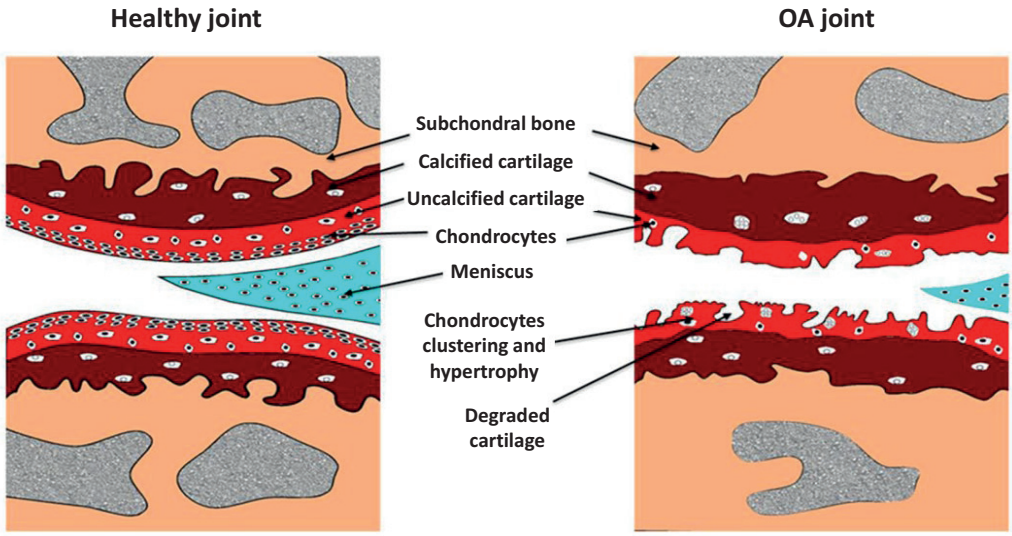

Figure 1. Schematic representation of a healthy and an OA knee joint. The healthy joint (on left) has smooth cartilage surface with normal chondrocyte distribution and the OA knee joint (on right) shows cartilage degeneration and subchondral bone changes (Ansari et al., 2020).

2.2) Chronic Pain

Pain is defined by the IASP as a one of the most relevant study parameters in medicine due to its great impact on society (Raja et al., 2020). Pain can be classified as acute or chronic. Pain is

considered as acute when it can be resolved spontaneously or in few time and chronic when a painful state remains for a long time (The Federation of State Medical Boards of The United States, 1999).

Chronic pain can be classified into different types: inflammatory (when it occurs in response to a surgery inflammation, for example, postoperative pain, trauma, arthritis, etc.), neuropathic (when painful stimulus is the result of nerve damage or might be associated with metabolic diseases or provoked by chemotherapy), visceral (when results from the nociceptors from pelvic, thoracic or abdominal viscera) and cancer pain (when painful stimulus is derived from some type of cancer) among others. Chronic OA pain is primarily generated by an imbalance between inflammatory responses/oxidative stress and the endogenous antioxidant system (Marchev et al., 2017). The main inflammatory responses involved in OA pain are characterized by increased levels of diverse cytokines (interleukins (IL), tumor necrosis factor (TNF-α), etc), nitric oxide (NO) and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) in the joints of OA patients (Goldring, 2000; Uchimura et al., 2016). This inflammatory process contributes to the degradation of articular cartilage generating OA pain manifested with the presence of allodynia or hyperalgesia and mechanical dysfunctions such as a decrease in grip strength force in the extremities.

Chronic OA pain is difficult to treat due to the central sensitization occurs, causing important morphological and biochemical changes in the central nervous system (CNS), that favoring the development and progression of pain (Covey et al., 2000; Woolf and Salter, 2000; Latremoliere and Woolf, 2009). The main brain areas involved in the regulation of pain are the periaqueductal gray matter (PAG), modulating the descending inhibitory pain system (Lee et al., 2000), the anterior cingulate cortex (ACC) regulating pain processing (Zhang et al., 2005; Fuchs et al., 2014) and the prefrontal cortex (PFC) receiving the ascending nociceptive inputs and controlling pain sensation (Ong et al., 2019). Moreover, people who suffer chronic pain also manifest high rates of affective and cognitive disorders such as anxiety- or depressive-like behaviors (Blackburn-Munro and Blackburn-Munro, 2001; Teh et al., 2009; Sharma et al., 2016) and memory deficits (Innes and Sambamoorthi, 2018), which at the same time can increase pain susceptibility establishing a positive correlation between pain and emotional disabilities (Bushnell et al., 2013).

There are different brain areas involved in the development of anxiety- and depressive-like behaviors. One is the amygdala (AMG), that modulates the emotional and cognitive components of pain (Neugebauer, 2020); the PFC is another area involved with the modulation of the emotional disorders associated with chronic pain, particularly, the medial PFC (mPFC) that is involved in the pathophysiology of depression (Russo and Nestler, 2013; Descalzi et al., 2017). The ACC is more implicated in the modulation of executive, attentional and decision-making processes (Hosking et al., 2014) (Figure 2).

For other hand, the permanence of pain also increases the risk to suffer different types of dementia (Scherder et al., 2009). Thus, clinical studies reported that patients with chronic pain often manifest poor memory, particularly on the most attentional-demanding memory processes, such as working memory and long-term memory (Berryman et al., 2013; Mazza et al., 2018). One of the most brain areas that could be affected by chronic pain is the hippocampus (HIP), an area mainly involved in the modulation of learning, memory, and depressive-like behaviors (Mokhtari et al., 2019). Previous studies reported that during chronic pain HIP suffers a decrease of neuroplasticity and neurogenesis process, as well as an increase on the levels of proinflammatory mediators causing alteration in its functions (Mokhtari et al., 2019). Furthermore, HIP sent different inputs to other brain regions, such as mPFC and AMG suggesting that the alteration of HIP may affected the correct functions of other brain areas favoriting the

appearance of additional emotional disturbances (Sah et al., 2003; Felix-Ortiz and Tye, 2014; Phillips et al., 2019).

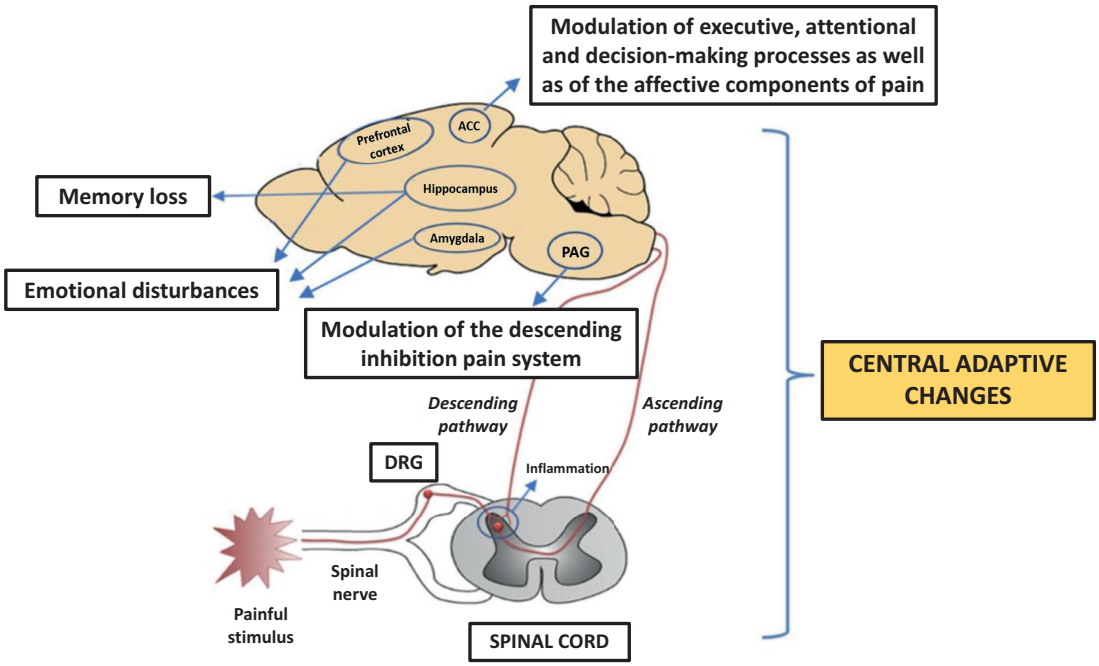

Figure 2. Chronic pain induces central adaptive changes. *Central adaptive changes are related to morphological and biochemical alterations in several brain areas (PFC, AMG, PAG or ACC) mainly implicated in the modulation of pain, memory, emotions, executive, attentional and decision-making processes (Bravo et al., 2019; Da Silva et al., 2020).*

In addition, since several neurochemical mechanisms implicated in the generation and progression of OA pain are also involved in the development of associated comorbidities, there is positive feedback between pain and the emotive and cognitive disabilities (Bushnell et al., 2013) (Figure 3).

Figure 3. Feedback between pain and emotive/cognitive dysfunctions*. Pain can have a negative impact on emotions/cognitive functions. At the same time, a negative emotional/cognitive state can increase pain, whereas a positive emotional/cognitive state can reduce it (Bushnell et al., 2013).*

2.3) Oxidative stress and chronic pain

Oxidative stress results when there is an imbalance between the levels of free radicals, such as reactive oxygen species (ROS), and the endogenous antioxidant system activated by the nuclear factor erythroid 2-related factor 2 (Nrf2) (Guo et al., 2018).

Under non-pathological conditions, the Nrf2 transcription factor, that regulates the expression of different phase II antioxidant enzymes, such as superoxide dismutase-1 (SOD-1), NAD(P)H:quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutathione S-transferase mu 1 (GSTM1), or glutathione S-transferase alpha 1 (GSTA1) (Ekuban et al., 2021),

exercises an important defensive role against oxidative damage (Chen et al., 2015). However, under several pathological conditions, the Nrf2 signaling pathway is down regulated, decreasing the capacity of the antioxidant enzymes to modulate ROS, thus favoring an oxidative stress state (Yu et al., 2019) (Figure 4).

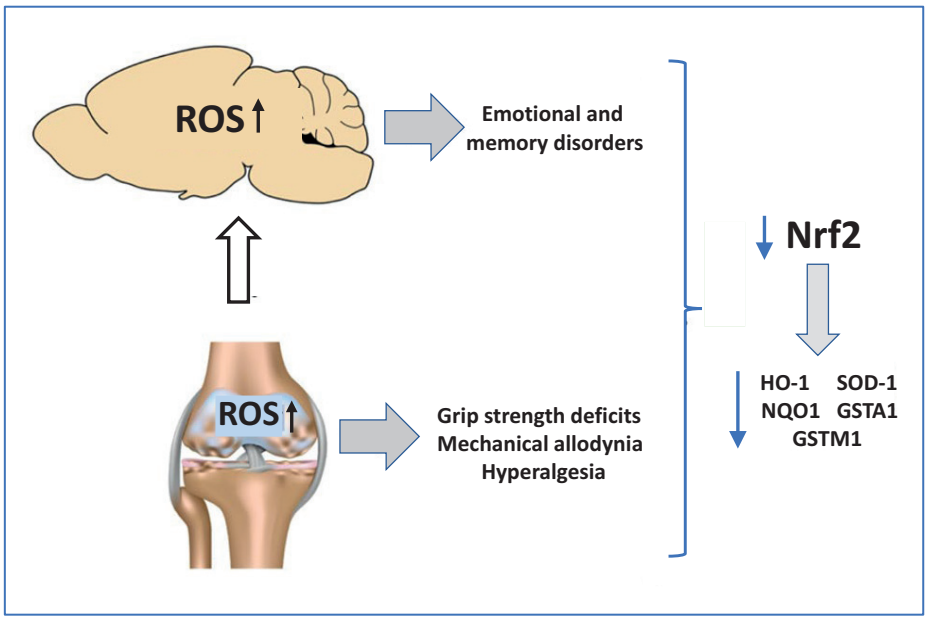

Figure 4. The OA induces an increased ROS expression and/or a down-regulation of the Nrf2 pathway in joints and CNS. Increased levels of ROS favorites the damage and apoptosis of articular cartilage contributing to the appearance of OA pain, manifested with mechanical allodynia and/or hyperalgesia, and grip strength deficits. Increased ROS levels are also observed in the CNS contributing to the appearance of emotional disorders, such as anxiety- or depressive-like behaviors, and/or memory dysfunctions. Moreover, during OA, the expression of phase II antioxidant enzymes' superoxide dismutase-1 (SOD-1), NAD(P)H:quinone oxidoreductase-1 (NQO1), heme oxygenase-1 (HO-1), glutathione S-transferase mu 1 (GSTM1), and/or glutathione S-transferase alpha 1 (GSTA1), might be down regulated due to the decrease expression of Nrf2.

Oxidative stress is very involved in the progression of OA (Li et al., 2012; Lepetsos and Papavassilou, 2016) because to its important role in cell apoptosis, thus causing joint damage (Zhuang et al., 2020). Oxidative stress by down regulating the Nrf2 triggered pathway and increasing the production of aldehydes, such as malondialdehyde and 4-hydroxy-2-nonenal (4-HNE) which also contribute to induce pain (Yang et al., 2003; Martins et al., 2021). In accordance, several studies have evaluated the effects of Nrf2 inducers in OA, revealing their effectivity in inhibiting cartilage degradation (Kim et al., 2009; Davidson et al., 2013). In addition, studies performed in animals with chronic peripheral inflammation or in diabetic mice revealed that the induction of Nrf2 reduced inflammatory pain and diabetic neuropathy thus demonstrating the important role played by this transcription factor in modulating chronic pain (McDonnell et al., 2017; Redondo et al., 2017).

The Nrf2 signaling pathway also plays a key role in mood disorders (Hashimoto, 2018). Nrf2 knockout mice displayed depressive-like behaviors (Yao et al., 2016) and the pharmacological induction of Nrf2 modulated the anxiety- and depressive-like behaviors associated with neuropathic pain in mice (Ferreira-Chamorro et al. 2018).

All these results suggest that the prevention of oxidative stress could be a way to avoid the progression of OA pain and its associated comorbidities.

2.4) Inflammation and OA pain

Inflammation plays an important function in the development of OA pain (Scanzello, 2017). Previous studies demonstrated that oxidative stress is related to inflammation through the activation of the redox sensitive transcription NF-kB, causing uncontrolled inflammatory responses (Yuan et al., 2014). When inflammation occurs, IKB kinases phosphorylates NF-kB (Okazaki et al., 2005) that induces its entrance into the nucleus, provoking the synthesis of pro-inflammatory cytokines: TNFα, IL-1β, IL-6, IL-15, IL-17 and IL-18, among others (Kapoor et al., 2011) (Figure 5).

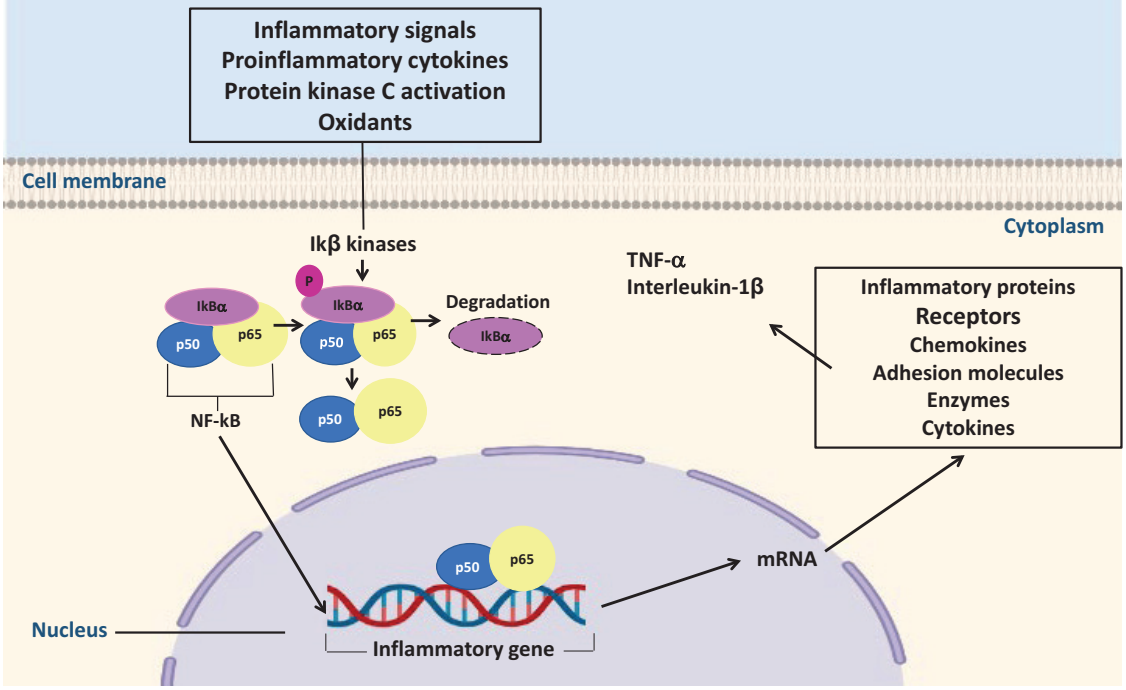

Figure 5. The activation of NF-kB induced by an inflammatory stimulus. The activation of NF-kB provoked by inflammatory stimuli allows its entrance to the nucleus, where it induces the transcription of different inflammatory mediators (Barnes and Karin, 1997).

For the other hand, the gaseous neurotransmitter NO, distributed in both the CNS and peripheral nervous system (PNS), also contributes to the inflammatory component of OA. NO is synthesized by three nitric oxide synthase (NOS) isoforms, the neuronal (NOS1), neuronal (NOS2) and endothelial (Stuehr, 1999). Previous studies performed in animals with peripheral inflammation revealed increased expression of NOS2 and NOS1 in some areas of the PNS (paw and dorsal root ganglia; DRG) and CNS (spinal cord, loci coeruleus) (Boettger et al., 2007; Negrete et al., 2011; Moreno et al., 2019). Moreover, clinical trials of OA patients have detected high concentrations of this gasotransmitter in the knee synovial fluid (Vuolteenaho et al., 2007; More et al., 2013) revealing the implication of NO in this inflammatory disease.

Due to the association between inflammation and pain, the implication of glial cells in the progression of pain has also been demonstrated. Both microglia and astroglia contribute to central sensitization. In neuropathic pain, microglia seem to be early activated than astrocytes, and their activation is related to the induction and maintenance of neuropathic pain, respectively (Yan et al., 2017). Similar results were observed in the spinal cord of animals with OA pain (Sagar et al., 2011), while other studies reported that the brain microglia activation is associated with all phases of the inflammatory pain (Raghavendra et al., 2004).

As consequence of these results, several treatments against OA pain are focused on decreasing inflammation, for instance, NOS2 inhibitors (Abramson, 2008; More et al., 2013), paracetamol (Neame et al., 2004) or non-steroidal anti-inflammatory drugs (NSAID) (Richette et al., 2015). However, despite these treatments have resulted useful in decreasing inflammation or oxidative stress, most of them do not effectively alleviate pain symptoms and produce several side effects (Seed et al., 2009; Makris et al., 2010).

Moreover, peripheral inflammation is also implicated in the development of emotional disorders. Thereby, the high levels of microglial NLRP3 inflammasome in PFC (Pan et al., 2014), TNFα in the HIP and AMG (Chen et al., 2013b; Fasick et al., 2015), or astrocyte activation in the ACC (Chen et al., 2012) contribute to the anxiety- and/or depressive-like behaviors associated with chronic pain, establishing a linking between inflammation, pain, and emotional disorders. However, more studies are required to clarify the molecular mechanisms involved in these processes.

2.5) PI3K/Akt pathway

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway is involved in the maintenance of cellular homeostasis, including cell growth, cell survival, inflammation, and metabolism. Inflammatory and neuropathic pain, in addition to oxidative and inflammatory responses, also share a common pathophysiology that involves the activation of PI3K/AKT pathway (Liu et al. 2018; Sun et al., 2020).

Studies performed in animals with OA demonstrated the involvement of PI3K/Akt pathway in the progression of OA (Chen et al., 2013a) and its inhibition of a target to attenuate post-traumatic OA (Lin et al., 2018) and restore the cartilage homeostasis by attenuating join damage (Sun et al., 2020). Activated PI3K/Akt have been also detected in the spinal cord and HIP of mice with neuropathic pain (Díaz et al., 2019), and its inhibition reduced the development of neuropathic pain (Guo et al., 2017), revealing the participation of this pathway in regulating neuropathic pain. Moreover, Xie et al. (2019) further revealed that the inhibition of the PI3K/AKT/NF-κB signal pathway attenuated the development of OA (Figure 6).

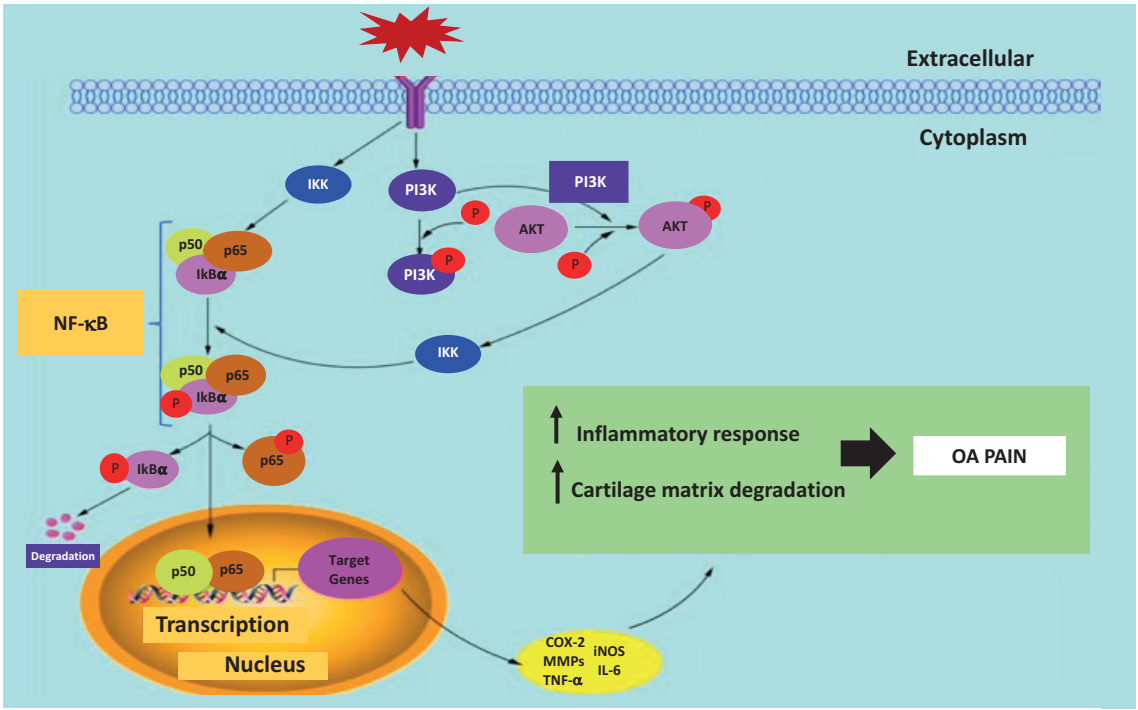

Figure 6. The pro-inflammatory effects of PI3K/AKT pathway on the articular cartilage degradation during OA pain. During OA, the activation of PI3K/AKT pathway induces the up-regulation of NF-kB signaling pathway causing the transcription of diverse pro-inflammatory mediators resulting in cartilage degradation and OA pain (Xie et al., 2019).

Other works demonstrated that different treatments ameliorated the anxiety and depressive-like behaviors, and/or the cognitive impairments associated with chronic pain by inhibiting the PI3K/Akt signaling pathway in the CNS and PNS (Díaz et al., 2019; Yuan et al., 2019; Jiang et al., 2021; Liu et al., 2022), thus revealing the involvement of this path in the development of mental disturbances associated with chronic pain. These results suggest that the PI3K/Akt pathway is an interesting target to modulate not only the progression of OA pain but also the development of the emotional comorbidities associated.

2.6) Apoptosis and OA progression

Apoptosis is a highly regulated and active process of cell death resulting from the disequilibrium between apoptotic and non-apoptotic factors and is involved in many processes, such as development, homeostasis, and aging. When apoptotic pathways are dysregulated, it causes different pathological states, such as cancer, developmental anomalies and/or degenerative diseases (Hwang and Kim, 2015). The pathogenesis of OA is favored by the increased chondrocytes apoptosis, establishing a positive correlation between apoptosis and cartilage degeneration (Aigner and Kim, 2002). There are a high number of signaling pathways regulating apoptosis. Among them, the mitogen-activated protein kinase (JNK) pathway which activation increases osteoblasts' apoptosis and the PI3K/Akt pathway that protects from apoptosis (Song et al., 2016). Thus, the equilibrium between the PI3K/Akt and JNK pathways which maintain the homeostasis under non-pathological conditions is disrupted by oxidative stress. Thus, increased ROS levels induce the over-production of JNK and the activation of 4-HNE, which ends inhibiting Bcl-2 expression, increasing BAX production and activating the caspases that cause apoptosis in osteoblasts and chondrocytes (Song et al., 2016; He et al., 2020) (Figure 7). The maintenance of chondrocyte death/survival is critical for OA (Salucci et al., 2022), therefore treatments capable of stopping or slowing down chondrocyte apoptosis in the joints of OA patients are plausible therapies for OA (Xiang et al., 2023).

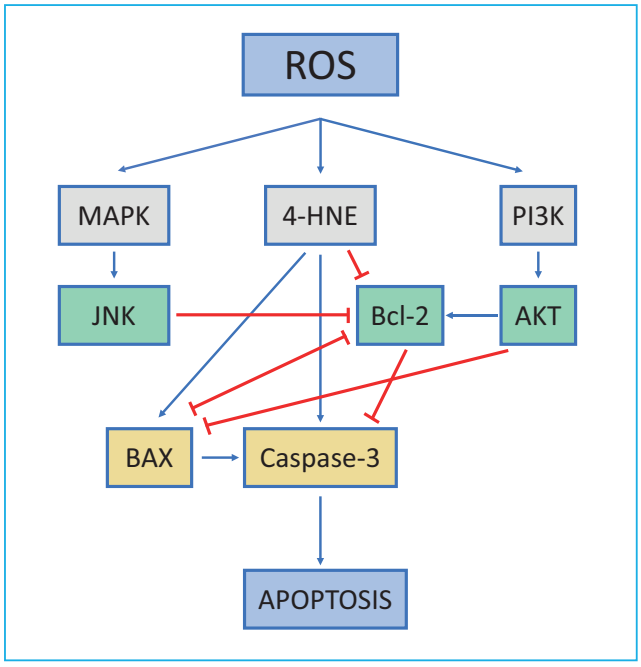

Figure 7. ROS signaling pathways in chondrocyte apoptosis. ROS induce the activation of JNK that inhibits the expression of Bcl-2 favoring the expression of caspase-3 causing apoptosis. ROS also increase the expression of 4-HNE that inhibits Bcl-2 and induces the activation of BAX favoring apoptosis. Finally, the anti-apoptotic pathway PI3K/AKT is also activated, favoring the expression of Bcl-2 and inhibiting the BAX/caspase-3-induced apoptosis (Lepetsos Papavassiliou, 2016; Song et al., 2016).

2.7) Treatments for OA pain

Due to the importance of ROS in the development of OA pain, several studies have evaluated the use of diverse Nrf2 inducers to attenuate OA pain (Pol, 2021, Gao et al., 2022) and proposed Nrf2 activators and HO-1 inducers as adjuvant treatments, since both can increase the analgesic effects of other drugs (Pol, 2021). There are other natural or synthetic antioxidants that have been proposed to modulate some aspects of OA pain, such as magnesium ions or vitamin C (Yao et al., 2020), eicosapentaenoic acid- and docosahexaenoic acid-derived resolvins (Souza and Norling, 2016), hyaluronic acid (Bannuru et a., 2011), nutritional supplement L-carnitine (Baghban et al., 2021) or the use of dietary polyphenols (Sirše, 2022) which include a wide range of compounds present in different types of food, such as fruits, vegetables, wine, tea, extra virgin olive oil, chocolate etc. In summary, antioxidant agents are an interest target to treat OA pain.

Moreover, several treatments against OA pain have been focused on decreasing inflammation. Thus, the administration of coenzyme Q10 alleviates OA pain and cartilage degradation by regulating NO and inflammatory cytokines production (Lee et al., 2013). The administration of selective NOS2 inhibitors, such as SD-6010, attenuates the progression of OA pain by averting the cartilage destruction associated with OA (Connor et al., 2012). Non-selective and selective NSAID have been used in some clinical trials to decrease OA pain by inhibiting cyclooxygenase-2 (COX-2) enzymes and the subsequent synthesis of prostaglandins (Fendrick and Greenberg, 2009; Cohen and Lee, 2015). Paracetamol is another treatment usually used to relieve OA pain (Zhang et al., 2004), however, due to its scarce effects on OA pain and the side effects derived from its administration for a long period, its use has been substantially reduced (Conaghan et al., 2019a). Finally, synthetic, and biologic disease-modifying antirheumatic drugs (DMARDs), such as methotrexate, are used to treat OA pain (Conaghan et al., 2019b), but its use remains controversial due to neither synthetic nor biological DMARDs relieve OA pain effectively (Persson et al., 2018). In addition, although more of these treatments alleviate OA pain, few can prevent the emotional and/or memory disorders accompanying it. Then our objective is to find new strategies to relieve, not only OA pain, but also the associated comorbidities, with low side effects.

2.8) Hydrogen sulfide

Hydrogen sulfide (H_2S), together with NO and carbon monoxide (CO), is a gaseous neurotransmitter amply distributed in the CNS and PNS which modulates several physiological processes (Paul and Snyder, 2015; Cirino et al., 2023) such as redox balance, apoptosis, and inflammatory processes (Fouad et al., 2020) and takes an important role in pain modulation (Guo et al., 2020).

H_2S is a colorless, flammable, water-soluble gas which smell as rotten eggs, synthesized in most cells and tissues by the enzymes L-cysteine, cystathionine-b-synthase and cystathionine γ-lyase, combined with the action of the enzymes 3-mercaptopyruvate sulfurtransferase and cysteine amino transferase, also known as L-cysteine:2-oxoglutarate aminotransferase, aspartate aminotransferase, or aspartate/cysteine amino transferase (Szabo and Papapetropoulos, 2017) (Figure 8).

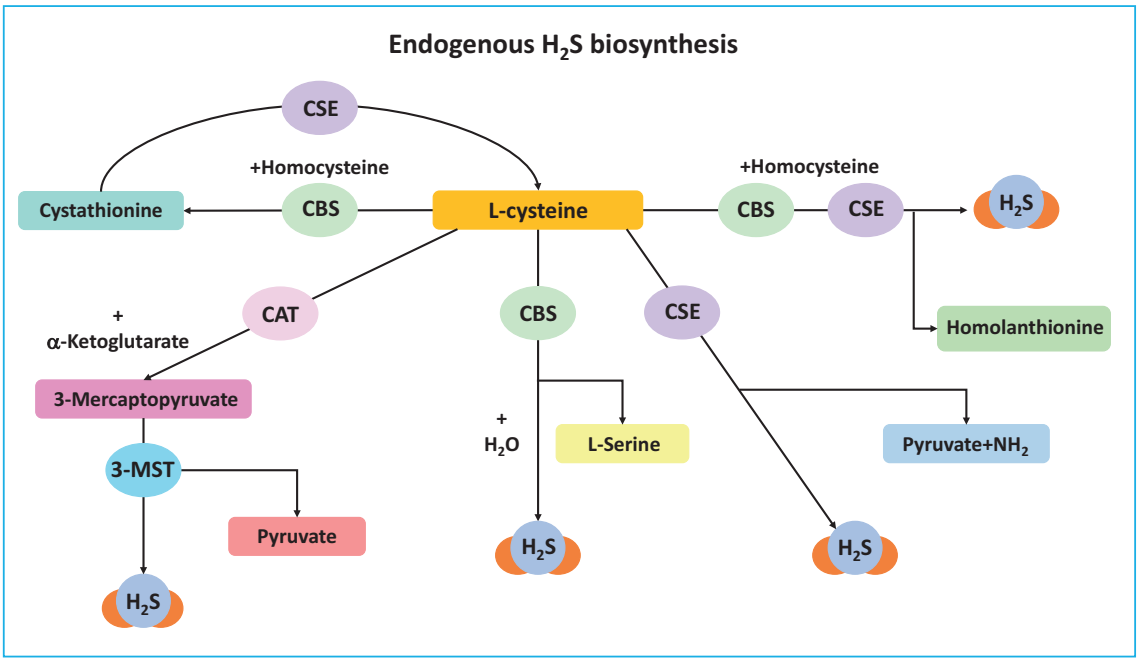

Figure 8. Schematic representation of the endogenous synthesis of H_2S. The endogenous production of H_2S in mammals is associated with the action of three tissue-specific enzymes: cystathionine-b-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3-MST). The substrate to generate endogenous H_2S is L-cysteine. CBS-driven H_2S generation is related to the condensation of homocysteine with L-cysteine to produce cystathionine and H_2S. CSE converts L-cysteine into pyruvate+NH3, and H_2S. cysteine amino transferase (CAT) generates 3-mercaptopyruvate via the reaction of L-cysteine with α-ketoglutarate. The 3-mercaptopyruvate is then metabolized by 3-MST to generate H_2S (Donnarumma et al., 2017).

For a long time, H_2S has been considered as a toxic gas and its toxicity has been extensively studied in both animal and human models (Szabo and Papapetropoulos, 2017). However, more recent studies revealed the powerful anti-inflammatory and antioxidant actions of H_2S (Burguera et al., 2017). The role of H_2S in pain modulation has shown contradictory results because it can be considered as a pro-nociceptive and antinociceptive depending on the dose and the type of H_2S-releasing donor used (Di Cesare Mannelli et al., 2017). Then, while the administration of fast H_2S -releasing donors, that release large amounts of H_2S in a short time and in an uncontrolled manner, can increase, inhibit, or not alter pain (Cunha et al., 2008; Ekundi-Valentim et al., 2010), substances that release H_2S slowly, simulating the physiologic conditions (Caliendo et al., 2010), exert potent anti-inflammatory effects (Huang et al., 2016), and relieve neuropathic and inflammatory pain efficiently (Di Cesare Mannelli et al., 2017; Porta et al., 2021). Moreover, the mode that the H_2S is given is an important factor to be considered, since previous studies suggested that local administration of H_2S may favorite pain while its systemic administration causes antinociceptive effects (Guo et al., 2020).

Regarding the pathways involved in the analgesic properties of slow-releasing H_2S donors, several studies displayed the participation of the voltage gated Kv7 potassium channels under inflammatory, visceral, and neuropathic pain conditions (Di Cesare Mannelli et al., 2017; Lucarini et al., 2018) and the Nrf2 signaling pathway in neuropathic and inflammatory pain (Porta et al., 2021; Roch et al., 2022). Moreover, slow H_2S-releasing donors can prevent cell apoptosis by inhibiting oxidative stress provoked cell death (Majid et al., 2013). Nonetheless, the effects of slow-releasing H_2S donors on the mechanical allodynia and grip strength deficits associated with OA, and the possible contribution of Kv7 potassium channels and/or the Nrf2 signaling pathway in these actions remain unknown.

The administration of slow H_2S-releasing donors also has anxiolytic and antidepressant effects in different animal models of anxiety or depression and in diabetic animals without conditioning locomotor activity (Tang et al., 2015). Moreover, the effects of H_2S on learning and memory have also been shown (Xuan et al., 2012; Yakovleva et al., 2020). These data open the possibility that the administration of slow H_2S releasing donors could be effective not only for alleviating OA pain, but also the accompanying emotional and cognitive disorders.

2.8.1) Slow H_2S-releasing compounds

The use of slow H_2S-releasing donors has been proposed as a suitable alternative to avoid the negative effects derived from the use of fast H_2S-releasing donors, which can easily exceed the physiological H_2S concentrations causing pro-inflammatory (Di Cesare Mannelli et al., 2017) and/or pro-nociceptive actions (Cunha et al., 2008). There are several types of slow H_2S releasing donors:

a) Phenyl isothiocyanate and allyl isothiocyanate

Isothiocyanates, many derivates from plants, are slow releasing H_2S compounds produced by enzymatic conversion of metabolites called glucosinolates. These compounds have shown beneficial effects in different models of CNS and PNS damaged, as well as in cancer and inflammatory diseases (Brunelli et al., 2010; Waterman et al., 2014). The therapeutic effects of isothiocyanates seem to be mediated by blocking the protein complex NF-kB and the adipokine/cytokine TNFα (Rose et al., 2005), both implicated in the development of inflammation (Heiss et al., 2001; Xu et al., 2005). In this work, we evaluated the effects of two isothiocyanates: allyl isothiocyanate (A-ITC) as a natural isothiocyanate (mustard oil) and phenyl isothiocyanate (P-ITC) as an artificial isocyanate.

b) Diallyl disulfide

Diallyl disulfide (DADS) is a major oil-soluble organosulfur ingredient compound derived from garlic (Amagase et al., 2001). DADS manifests different antioxidant, anti-inflammatory and anti-apoptotic properties (Sheen et al., 2001; Lee et al., 2014; Hosseinzadeh et al., 2017). Its therapeutic effects seem to be mediated by decreasing the production of different pro-inflammatory cytokines such as IL-1 β and TNFα, by deactivating the NF-kB transcription factor, decreasing COX-2 and NOS2 expression with the subsequent decrease of NO production (Park et al., 2012; Shin et al., 2013; Saud et al., 2016). Furthermore, previous reports indicated that DADS could enhance the activity of NQO1, GST and SOD-1 via activation the Nrf2/HO-1 pathway (Lee et al., 2014; Shan et al., 2016).

c) GYY4137

GYY4137 (morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex) is another slow H_2S-releasing donor. This donor has the edge that is water soluble and easy to synthesize chemically (Rose et al., 2015). This donor can exert different antioxidant, anti-inflammatory, and anti-apoptotic effects via inhibiting NF-kB signaling pathways and decreasing the expression of different pro-inflammatory mediators (IL-1ß, IL-6, TNFα), prostaglandin E2, COX-2 and NOS2 (Whiteman et al., 2010; Burguera et al., 2014).

Therefore, considering the antioxidant and anti-apoptotic properties of these slow-releasing H_2S donors, they can be proposed as an interesting treatment for OA pain.

2.9) Carbon monoxide

CO is a gaseous neurotransmitter with important functions in pain modulation (Fan et al., 2011; Pol, 2021). In a low concentration, CO has relevant physiologic and cytoprotective effects, such as anti-proliferative, anti-apoptotic and anti-inflammatory properties (Takagi et al., 2011; Wen et al., 2013). Several studies demonstrated its anti-inflammatory effects and its implication in the modulation of neuropathic (Hervera et al. 2012) and inflammatory pain (Negrete et al., 2014).

CO is generated through the action of HO enzymes (Ryter and Otterbein, 2004). There are two distinct isoenzymes of HO: the inducible (HO-1), which is synthesized under cellular or systemic stress situations and exert potent antinociceptive effects (Liu et al., 2016), and the constitutive (HO-2), which is focus on cellular protection and oxygen response (Muñoz-Sánchez and Chánez-Cárdenas, 2014) (Figure 9).

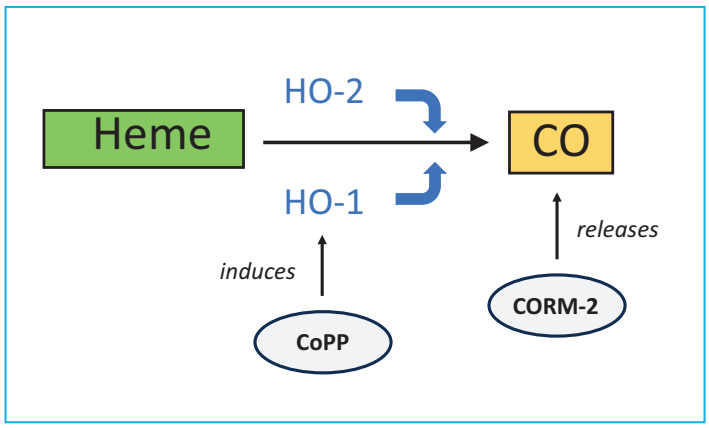

***Figure 9. Schematic representation of the synthesis of CO**. The CO synthesis involves the action of HO-1 and HO-2 enzymes. The cobalt protoporphyrin IX (CoPP) potentiates the synthesis of HO-1 and the tricarbonyldichlororuthenium (II) dimer (CORM-2), the CO release.*

Therefore, while the over-expression of the HO-1 is associated with potent anti-inflammatory and antinociceptive effects during acute and chronic pain (Rosa et al., 2008; Egea et al., 2009; Pol, 2021), the HO-2 over expression is correlated with pro-nociceptive effects (Gordh et al., 2000; Li and Clark, 2000). In fact, the HO-1 inducer compound, cobalt protoporphyrin IX (CoPP) or the CO-releasing molecule tricarbonyldichlororuthenium (II) dimer (CORM-2), both inhibit different types of pain, such as visceral, neuropathic, or inflammatory (Hervera et al., 2013; Negrete et al., 2014; Riego et al., 2018) and also reduce the anxiety- and depressive-like behaviors associated with neuropathy induced by chemotherapy (Roch et al., 2022). However, the effects of CORM-2 and CoPP on the mechanical allodynia and grip strength deficits associated with OA pain have not been evaluated.

In addition, and given the fact that H_2S and CO activate similar biochemical pathways and previous studies reported the existence of interactions between both gasotransmitters in diverse pathological circumstances (Han et al., 2006; Magierowski et al., 2016; Glowacka et al., 2020), it raises the possibility that there might be an interaction between H_2S and CO in modulating OA pain. A possible positive interaction between both gasotransmitters in modulating OA pain would open the possibility of using low doses of slow H_2S-releasing donors and of HO-1 inducers/CO releasing compounds for alleviating the grip strength deficits and mechanical allodynia originated by OA , thus reducing the risk of adverse effects associated with the use of high doses of these compounds (Figure 10).

On the other hand, although previous studies have demonstrated the participation of the Nrf2 signaling pathway in the analgesic actions of H_2S donors during inflammatory pain (Porta et al., 2021), it is unknown if this occurs during OA pain.

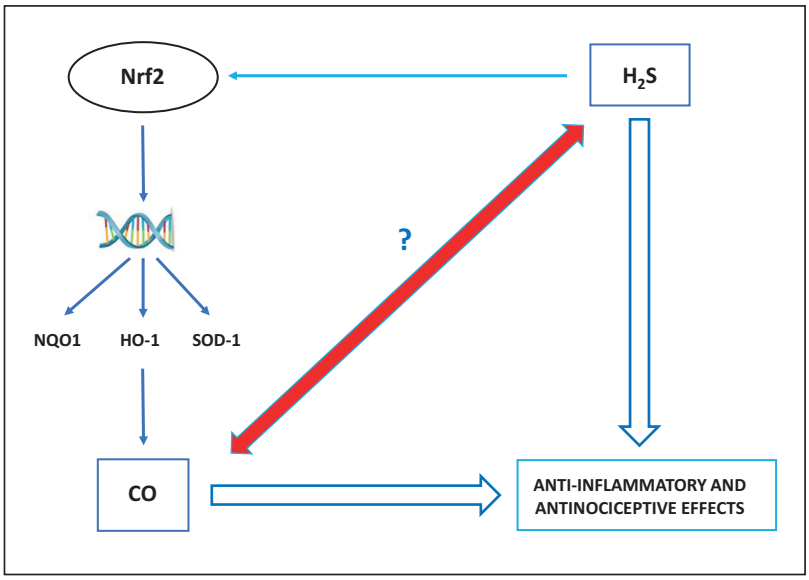

Figure 10. The interaction between H_2S and CO systems. *The transcription factor Nrf2 is involved in the synthesis of different antioxidant agents, such as NQO1, SOD-1 and HO-1. At the same time, HO-1 is implicated in the synthesis of CO that has anti-inflammatory and antinociceptive properties. On the other hand, H_2S can up regulate the Nrf2 expression, establishing an indirect interaction between H_2S and HO-1/CO systems. However, a possible direct interaction between H_2S and CO during OA pain remains unknown.*

3. Objectives

In an OA pain model induced by the knee intra-articular injection of monosodium iodoacetate (MIA) in female mice, the main aims of this thesis are to evaluate:

1. The role played by H_2S in the modulation of the mechanical allodynia and grip strength deficits provoked by OA and the mechanisms implicated, using slow-releasing H_2S donors (A-ITC, P-ITC, DADS and GYY4137).

2. The impact of H_2S in regulating the anxiety- and depressive-like behaviors and memory deficits associated with OA pain, and the main pathways involved in these actions in several brain areas.

3. The effects of HO-1 (CoPP) and CO (CORM-2) on the allodynia and/or functional deficits induced by the OA and their interaction with H_2S at pharmacological and biochemical level.

4. Manuscripts

4.1. The Inhibitory Effects of Slow-Releasing Hydrogen Sulfide Donors in the Mechanical Allodynia, Grip Strength Deficits, and Depressive-Like Behaviors Associated with Chronic Osteoarthritis Pain.
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The Inhibitory Effects of Slow-Releasing Hydrogen Sulfide Donors in the Mechanical Allodynia, Grip Strength Deficits, and Depressive-Like Behaviors Associated with Chronic Osteoarthritis Pain

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Abstract: Osteoarthritis and its associated comorbidities are important clinical problems that have a negative impact on the quality of life, and its treatment remains unresolved. We investigated whether the systemic administration of slow-releasing hydrogen sulfide (H_2S) donors, allyl isothiocyanate (A-ITC) and phenyl isothiocyanate (P-ITC), alleviates chronic osteoarthritis pain and the associated emotional disorders. In C57BL/6 female mice with osteoarthritis pain induced by the intra-articular injection of monosodium iodoacetate, we evaluated the effects of repeated administration of A-ITC and P-ITC on the (i) mechanical allodynia and grip strength deficits; (ii) emotional conducts; and (iii) glial activity and expression of inducible nitric oxide synthase (NOS2), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and antioxidant enzymes (heme oxygenase 1, NAD(P)H:quinone oxidoreductase-1, glutathione S-transferase mu 1 and alpha 1) in the hippocampus. The administration of A-ITC and P-ITC inhibited the mechanical allodynia, the grip strength deficits, and the depressive-like behaviors accompanying osteoarthritis. Both treatments inhibited microglial activation, normalized the upregulation of NOS2 and PI3K/p-Akt, and maintained high levels of antioxidant/detoxificant enzymes in the hippocampus. Data suggest that treatment with low doses of slow-releasing H_2S donors might be an interesting strategy for the treatment of nociception, functional disability, and emotional disorders associated with osteoarthritis pain.

Keywords: analgesia; anxiety; depression; grip strength; hydrogen sulfide donors; inflammation; microglia; osteoarthritis pain; oxidative stress

1. Introduction

Osteoarthritis is one of the most prevalent diseases affecting more than 100 million people worldwide. Osteoarthritis is a chronic degenerative joint disorder characterized by the destruction of articular cartilage causing subchondral bone alterations, inflammation, and intense pain [1]. Chronic osteoarthritis pain, which is characterized by persistent pain with inflammatory and neuropathic components, causes a physical inability to perform daily tasks, difficulty walking, etc. [2]. It is also accompanied by affective disorders such as anxiety and depression, which further contribute to the impairment in the quality of life of patients and exert a negative influence on the perception of pain, creating a vicious circle that leads to unwanted results [3].

At present, the treatment of chronic osteoarthritis pain remains a challenge. Therapies to treat osteoarthritis pain are limited, with modest efficacy and important adverse effects. Furthermore, it is important to note that while some treatments may relieve pain, few are able to reduce the emotional disorders associated with chronic osteoarthritis pain. Therefore, an investigation of new strategies that effectively relieve chronic osteoarthritis pain and the associated comorbidities, such as anxiety and depression, is essential.

Hydrogen sulfide (H_2S), together with nitric oxide and carbon monoxide, is an integral part of the triad of neurotransmitter gases with important physiological and pathophysiological functions. H_2S is widely distributed in the central and peripheral nervous system and plays an important role in redox balance, apoptosis, and inflammatory processes [4]. However, despite numerous studies revealing that the exogenous administration of H_2S exerts powerful anti-inflammatory and antioxidant actions [5], the role of H_2S in pain modulation is controversial. Thus, conflicting data suggest that H_2S can be both pronociceptive and antinociceptive, depending on the type of H_2S-releasing substances used [6]. That is, compounds that release large amounts of H_2S in a short time and in an uncontrolled manner can relieve pain, not alter it, or even increase it, depending on the dose, the route of administration, and the time of the evaluation of their effects [7–10]. In contrast, more recent studies show that the administration of substances that are capable of releasing H_2S slowly, simulating the conditions of H_2S release in vivo, in addition to exerting potent anti-inflammatory effects [11], are also able to relieve neuropathic pain induced by the administration of antineoplastic drugs in animals [12]. Nevertheless, the effect of the administration of low doses of two slow-releasing H_2S compounds, such as allyl isothiocyanate (A-ITC) and phenyl isothiocyanate (P-ITC), on the mechanical allodynia and grip strength deficits induced by chronic osteoarthritis in mice has not yet been studied.

Several studies also show that the exogenous administration of H_2S exerts anxiolytic and antidepressant effects in different animal models of anxiety and depression [13], as well as in the anxiety-like behavior associated with diabetes [14], without affecting locomotor activity. Thus, these data suggest that treatment with slow-releasing H_2S compounds may be a good approximation for the treatment of the anxiety-like and depressive-like behaviors associated with chronic osteoarthritis pain.

The mechanisms implicated in the development of osteoarthritis are not completely known. Inflammation and oxidative stress are closely integrated in the osteoarthritis pathology. The levels of several inflammatory mediators, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), are augmented in the joint tissue of osteoarthritis patients [15]. Moreover, IL-1β and TNFα also stimulate the synthesis of prostaglandins, nitric oxide, phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), nuclear factor κB (NF-κB), and diverse mitogen-activated protein kinase (MAPK) signaling pathways further contributing to osteoarthritis pain [16]. Accordingly, the PI3K/Akt/NF-κB and MAPK signaling pathways are activated in the spinal cord and different brain areas, such as the hippocampus and/or prefrontal cortex, of animals with chronic pain [17,18], and their inhibition reduced the development of osteoarthritis and neuropathic pain [19,20].

Microglia are likewise implicated in the progression of chronic pain, and their activation in the supraspinal areas is in part responsible for the molecular neuroplasticity changes associated with chronic pain [21] as well as the emotional disorders accompanying it [3]. Thus, microglial activation has been demonstrated in the medial prefrontal cortex and the hippocampus of animals with depressive-like conducts associated with osteoarthritis or neuropathic pain [18,22] as well as in several brain regions of depressive patients suffering chronic pain [23]. Consequently, the depressive-like behaviors accompanying chronic pain diminished with the administration of microglial inhibitors [24,25].

Oxidative stress also plays an essential role in the pathogenesis of osteoarthritis. High levels of reactive oxygen species have been demonstrated in the synovial tissue, chondrocytes, and fibroblasts of the joint, thus promoting the structural and functional damage of the cartilage [26], which can also potentiate the inflammatory responses by activating the production of nitric oxide synthetized by inducible nitric oxide synthase (NOS2). Therefore, in addition to the well-known positive effects of anti-inflammatory agents, treatment with antioxidant compounds, for example, inducers

of nuclear factor erythroid 2-related factor 2 (Nrf2) and/or other enzymes activated by Nrf2, such as heme oxygenase 1 (HO-1), also attenuates the development of osteoarthritis by regulating redox homeostasis [27,28]. Nevertheless, the effects of knee osteoarthritis and those of the systemic treatment with slow-releasing H_2S compounds in the endogenous antioxidant system in the central nervous system have not been evaluated.

Lastly and taking account that women are more likely to suffer chronic pain than men and because most of the pain symptoms and disorders associated with chronic osteoarthritis pain are more prevalent in women [29], this study was performed in female mice.

In female mice with chronic osteoarthritis pain induced by the intra-articular administration of monosodium iodoacetate (MIA), at 29 days after injection, we assessed the effects of the repeated administration of 4.4 μmol/kg A-ITC or 13.3 μmol/kg P-ITC on (1) the mechanical allodynia; (2) the grip strength deficits; (3) the emotional behaviors associated with chronic osteoarthritis pain; (4) the inflammatory and molecular changes induced by MIA in the central nervous system by evaluating the protein levels of CD11b/c (a microglial marker), glial fibrillary acidic protein (GFAP, an astroglial marker), NOS2, and PI3K/p-Akt; and (6) the expression of antioxidant and detoxificant proteins such as HO-1, NAD(P)H:quinone oxidoreductase-1 (NQO1), glutathione S-transferase mu 1 (GSTM1), and glutathione S-transferase alpha 1 (GSTA1) in the hippocampus.

2. Materials and Methods

2.1. Animals

We used female mice of 6–8 weeks of age acquired from Envigo laboratories (Barcelona, Spain). All mice weighed between 21 and 25 g and were accommodated in a room with 12/12 h of light/dark and a controlled temperature of 22 °C and humidity of 66%. The animals had free access to food and water, and experiments started at 7 days after acclimatization to the housing conditions. All the proposed experiments were conducted between 9:00 a.m. and 5:00 p.m. and were carried out in accordance with the guidelines of the European Commission's directive (2010/63/EC) and the Spanish Law (RD 53/2013) regulating animal research, and they were approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona (number: 1325R5). Maximum efforts were made to minimize the number of animals used and their suffering.

2.2. Induction of Osteoarthritis Pain

Osteoarthritis pain was induced in anesthetized mice with isoflurane (2%) by the intra-articular injection of MIA (Sigma-Aldrich, St. Louis, MO, USA). The right knee joint was shaved and flexed at a 90° angle, and 10 μl of MIA (15 mg/mL) dissolved in saline solution (NaCl 0.9%; SS) was intra-articularly injected with a 30-gauge needle. Control mice received the same volume of SS.

2.3. Mechanical Allodynia

Mechanical allodynia was evaluated by measuring the hind paw withdrawal response to von Frey filament stimulation. For this purpose, mice were placed in methacrylate cylinders (20 cm high and 9 cm in diameter) on a lifted wire grid through which the von Frey filaments (North Coast Medical, Inc., San Jose, CA, USA) with a bending force range of 0.008 to 3.5 g were applied to each hind paw, using the up–down paradigm reported by Chaplan et al. [30]. The test was started with the 0.4 g filament, and the strength of the next filament was increased or decreased in accordance with the response. Finally, the threshold of the response was calculated from the sequence of filament strength using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) that includes the adjustment of the data curve. Both ipsilateral and contralateral hind paws were assessed, and animals were habituated for 1 h before starting the test to allow appropriate behavioral immobility.

2.4. Measurement of Grip Strength

Grip strength was measured with a computerized grip strength meter (Model 47200, Ugo Basile, Varese, Italy) according to the method reported by [31]. To measure grip strength in the hind paws, the experimenter held the animal by the base of the tail, allowing the mice to grasp the metal bar of the grip strength meter with its hind paws. The metal bar was connected to a force transducer that automatically recorded the peak force of each measurement in grams. For each mouse, the grip strength of the hind limbs was measured in triplicate. To prevent the mice from gripping the metal bar with their forepaws during the test, the animals were first allowed to grasp a wire mesh cylinder with their forepaws. Baseline grip strength values were recorded for each mouse as the average of three determinations before the administration of MIA or SS. This value was considered 100% of grip strength and was used as a reference for the following determinations.

2.5. Measurement of Anxiety-Like Behaviors

The anxiety-like behaviors were evaluated using the elevated plus maze (EPM) and the open field (OF) tests.

The EPM is an apparatus with 4 arms 5 cm wide and 35 cm long, two of which are open and two closed with walls 15 cm high. The distance of the EPM to the ground is 45 cm. The animal was placed in the central square of the maze facing one of the open arms, and its behavior was recorded by a digital camera for 5 min, according to the method described by [32]. The number of entries in the open and closed arms, as well as the percentage of time spent in the open arms, was calculated for each animal.

The OF test was also used to evaluate the anxiety-like behavior of animals according to the method used by [33]. Mice were placed on a 44 × 44 cm box with a gray nonreflecting base and walls, and their behavior was recorded by a digital camera for 5 min. At the beginning of the test, mice were placed in the center of the arena and 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 determined for each animal.

2.6. Measurement of Depressive-Like Behaviors

The evaluation of the depressive-like behaviors was realized using the tail suspension test (TST) in accordance with the procedures described by [34] and the forced swimming test (FST) according to the method described by [35].

In the TST, each mouse was suspended 35 cm above the floor with an adhesive tape attached to the tip of its tail. The entire experiment was recorded with a digital camera, and the immobility time was measured over a period of 6 min. The mice were considered immobile when they remained completely quiet.

In the FST, each mouse was placed in a transparent Plexiglas cylinder (25 cm high × 10 cm diameter) 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.

All the behavioral experiments were executed by an experimenter blinded to the treatment applied.

2.7. Western Blot Analysis

Animals were euthanized by cervical dislocation at 29 days after injection (MIA or SS). Tissues from the contralateral hippocampus were extracted quickly after killing, frozen, and maintained at −80°C until use. The protein levels of CD11b/c, GFAP, NOS2, PI3K, p-Akt, HO-1, NQO1, GSTM1, and GSTA1 were analyzed. The homogenization of the tissues was performed in ice-cold lysis buffer (50 mM Tris-Base, 150 nM NaCl, 1% NP-40, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 Triton X-100, 0.1% sodium dodecyl sulfate, 1 mM Na3VO4, 25 mM NaF, 0.5% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail). All reagents were acquired from Sigma-Aldrich (St. Louis,

MO, USA), except for NP-40, which was purchased from Calbiochem (Darmstadt, Germany). After solubilization of crude homogenate for 1 h at 4 °C, it was sonicated for 10 s and centrifuged at 4 °C for 15 min at 700× *g*.

Then, the supernatants (60 μg of total protein) were mixed with 4 × Laemmli loading buffer and loaded onto 4% stacking/10% separating sodium dodecyl sulfate polyacrylamide gels. Proteins were electrophoretically transferred onto a polyvinylidene fluoride membrane for 120 min and blocked with phosphate-buffered saline plus 5% nonfat dry milk or Tris-buffered saline with Tween 20 plus 5% nonfat dry milk or 5% bovine serum albumin for 1 h and 15 min and then incubated with specific rabbit primary anti CD11b/c (1:200), GFAP (1:5000), GSTM1 (1:150), and GSTA1 (1:150) from Novus Biologic, Litton, CO, (USA); NOS2 (1:200) and PI3K (1:200) from Abcam, Cambridge (United Kingdom); p-Akt (1:200) and Akt (1:200) from Cell Signaling Technology, Danvers, MA (USA); HO-1 (1:150) from Enzo Life Sciences, Lausen (Switzerland); and NQO1 (1:250) from Sigma-Aldrich, St. Louis, MO (USA) antibodies overnight at 4 °C. Blots were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, United Kingdom) to detect proteins, which were then visualized by chemiluminescence reagents (ECL kit; GE Healthcare, Little Chalfont, United Kingdom) and exposure to Kodak film. Densitometric analysis was done using Image-J program (National Institutes of Health, Bethesda, MD, USA). We used a rabbit anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:5000; Merck, Billerica, MA, USA) as a loading control.

2.8. Experimental Procedures

In the first experiments, baseline responses for the von Frey filaments and grip strength were established. After that, osteoarthritis pain was induced, and animals were tested again on days 19, 20, 22, 25, 26, 27, and 29 after MIA injection. SS-injected mice were used as controls (six animals per group).

In other groups of animals, we investigated the effects of the intraperitoneal daily administration of 4.4 μmol/kg A-ITC or vehicle from day 25 to day 29 after MIA or SS injection and the effects of the intraperitoneal daily administration of 13.3 μmol/kg P-ITC or vehicle from day 19 to day 29 after MIA or SS injection on the mechanical allodynia and the grip strength deficits induced by MIA (n = six animals per group). The effects of A-ITC or the vehicle were evaluated on days 26, 27, and 29 post-MIA or SS injection, while the effects of P-ITC or the vehicle were measured on days 20, 22, 25, and 29 post-MIA or SS injection at 30 min after drug or vehicle injection.

We also evaluated the effects of the treatment with 4.4 μmol/kg A-ITC or vehicle during 4 consecutive days (25 to 29 after MIA or SS injection) and the effects of 13.3 μmol/kg P-ITC or vehicle administered during 10 consecutive days (19 to 29 after MIA or SS injection) on the anxiety- and depressive-like behaviors associated with chronic osteoarthritis pain at 29 days after MIA injection. The anxiety-like behaviors were evaluated in the EPM and OF tests and the depressive-like behaviors in the TST and FST (n = 10 animals per group).

The involvement of Kv7 potassium channels in the inhibition of the allodynia, grip strength deficits, and depressive-like behaviors produced by the administration of 4.4 μmol/kg A-ITC or 13.3 μmol/kg P-ITC during 4 days or 10 consecutive days was also studied by evaluating the reversion of these effects with the administration of 8.0 μmol/kg of the selective Kv7 potassium channel blocker, XE-991 [36].

Finally, at day 29 after MIA injection and at 4 (A-ITC) or 10 days (P-ITC) of drug or vehicle administration, mice were euthanized by cervical dislocation, and the protein levels of CD11b/c, GFAP, NOS2, PI3K, p-Akt, HO-1, NQO1, GSTM1, and GSTA1 in the hippocampus were evaluated by Western blot. In these experiments, SS-vehicle-treated mice were used as controls (n = 4 samples per group).

2.9. Drugs

A-ITC and P-ITC, obtained from Sigma-Aldrich (St. Louis, MO, USA) and XE-991, purchased in Tocris Bioscience (Ellisville, MO, USA) were dissolved in SS. All drugs were freshly prepared before use and intraperitoneally administered in a final volume of 10 mL/kg, 30 min, and 45 min before testing,

in accordance with our preliminary studies and other work [12]. For each group treated with a drug, the respective control group received the same volume of vehicle.

2.10. Statistical Analyses

All data are expressed as the mean values ± standard error of the mean (SEM). The statistical results indicate the F value, the degrees of freedom Fx,y, and the p value of the ANOVA. Statistical analysis was carried out using the SPSS program (version 13 for Windows, IBM, Madrid, Spain). We used the three-way repeated measures analysis of variance (ANOVA) with injection, treatment, and time as the factors of variation, followed by one-way ANOVA and the Student–Newman–Keuls test to evaluate the effects of the repetitive treatment with A-ITC and P-ITC and their corresponding vehicle on the mechanical allodynia and grip strength deficits induced by MIA.

The effects of the repetitive treatment with A-ITC and P-ITC on the anxiety-like and depressive-like behaviors associated with osteoarthritis pain were assessed using a two-way ANOVA followed by the corresponding one-way ANOVA and the Student–Newman–Keuls test. The reversion of the antinociceptive and antidepressant effects of A-ITC and P-ITC with XE-991were evaluated using a one-way ANOVA and the Student–Newman–Keuls test.

Variations in the protein levels were also analyzed with a one-way ANOVA followed by the Student–Newman–Keuls test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Treatment with A-ITC Reverses the Mechanical Allodynia and the Grip Strength Deficits Induced by the Intra-Articular Injection of MIA in Mice

The three-way repeated measures ANOVA revealed significant effects among the injection ($F_{1,5} = 211.56$, $p < 0.001$), treatment ($F_{1,5} = 50.67$, $p < 0.001$), and time ($F_{3,15} = 5.37$, $p < 0.010$), and interactions between injection and treatment ($F_{1,5} = 79.12$, $p < 0.001$), injection and time ($F_{3,15} = 5.51$, $p < 0.009$), treatment and time ($F_{3,15} = 4.26$, $p < 0.023$), and among the three factors ($F_{3,15} = 3.41$, $p < 0.045$) for the mechanical allodynia. These results confirmed that MIA injection reduces the threshold of the ipsilateral hind paw withdrawal to the von Frey filament stimulation from days 25 to 29 after MIA injection ($p < 0.001$, one-way ANOVA versus the corresponding SS-injected mice treated with vehicle; Figure 1A, Table 1). The administration of A-ITC decreased the mechanical allodynia induced by MIA after one day of treatment. Thus, the threshold of the ipsilateral paw withdrawal to a mechanical stimulus in MIA-injected mice treated with A-ITC was similar to that obtained in SS-injected animals treated with vehicle or A-ITC for one day (Figure 1A). The intraperitoneal administration of A-ITC or vehicle did not produce any significant effect on the mechanical allodynia in either the ipsilateral paw of the SS-injected mice (Figure 1A) or the contralateral paw of the MIA-injected or SS-injected animals (data not shown).

In the grip strength test, the three-way repeated measures ANOVA also revealed significant effects of the injection ($F_{1,5} = 55.69$, $p < 0.001$) and time ($F_{3,15} = 3.75$, $p < 0.034$) and the interaction between injection and treatment ($F_{1,5} = 21.42$, $p < 0.006$). The decreased hind limb grip strength induced by MIA from days 25 to 29 after injection ($p < 0.009$, one-way ANOVA versus the corresponding SS-injected mice treated with vehicle; Figure 1B, Table 1) was inhibited by A-ITC treatment in a time-dependent manner. The grip strength deficits induced by MIA were completely reversed after 4 days of treatment with A-ITC (Figure 1B).

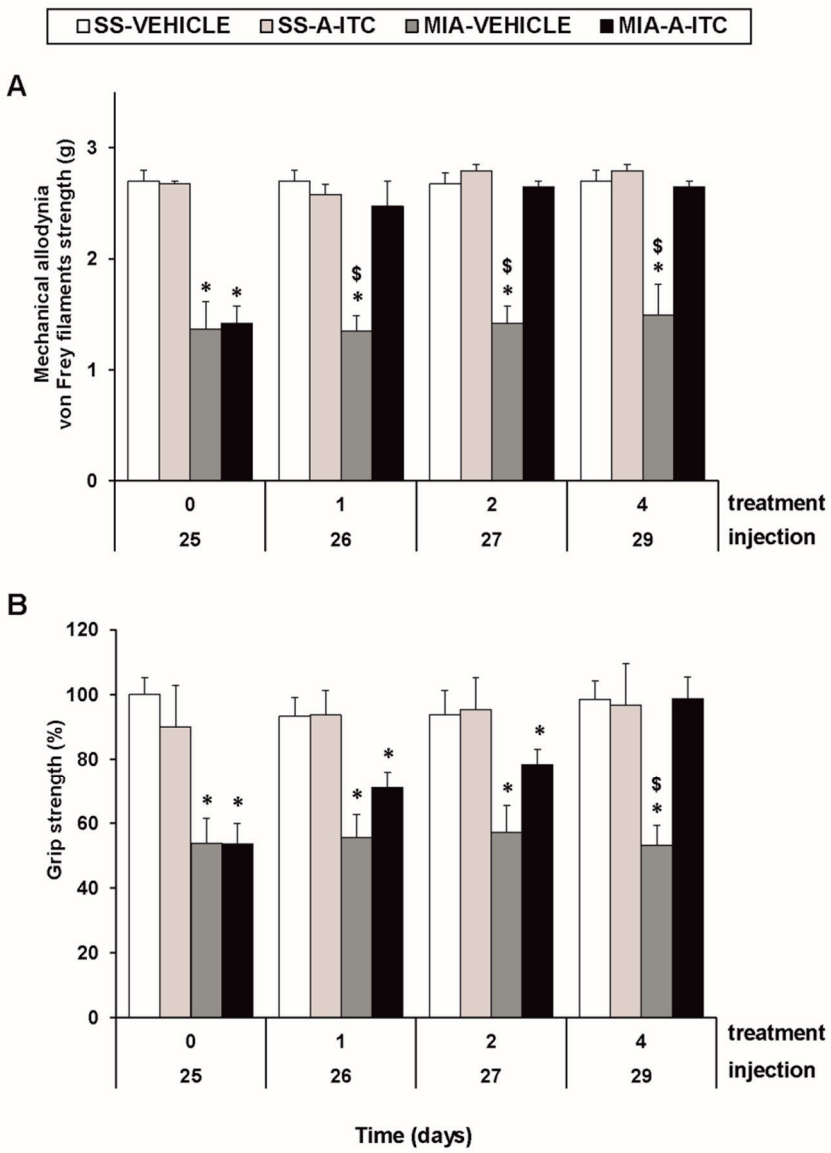

Figure 1. Treatment with allyl isothiocyanate (A-ITC) reduces the mechanical allodynia and the grip strength deficits induced by the intra-articular injection of MIA. The development of (**A**) mechanical allodynia in the ipsilateral paw and (**B**) grip strength deficits in the hind paws of the MIA- or SS-injected mice treated with A-ITC or vehicle for 4 consecutive days are shown. The effects of A-ITC were evaluated at days 26, 27, and 29 after MIA or SS injection. For each test and time evaluated, * denotes significant differences vs. their respective SS-injected mice, and $ denotes significant differences vs. MIA-injected mice treated with A-ITC ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are shown as the mean values ± SEM; $n = 6$ animals per experimental group.

Table 1. Summary of the one-way ANOVA's performed with the results obtained for the mechanical allodynia and grip strength deficits at 0, 1, 2, and 4 days after the administration of allyl isothiocyanate (A-ITC) or vehicle in saline (SS) and monosodium iodoacetate (MIA)-injected mice.

	Time of Treatment (days)			
	0	**1**	**2**	**4**
Mechanical allodynia	$F_{3,20} = 25.15$ $p < 0.001$	$F_{3,20} = 18.27$ $p < 0.001$	$F_{3,20} = 52.24$ $p < 0.001$	$F_{3,20} = 17.98$ $p < 0.001$
Grip strength	$F_{3,20} = 7.97$ $p < 0.001$	$F_{3,20} = 8.26$ $p < 0.001$	$F_{3,20} = 5.05$ $p < 0.009$	$F_{3,20} = 7.09$ $p < 0.002$

3.2. Treatment with P-ITC Reverses the Mechanical Allodynia and the Grip Strength Deficits Induced by the Intra-Articular Injection of MIA in Mice

For mechanical allodynia, the three-way repeated measures ANOVA demonstrated significant effects of the injection ($F_{1,5}$ = 679.25, $p < 0.001$) and treatment ($F_{1,5}$ = 12.58, $p < 0.016$). A significant interaction among injection and treatment ($F_{1,5}$ = 6.59, $p < 0.050$), injection and time ($F_{4.20}$ = 3.35, $p < 0.030$), and the three factors ($F_{4.20}$ = 3.54, $p < 0.024$) was also demonstrated. As a consequence, the reduced threshold of the ipsilateral hind paw withdrawal to von Frey filament stimulation from days 19 to 29 after MIA injection ($p < 0.001$, one-way ANOVA versus the corresponding SS-injected mice treated with vehicle; Figure 2A, Table 2) was completely reversed after 3 days of treatment with P-ITC. The intraperitoneal administration of P-ITC did not have any significant effects on the mechanical allodynia in either the ipsilateral paw of the SS-injected mice (Figure 2A) or the contralateral paw of the MIA-injected or SS-injected animals (data not shown).

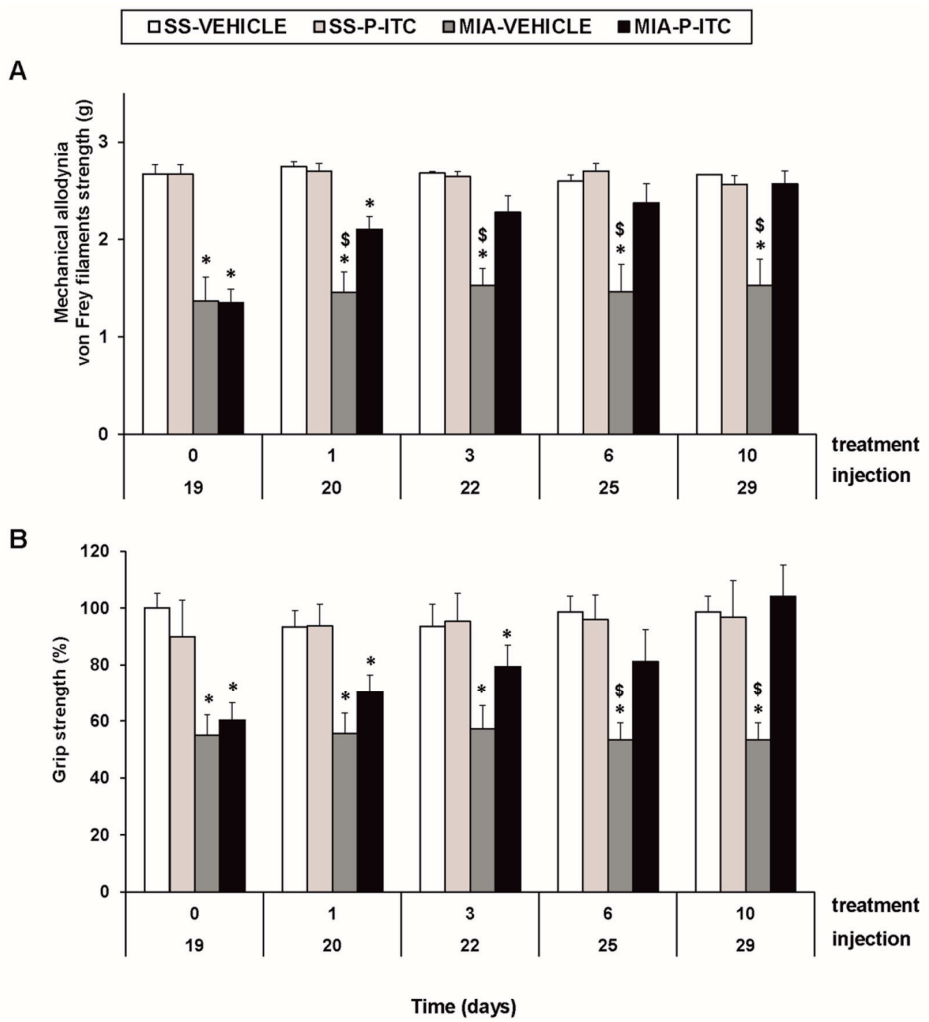

Figure 2. Treatment with phenyl isothiocyanate (P-ITC) decreases the mechanical allodynia and the grip strength deficits induced by the intra-articular injection of MIA. The development of (**A**) the mechanical allodynia in the ipsilateral paw and (**B**) the grip strength deficits in the hind paws of the MIA- or SS-injected mice treated with P-ITC or vehicle for 10 consecutive days are presented. The effects of P-ITC were assessed at days 20, 22, 25, and 29 after MIA or SS injection. For each test and time evaluated, * denotes significant differences vs. their respective SS-injected mice, and $ denotes significant differences vs. MIA-injected mice treated with P-ITC ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are shown as the mean values ± SEM; n = 6 animals per experimental group.

Table 2. Summary of the one-way ANOVA's performed with the results obtained for the mechanical allodynia and grip strength deficits at 0, 1, 3, 6, and 10 days after the administration of P-ITC or vehicle in SS and MIA-injected mice.

	Time of Treatment (days)				
	0	**1**	**3**	**6**	**10**
Mechanical allodynia	$F_{3,20} = 21.29$ $p < 0.001$	$F_{3,20} = 20.36$ $p < 0.001$	$F_{3,20} = 18.33$ $p < 0.001$	$F_{3,20} = 9.92$ $p < 0.001$	$F_{3,20} = 10.53$ $p < 0.001$
Grip strength	$F_{3,20} = 6.84$ $p < 0.001$	$F_{3,20} = 7.65$ $p < 0.001$	$F_{3,20} = 4.44$ $p < 0.015$	$F_{3,20} = 6.27$ $p < 0.004$	$F_{3,20} = 6.13$ $p < 0.004$

In the grip strength test, the three-way repeated measures ANOVA also revealed a significant effect of the injection ($F_{1,5} = 53.18$, $p < 0.001$) and treatment ($F_{1,5} = 6.06$, $p < 0.050$) and the interaction between injection and treatment ($F_{1,5} = 20.10$, $p < 0.006$). The grip strength deficits induced by MIA from days 19 to 29 after injection ($p < 0.015$, one-way ANOVA versus the corresponding SS-injected mice treated with vehicle; Figure 2B, Table 2) were inhibited by P-ITC treatment in a time-dependent manner. The decrease in the grip strength induced by MIA was completely reversed after 10 days of treatment with P-ITC (Figure 2B).

3.3. The Administration of A-ITC or P-ITC did not Inhibit the Anxiety-Like Behaviors Associated with Chronic Osteoarthritis Pain in Mice

The effects of A-ITC and P-ITC treatments on the anxiety-like behaviors accompanying osteoarthritis pain were evaluated in the EPM and OF tests at 29 days after the intra-articular injection of MIA.

In the EPM test, the two-way ANOVA revealed significant effects of the injection in the number of entries in the open arms ($F_{1,54} = 49.78$, $p < 0.001$) and in the time spent in the open arms ($F_{1,54} = 38.19$, $p < 0.001$). The significant reduction in the number of entries ($F_{5,54} = 10.33$, $p < 0.001$, one-way ANOVA versus SS-injected mice treated with vehicle; Figure 3A) and in the time spent in the open arms ($F_{5,54} = 7.97$, $p < 0.001$, one-way ANOVA versus SS-injected mice treated with vehicle; Figure 3B) observed in MIA-injected mice treated with vehicle demonstrated the anxiety-like behavior associated with chronic osteoarthritis pain. Treatment with A-ITC or P-ITC did not normalize the anxiety-like responses induced by MIA. Moreover, no differences in the number of entries into the closed arms were observed between MIA and SS-injected mice treated with A-ITC, P-ITC, or vehicle (Figure 3C).

In the OF test, the two-way ANOVA also revealed significant effects of the injection in the number of entries in the central area ($F_{1,54} = 35.69$, $p < 0.001$) and in the time spent in it ($F_{1,54} = 36.30$, $p < 0.001$). The significant reductions in the number of entries into the central area ($F_{5,54} = 7.69$, $p < 0.001$, ANOVA versus SS-injected mice treated with vehicle; Figure 3D) and in the time spent in it ($F_{5,54} = 7.30$, $p < 0.001$, ANOVA versus SS-injected mice treated with vehicle; Figure 3E) caused by MIA were not reversed by the repeated administration of A-ITC or P-ITC. Thus, confirming that both treatments are not capable of normalizing the anxiety-like responses induced by MIA. Finally, no differences in the number of squares crossed in the OF test were detected between MIA and SS-injected mice treated with A-ITC, P-ITC, or vehicle (Figure 3F).

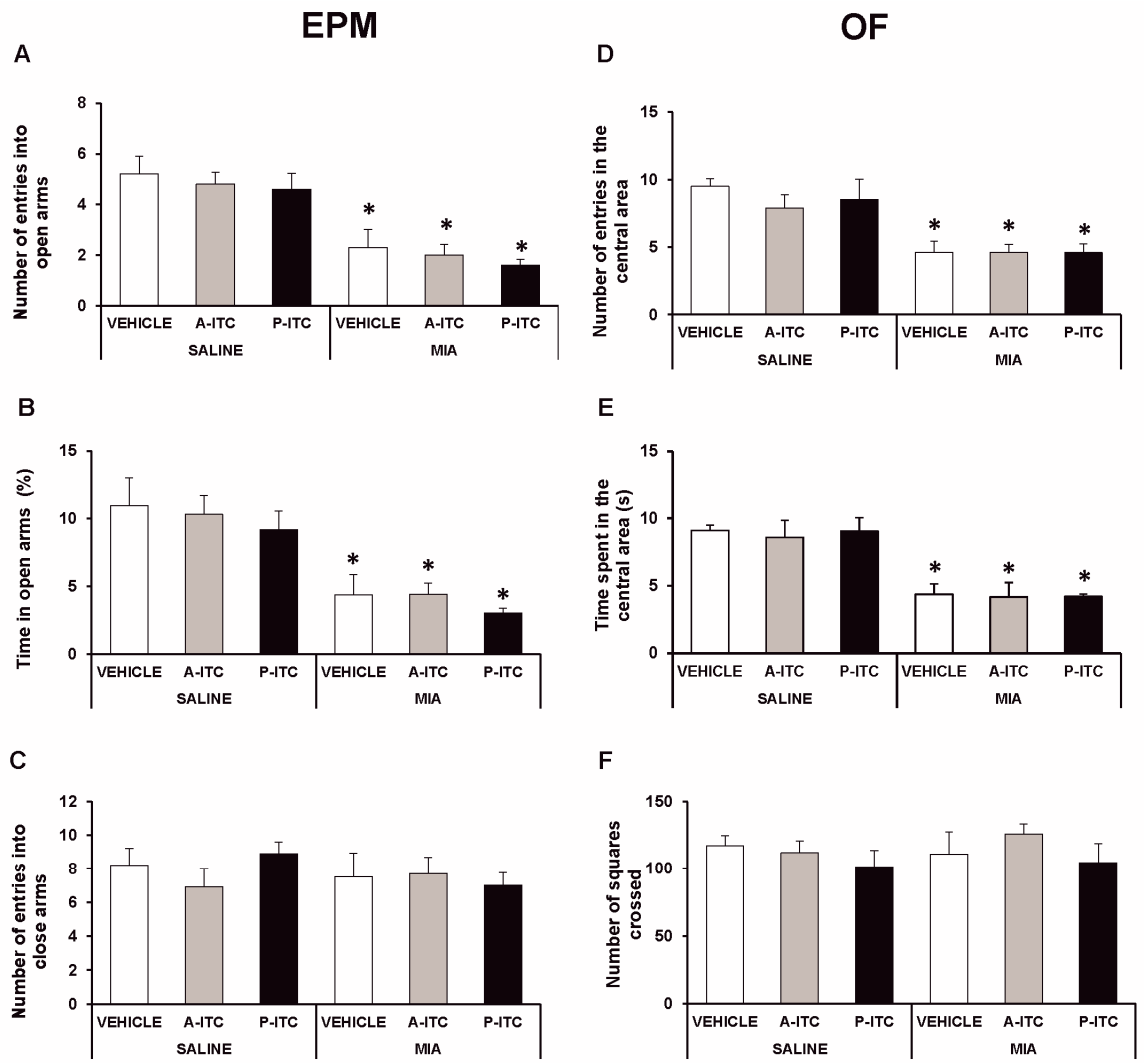

Figure 3. Treatment with A-ITC or P-ITC does not alter the anxiety-like behaviors associated with chronic osteoarthritis pain. The anxiety-like behaviors were evaluated on day 29 after MIA or SS injection and at 4 or 10 days of treatment with A-ITC or P-ITC in the elevated plus maze (EPM) and open field (OF) tests. In the EPM, (**A**) the number of entries into the open arms, (**B**) percentage of time spent in the open arms, and (**C**) the number of entries into the closed arms are shown. For OF, (**D**) the number of entries into the central area, (**E**) time spent in the central area (s), and (**F**) the number of squares crossed are shown. For each test evaluated, * denotes significant differences vs. their respective SS-injected mice ($p < 0.05$; one-way ANOVA followed by the Student-Newman-Keuls test). The results are presented as the mean ± SEM; $n = 10$ animals per experimental group.

3.4. The Administration of A-ITC and P-ITC Inhibits the Depressive-Like Behaviors Associated with Chronic Osteoarthritis Pain

Depressive-like behaviors were assessed in the TST and FST at 29 days after the intra-articular injection of MIA. The two-way ANOVA revealed significant effects of the injection ($F_{1,54} = 30.16$, $p < 0.001$) and treatment ($F_{2,54} = 30.86$, $p < 0.001$) in the TST. Thus, osteoarthritis induced a depressive-like behavior evidenced by the significant increase in the immobility time ($F_{5,54} = 19.32$, $p < 0.001$, one-way ANOVA versus SS-injected mice treated with vehicle, Figure 4A), which was reduced with the administration of A-ITC or P-ITC. Moreover, a significant effect of the injection ($F_{1,54} = 23.47$, $p < 0.001$) and treatment ($F_{2,54} = 10.70$, $p < 0.001$) was also observed in the FST, and both treatments reversed the increased immobility time observed in MIA-injected animals ($F_{5,54} = 9.18$, $p < 0.001$, one-way ANOVA versus SS-injected mice treated with vehicle, Figure 4B). In the TST and FST, both treatments

also reduced the immobility time in SS-injected mice (Figure 4). These results demonstrated the antidepressant effects of these drugs in animals with and without chronic pain.

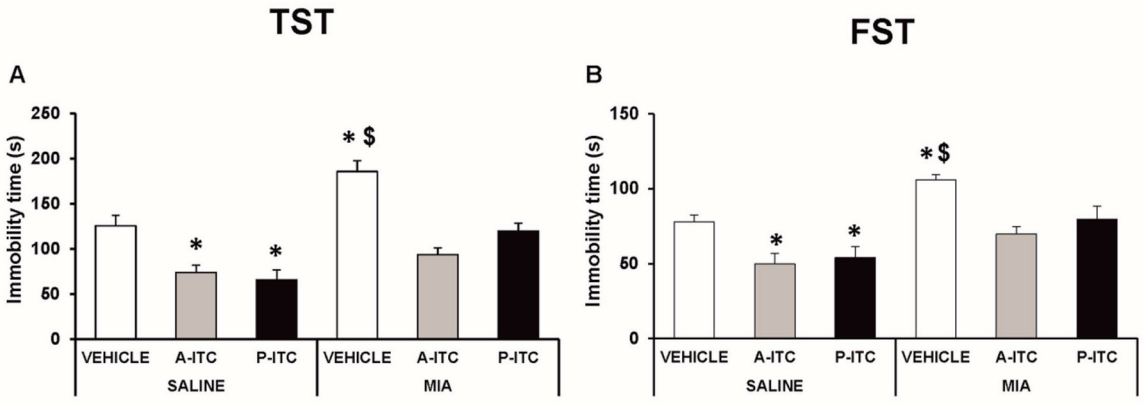

Figure 4. Treatment with A-ITC or P-ITC decreases the depressive-like behaviors associated with chronic osteoarthritis pain. The immobility time (s) evaluated with the (**A**) tail suspension test (TST) and (**B**) forced swimming test (FST) at 29 days after MIA or SS injection in mice treated for 4 consecutive days with A-ITC or for 10 days with P-ITC is shown. For each test evaluated, * denotes significant differences vs. SS-injected mice treated with vehicle, and $ denotes significant differences vs. MIA-injected mice treated with a drug ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean values ± SEM; $n = 10$ animals per experimental group.

3.5. The Administration of XE-991 Reverses the Inhibition of the Mechanical Allodynia, Grip Strength Deficits, and Depressive-Like Behaviors of A-ITC and P-ITC during Osteoarthritis Pain

To assess the involvement of H_2S in the effects produced by the repetitive administration of 4.4 µmol/kg A-ITC during 4 days or 13.3 µmol/kg P-ITC during 10 days in animals with osteoarthitis pain, the reversion of their effects with the selective Kv7 potassium channel blocker, XE-991, administered at 8.0 µmol/kg over one day was evaluated. Our results showed that the administration of XE-991 reversed the inhibition of the mechanical allodynia ($F_{6,35} = 16.70$, $p < 0.001$, one-way ANOVA versus MIA saline-injected mice treated with vehicle, Figure 5A) and the grip strength deficits induced by A-ITC and P-ITC treatments ($F_{6,35} = 11.32$, $p < 0.001$, one-way ANOVA versus MIA-saline-injected mice treated with vehicle, Figure 5B). Moreover, the antidepressant effects of A-ITC and P-ITC in the TST ($F_{6,42} = 8.63$, $p < 0.001$, one-way ANOVA versus MIA saline-injected mice treated with vehicle, Figure 6A) and FST ($F_{6,42} = 9.50$, $p < 0.001$, one-way ANOVA versus MIA saline-injected mice treated with vehicle, Figure 6B) were reversed with XE-991. The administration of XE-991 alone (Figures 5 and 6) did not produce any significant effect.

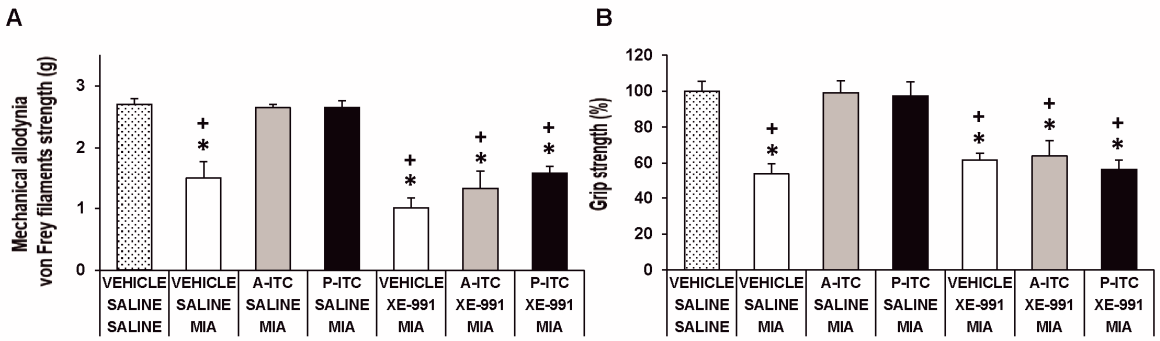

Figure 5. The administration of XE-991 reverses the inhibition of the mechanical allodynia and grip strength deficits of A-ITC and P-ITC during chronic osteoarthritis pain. The mechanical allodynia in the ipsilateral paw (**A**) and grip strength deficits in the hind paws (**B**) of the MIA-injected mice treated

with A-ITC or P-ITC during 4 or 10 days alone and combined with the selective Kv7 potassium channel blocker XE-991 are shown. The effects of XE-991 administered alone are also represented. For each test evaluated, * denotes significant differences vs. saline-saline-injected mice treated with vehicle and + denotes significant differences vs. MIA saline-injected mice treated with a drug ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are shown as the mean values ± SEM; $n = 6$ animals per experimental group.

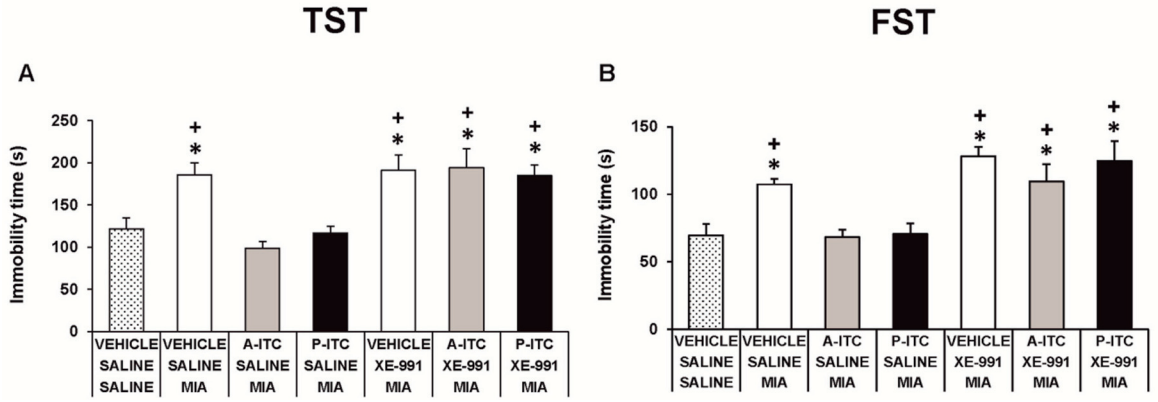

Figure 6. The administration of XE-991 reverses the antidepressant effects of A-ITC and P-ITC in mice with chronic osteoarthritis pain. The immobility time (s) evaluated with the (**A**) TST and (**B**) FST in mice treated for 4 or 10 consecutive days with A-ITC or P-ITC alone and combined with the selective Kv7 potassium channel blocker XE-991 is shown. The effects of XE-991 administered alone are also represented. For each test evaluated, * denotes significant differences vs. saline–saline-injected mice treated with vehicle and + denotes significant differences vs. MIA–saline-injected mice treated with a drug ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are shown as the mean values ± SEM; $n = 6$–8 animals per experimental group.

3.6. Effect of A-ITC and P-ITC on the Expression of CD11b/c, GFAP, NOS2, PI3K, p-Akt, HO-1, NQO1, GSTM1, and GSTA1 in the Hippocampus of MIA-Injected Mice

Our results showed that the intra-articular injection of MIA caused a significant increase in the expression of CD11b/c ($F_{3,12} = 4.39$, $p < 0.026$, one-way ANOVA; Figure 7A), NOS2 ($F_{3,12} = 13.34$, $p < 0.001$, one-way ANOVA; Figure 7C), PI3K ($F_{3,12} = 5.63$, $p < 0.012$, one-way ANOVA; Figure 8A), p-Akt ($F_{3,12} = 8.60$, $p < 0.003$, one-way ANOVA; Figure 8B), HO-1 ($F_{3,12} = 9.87$, $p < 0.001$, one-way ANOVA; Figure 9A), NQO1 ($F_{3,12} = 8.87$, $p < 0.002$, one-way ANOVA; Figure 9B), GSTM1 ($F_{3,12} = 6.54$, $p < 0.007$, one-way ANOVA; Figure 9C), and GSTA1 ($F_{3,12} = 3.75$, $p < 0.041$; Figure 9D) in the hippocampus. Treatment with A-ITC and P-ITC normalized the enhanced protein levels of CD11b/c, NOS2, PI3K, and p-Akt in the hippocampus (Figures 7 and 8). Moreover, while both treatments maintained the increased protein levels of HO-1 and GSTA1 in the hippocampus of MIA-injected mice, the increased expression of NQO1 and GSTM1 induced by MIA was only maintained in A-ITC and P-ITC-treated animals, respectively (Figure 9). No changes in the expression of GFAP were detected in any of the groups evaluated (Figure 7B).

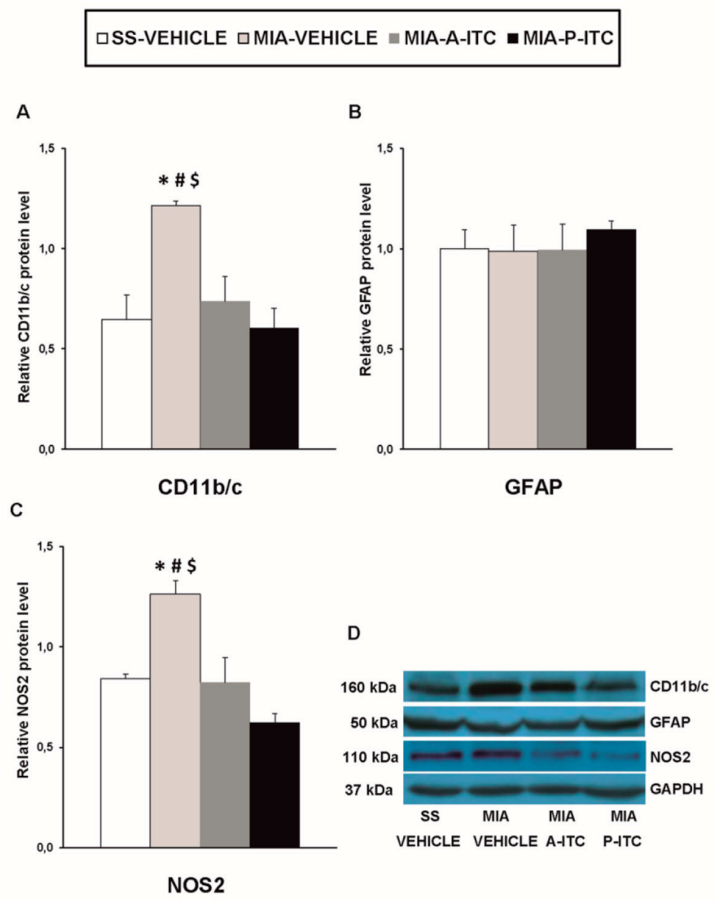

Figure 7. A-ITC and P-ITC treatments inhibit the overexpression of CD11b/c and NOS2 in the hippocampus of mice with osteoarthritis pain. The relative protein levels of (**A**) CD11b/c, (**B**) GFAP, and (**C**) NOS2 in the hippocampus of MIA-injected mice treated with A-ITC, P-ITC, or vehicle are presented. The SS-injected mice treated with vehicle were used as controls. (**D**) Representative blots for CD11b/c (160 kDa), GFAP (50 kDa), NOS2 (110 kDa), and GAPDH (37 kDa). All proteins are expressed relative to GAPDH levels. In all panels, * denotes significant differences vs. SS-injected mice treated with vehicle, # vs. MIA-injected mice treated with A-ITC and $ vs. MIA-injected mice treated with P-ITC ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean ± SEM; $n = 4$ samples per experimental group.

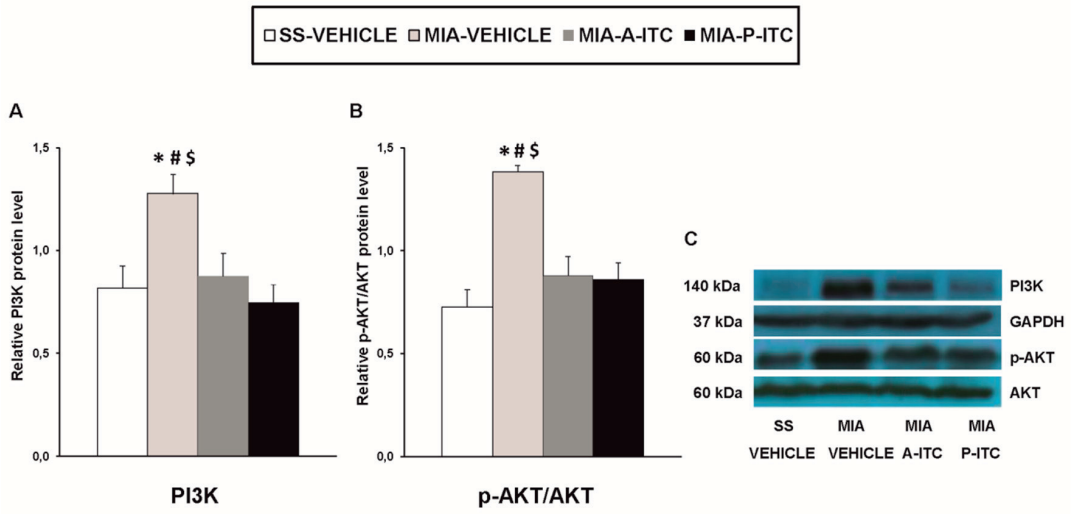

Figure 8. A-ITC and P-ITC treatments inhibit the overexpression of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (p-Akt) in the hippocampus of mice with osteoarthritis pain. The relative

protein levels of (**A**) PI3K and (**B**) p-Akt/Akt in the hippocampus of MIA-injected mice treated with A-ITC, P-ITC, or vehicle are presented. The SS-injected mice treated with vehicle were used as controls. (**C**) Representative blots for PI3K (140 kDa), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (37 kDa), p-Akt (60 kDa), and Akt (60 kDa). PI3K is expressed relative to GAPDH levels and p-Akt relative to total Akt. In all panels, * denotes significant differences vs. SS-injected mice treated with vehicle, # vs. MIA-injected mice treated with A-ITC and $ vs. MIA-injected mice treated with P-ITC ($p < 0.05$; one-way ANOVA and Student–Newman–Keuls test). Results are presented as the mean ± SEM; $n = 4$ samples per experimental group.

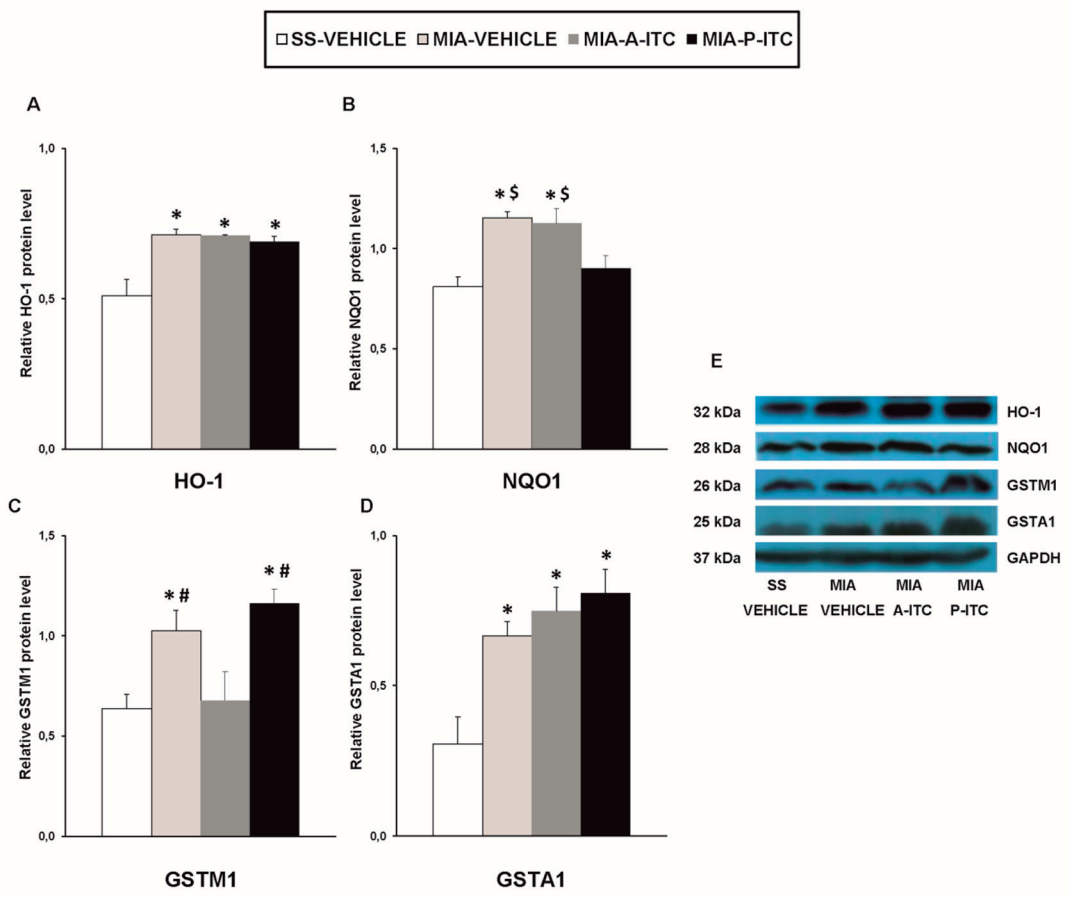

Figure 9. A-ITC and/or P-ITC treatments maintain the overexpression of heme oxygenase 1 (HO-1), quinone oxidoreductase-1 (NQO1), glutathione S-transferase mu 1 (GSTM1), and/or glutathione S-transferase alpha 1 (GSTA1) in the hippocampus of mice with osteoarthritis pain. The relative protein levels of (**A**) HO-1, (**B**) NQO1, (**C**) GSTM1, and (**D**) GSTA1 in the hippocampus of MIA-injected mice treated with A-ITC, P-ITC, or vehicle are presented. The SS-injected mice treated with vehicle were used as controls. (**E**) Representative blots for HO-1 (32 kDa), NQO1 (28 kDa), GSTM1 (26 kDa), GSTA1 (25 kDa), and GAPDH (37 kDa). All proteins are expressed relative to GAPDH levels. In all panels, * denotes significant differences vs. SS-injected mice treated with vehicle, # vs. MIA-injected mice treated with A-ITC and $ vs. MIA-injected mice treated with P-ITC ($p < 0.05$; one-way ANOVA and Student–Newman–Keuls test). The results are presented as the mean ± SEM; $n = 4$ samples per experimental group.

4. Discussion

This study reveals that the repeated administration of low doses of A-ITC or P-ITC inhibited the mechanical allodynia and grip strength deficits induced by the intra-articular injection of MIA as well as the depressive-like behaviors associated with chronic osteoarthritis pain. Both treatments also

normalized microglial activation, NOS2 overexpression, and PI3K/Akt phosphorylation induced by MIA and maintained high levels of the antioxidant enzymes in the hippocampus of mice with osteoarthritis.

The role of H_2S in pain modulation is controversial. It can produce pronociceptive or antinociceptive effects depending on the type of H_2S donor, the doses and pain models used, etc. [6]. Thus, while the fast donors of H_2S cannot alter or even increase pain [7–10], more recent studies show that the administration of substances capable of releasing H_2S slowly, simulating the conditions of H_2S release in vivo, in addition to exert potent anti-inflammatory effects [11], are also able to relieve neuropathic pain induced by the administration of antineoplastic drugs in animals [12]. In accordance, our results showed that the administration of A-ITC or P-ITC, two slow-releasing H_2S donors, inhibited the mechanical allodynia induced by the intra-articular administration of MIA with different effectiveness levels. That is, while one day of treatment with 4.4 μmol/kg A-ITC is enough to block the mechanical allodynia induced by osteoarthritis, three days of treatment with 13.3 μmol/kg of P-ITC are required to abolish it. Our results are in agreement with the inhibitory effects of A-ITC and P-ITC in the mechanical allodynia provoked by chemotherapy [12] and showed, for the first time, the antiallodynic properties of these compounds in female mice with chronic osteoarthritis pain at 29 days after MIA injection.

Chronic osteoarthritis pain, in addition to inducing important hypersensitivity in patients, also causes alterations in physical functioning that negatively influence several aspects of daily life, such as difficulty walking, inability to perform daily tasks, etc. [2]. Thus, it is important to measure the effect of treatments on the physical function of the subject, and grip strength evaluation is habitually utilized as a functional measure in patients with joint inflammation. Consequently, in this study, we evaluated the effects of A-ITC and P-ITC treatments on the functional deficits induced by osteoarthritis by measuring the grip strength of these animals. Our results demonstrated that the intra-articular injection of MIA decreased the hind limb grip strength from days 19 to 29 after the induction of osteoarthritis, corroborating and expanding the results obtained at 21 days after MIA injection [37]. Our data also revealed that while in complete Freund's adjuvant (CFA)-induced joint inflammation the grip strength deficit returns to normal values at 21 days after induction [31], in MIA-induced osteoarthritis, the grip strength deficit remains constant for at least 29 days. These differences may be related to the fact that MIA provokes cartilage destruction, and this effect is more persistent over time. Our results further demonstrated that the repeated administration of A-ITC and P-ITC reduced the grip strength deficits induced by MIA. However, as occurs in mechanical allodynia, their effects are produced at different times according to the treatment. Thus, while 4 days of treatment with 4.4 μmol/kg A-ITC completely reversed the grip strength deficit induced by MIA, 10 days of treatment with 13.3 μmol/kg P-ITC were required to block it. These results suggested that treatment with A-ITC is more effective than treatment with P-ITC at reversing the mechanical allodynia and the grip strength deficits generated by osteoarthritis. Our findings also showed that both compounds produce a more rapid recovery of the mechanical allodynia than the recovery of the grip strength deficits caused by osteoarthritis. These results are in contrast with the equipotency of oxycodone in reversing the allodynia and physical function but agree with the effects produced by other drugs, such as morphine, which is also less potent in the reversion of the grip strength deficits than the mechanical allodynia caused by CFA-induced joint inflammation in female mice [31].

Chronic osteoarthritis pain is also accompanied by emotional disorders such as depression and anxiety. Thus, the evaluation of the effects induced by a treatment on the affective manifestations associated with chronic osteoarthritis pain is fundamental. Several studies have demonstrated that the administration of some H_2S donors produced anxiolytic effects [13,38]. In this study, we evaluated the possible anxiolytic effects induced by treatment with slow-releasing H_2S donors in animals with chronic osteoarthritis pain using the EPM and OF tests. Our results confirmed the anxiety-like behaviors induced by the intra-articular injection of MIA, as demonstrated by the reduced number of entries and the time spent in the open arms in the EPM test as well as by the diminished number of entrances into the central area and the time spent in it in the OF test. We further demonstrated that the administration

of A-ITC or P-ITC did not alter the anxiety-like behaviors in mice with osteoarthritis pain. These results are in contrast with the anxiolytic effects induced by other fast-releasing H_2S donors, such as sodium hydrosulfide [13] or sodium sulfide in control animals and diabetic rats [14,38], thus displaying the different properties of H_2S in accordance with the type of donor used. Finally, treatment with A-ITC or P-ITC did not alter the number of entries into the closed arms in the EPM test or the number of squares crossed in the OF test, revealing that these compounds do not affect the locomotor activity of animals with or without osteoarthritis pain.

Chronic osteoarthritis pain is also associated with the development of depression, which is difficult to treat with conventional analgesics. In agreement with other studies, our results confirmed that osteoarthritis induced by MIA causes depressive-like behaviors [22]. In contrast to anxiety, the administration of A-ITC or P-ITC inhibited the depressive-like responses promoted by osteoarthritis. Thus, the increase in the immobility time observed in the TST and the FST at 29 days after MIA injection was inhibited by treatment with A-ITC or P-ITC. These results demonstrated the antidepressant effects of both slow-releasing H_2S donors in animals with chronic osteoarthritis pain. Moreover, and in accordance with the antidepressant properties of other H_2S donors [39], the reduction in the immobility time induced by A-ITC and P-ITC in control mice also evidenced their antidepressant properties in the absence of pain.

In this study, we also evaluated the possible involvement of Kv7 potassium channels in the inhibitory effects of A-ITC and P-ITC by assessing the reversion of their effects with the selective Kv7 potassium channel blocker, XE-991 [36]. Our results showed that the inhibition of the mechanical allodynia, grip strength deficits, and the depressive like-behaviors produced by A-ITC and P-ITC treatments was reversed with the administration of XE-991. These data agree with the reversion of the antinociceptive effects of A-ITC, P-ITC, and other H_2S donors (NaHS, glucoraphanin, and GYY4137) by selective Kv7 potassium channel blockers during chronic pain [12,40].

It is well recognized that the activation of several brain areas, for example the hippocampus, modulates pain and the emotional manifestations of pain. In this study, we evaluated the effects of the administration of A-ITC and P-ITC on microglial activation and the inflammatory and/or biochemical changes induced by chronic osteoarthritis in this brain region. Microglia play a critical role in the development and maintenance of chronic pain as well as in depression. Several works demonstrated microglial activation in the spinal cord [41] and the medial prefrontal cortex of animals with osteoarthritis at 25–26 days after MIA injection [22]. In this study, the increased expression of CD11b/c promoted by MIA in the hippocampus at 29 days after injection supported these findings and further demonstrated microglial activation in this brain area. Moreover, considering that treatment with minocycline relieves the depressive-like behaviors associated with chronic cancer pain by inhibiting microglia activation in the hippocampus [25]; the inhibition of microglial activation induced by A-ITC and P-ITC might explain their antidepressant effects during chronic osteoarthritis pain. In contrast to microglia, nonchanges in the expression of the astroglial marker (GFAP) were observed in the hippocampus. These results agree with the absence of changes in the spinal astrocyte expression reported by other studies performed in animals with chronic osteoarthritis pain [42,43]. Nonetheless, our treatments did not alter the astrocyte expression in the hippocampus.

Nitric oxide also contributes to the pathophysiology of osteoarthritis. High concentrations of nitric oxide synthesized by NOS2 were detected in the synovial fluid, and treatment with specific NOS2 inhibitors attenuated the osteoarthritis pain induced by MIA by reducing nitric oxide production [44]. Other studies also indicated that the cytoprotective effects of H_2S in osteoarthritis were mediated via inhibiting nitric oxide synthesis. To elucidate whether NOS2 is also involved in the effects produced by A-ITC and P-ITC during osteoarthritis, we investigated its expression in the hippocampus. Our results showed that the increased protein levels of NOS2 in this supraspinal region of animals with knee osteoarthritis were inhibited by the administration of slow-releasing H_2S donors. The fact that chronic inflammatory pain is inhibited in mice lacking NOS2 [45] supports the hypothesis that the antinociceptive effects induced by A-ITC and P-ITC during osteoarthritis might also be due to

modulating the expression of NOS2 in the hippocampus. Nevertheless and taking into account that several authors have demonstrated that the exposition to H_2S actives the synthesis of the endothelial nitric oxide synthase (NOS3) in different cell types [46], we cannot discard that the antinociceptive and/or antidepressant effects of the slow-releasing H_2S donors can be also produced via modulating NOS3.

The intracellular signaling pathway PI3K/p-Akt also regulates the inflammatory processes in the pathogenesis of osteoarthritis [47]. In accordance with the activated PI3K/Akt pathway observed in the spinal cord and hippocampus of animals with chronic neuropathic pain [18], our results further demonstrated that knee osteoarthritis also activates this signaling pathway in the hippocampus, which was inhibited by both A-ITC and P-ITC treatments. Thus, taking account that the inhibition of PI3K/p-Akt alleviates the mechanical allodynia induced by chronic pain [19], the antiallodynic effects of A-ITC and P-ITC might also be mediated via the hippocampal regulation of this pathway.

Finally, it is well known that oxidative stress is another key factor in the onset and progression of osteoarthritis, and the Nrf2 transcription factor activates the expression of several antioxidant and detoxificant enzymes to protect the organism against oxidative stress [48]. Consequently, while the inhibition of Nrf2 or HO-1 results in increased osteoarthritis severity [49], its induction evokes favorable effects during osteoarthritis [50]. In this study, we evaluated the effects of treatment with A-ITC and P-ITC on the protein levels of the antioxidant enzymes HO-1, NQO1, GSTM1, and GSTA1 in the hippocampus of animals with osteoarthritis pain. Our data indicate that the expression of these antioxidant/detoxificant enzymes is significantly increased in the hippocampus of MIA-injected mice and that treatment with A-ITC and P-ITC maintains its high levels in this brain area. These findings are in accordance with the overexpression of Nrf2 and HO-1 detected in the cartilage of patients and animals with osteoarthritis [51,52], suggesting that the high expression of the antioxidant/detoxificant proteins detected in the hippocampus of mice with knee osteoarthritis might act as an endogenous protective response against the oxidative stress generated by this pathology. Furthermore, the fact that treatment with A-ITC and/or P-ITC maintained the elevated levels of HO-1, NQO1, GSTM1, and/or GSTA1 supported the idea that the antinociceptive/anti-inflammatory effects induced by these slow-releasing H_2S donors during chronic osteoarthritis might also be produced by keeping the endogenous antioxidant system activated.

5. Conclusions

In conclusion, this study demonstrated that treatment with A-ITC and P-ITC inhibits the mechanical allodynia and grip strength deficits caused by osteoarthritis as well as the depressive-like behaviors associated with chronic osteoarthritis pain. Both treatments also modulate microglial activation and the inflammatory/nociceptive reactions induced by chronic osteoarthritis in the hippocampus and maintain the endogenous antioxidant system activated. Our data suggest that treatment with low doses of slow-releasing H_2S donors might be an interesting strategy for the treatment of nociception, functional disability, and depressive-like conducts related to chronic osteoarthritis pain.

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4.2. The Recovery of Cognitive and Affective Deficiencies Linked with Chronic Osteoarthritis Pain and Implicated Pathways by Slow-Releasing Hydrogen Sulfide Treatment.

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The Recovery of Cognitive and Affective Deficiencies Linked with Chronic Osteoarthritis Pain and Implicated Pathways by Slow-Releasing Hydrogen Sulfide Treatment

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Abstract: Chronic osteoarthritis pain is accompanied by several comorbidities whose treatment has not been completely resolved. The anti-inflammatory, analgesic, and antidepressant effects of slow-releasing hydrogen sulfide (H_2S) donors during osteoarthritic pain have been shown, but their actions in the accompanying memory impairment and anxious-like behaviors have not yet been demonstrated. Using female mice with chronic osteoarthritic pain, the effects of natural, diallyl disulfide (DADS) or synthetic, morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex (GYY4137) slow-releasing H_2S donors, on associated cognitive and grip strength deficits and anxiodepressive-like behaviors, were assessed. Their effects on specific brain areas implicated in the modulation of pain and emotional responses were also determined. Results demonstrated an improvement in memory and grip strength deficits, as well as in the anxious-like behaviors associated with chronic pain in GYY4137 and/or DADS treated mice. The painkiller and antidepressant properties of both treatments were also established. Treatment with DADS and/or GYY4137 inhibited: oxidative stress in the amygdala; phosphoinositide 3-kinase overexpression in the amygdala, periaqueductal gray matter, and anterior cingulate cortex; protein kinase B activation in the amygdala and infralimbic cortex; up-regulation of inducible nitric oxide synthase in the amygdala, periaqueductal gray matter and infralimbic cortex and apoptotic responses in the amygdala. These results might explain the recovery of memory and grip strength and the inhibition of allodynia and associated anxiodepressive-like behaviors by these treatments. In conclusion, this study revealed new properties of slow-releasing H_2S donors in cognitive impairment and affective disorders linked with chronic osteoarthritis pain and their effects on the central nervous system.

Keywords: anxiety; apoptosis; cognitive deficits; depression; grip strength impairments; hydrogen sulfide; osteoarthritic pain; oxidative stress

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1. Introduction

Osteoarthritis is a degenerative disorder and one of the most common forms of arthritis. It is also one of the most frequent sources of chronic pain, affecting more than 100 million people worldwide. However, despite its high incidence in society, especially in women (35%), its etiology and treatment have not been fully solved [1].

Osteoarthritis is characterized by the progressive loss of articular cartilage, bone, and synovial remodeling, thus leading to subchondral bone alterations and inflammation, and resulting in severe pain and loss of joint function [2,3]. In addition, and as a consequence of the pain and suffering, patients also experience related cognitive and affective disorders, including memory dysfunction and depressive-like and anxiety-like behaviors [4]. These

emotional comorbidities further potentiate hypersensitivity and a more rapid cartilage degradation and disability, thus contributing to a worse quality of life in patients [5]. Different studies have suggested that the underlying mechanisms involved in chronic pain processes are also implicated in memory deficits, as seen in patients with fibromyalgia or severe pain who have exhibited relevant alterations in working memory [6–8]. Moreover, osteoarthritis and several emotional and cognitive deficits share a common pathophysiology that involves the activation of the phosphatidylinositol 3 kinase (PI3K)-protein kinase B (AKT) pathway, oxidative stress, and increased proinflammatory responses [9–12]. The PI3K/p-AKT axis is involved in several cellular processes essential to maintaining homeostasis, including cell growth, cell survival, inflammation, and metabolism [13,14]. This pathway is also implicated in the progression of osteoarthritis pain, and its inhibition has been found to attenuate joint damage by restoring cartilage homeostasis, enhancing autophagy, and suppressing inflammation [10]. The PI3K/p-AKT pathway is also associated with negative mood disorders and may be an important target in the neuroprotection for depressive and anxiety syndromes [15,16]. Furthermore, the inactivation of this pathway has been found to additionally improve the cognitive alterations associated with chronic pain [17].

Several studies revealed that oxidative stress takes part in the pathogenesis of osteoarthritis [18,19]. Therefore, oxidative stress as a result of high reactive oxygen species synthesis, modest antioxidant defenses and lipid-rich formation is a negative factor for certain neuropsychological illnesses, such as alterations in learning and memory functions [11], anxiety [20], depression [21], and nociception [22]. Indeed, oxidative stress blockage can prevent the appearance of these behavioral syndromes, and the emotional and cognitive impairments have been manifested under low levels of antioxidant defenses, thus supporting the relationship between oxidative stress and emotional/memory deficits [11,23,24].

It has been demonstrated that in the progress of osteoarthritis, affected chondrocytes and synovial cells overproduce inflammatory mediators such as interleukin-1β and nitric oxide, which accelerate the degradation of cartilage [25]. Nitric oxide synthetized by the inducible nitric oxide synthase (NOS2) is one of the major proinflammatory and destructive mediators in osteoarthritis [26]. Indeed, high levels of NOS2 have been found in the synovial membrane of individuals with osteoarthritis [27]. Moreover, experiments performed on animals have supported this idea by showing that NOS2 inhibition could be an attractive way to treat osteoarthritis [26]. Finally, the excess production of reactive oxygen species and nitric oxide has been linked with apoptotic responses during osteoarthritis [19]. Therefore, the up-regulation of 4-HNE, a marker of oxidative damage, ends up causing increased BAX levels, leading to cellular apoptosis [18]. In this study, we evaluated the expression of 4-HNE and BAX as markers of oxidative stress and apoptosis.

Hydrogen sulfite (H_2S) is an endogenous gaseous neurotransmitter that takes part in several physiological processes in the central and peripheral nervous systems [28,29]. Accordingly, H_2S releasing compounds, especially slow-releasing compounds, have been explored as therapeutic agents in several diseases [30,31]. Numerous findings have demonstrated the beneficial anti-inflammatory and protective actions induced by the exogenous administration of H_2S in the cartilage and chondrocytes in cell cultures [32,33]. Other works have also proven the positive effects of the intra-articular administration of H_2S donors on rheumatic disease progression [34], cartilage destruction, and oxidative damage in rats [35]. The antinociceptive and antidepressant effects of the systemic administration of H_2S donors, such as isothiocyanates, in mice with knee osteoarthritis have also been shown [2,36]. Nevertheless, these compounds have failed to inhibit the anxiolytic-like behaviors concomitant with osteoarthritic or neuropathic pain [24,36].

Our objective is to find new treatments that can palliate the memory and grip strength impairments and the anxiodepressive-like behaviors accompanying chronic osteoarthritic pain, and to establish the principal signaling pathways implicated in these actions in specific areas of the central nervous system. Thus, we evaluated the effects of the systemic administration of two slow-releasing H_2S donors, a natural garlic bioactive component

(diallyl disulfide, DADS) and a synthetic compound (morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate, GYY4137), on nociceptive, grip strength, and memory deficits, as well as on the anxiodepressive symptoms provoked by the intra-articular administration of monosodium acetate (MIA). To examine the probable pathways involved in these actions, we analyzed their effects on the amygdala, periaqueductal gray matter, infralimbic cortex, and anterior cingulate cortex, all of which are highly involved in the modulation of nociception, memory, and emotional disorders [37–40].

2. Materials and Methods

2.1. Animals

Experiments were performed with female C57BL/6 mice (21–26 g; 5–6 weeks old), acquired from Envigo Laboratories (Barcelona, Spain). Animals were accommodated under standard light/dark (12/12 h), temperature (22 °C), and humidity (66%) requirements, with free access to food and water. Experiments were done following 7 days of acclimatization to the environmental conditions, performed between 9:00 a.m. and 5:00 p.m., and in compliance with the guidelines of the European Commission's 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 (ethical code: 9863). Every effort was made to reduce the amount and suffering of the animals used.

2.2. Induction of Osteoarthritis Pain

Osteoarthritis pain was induced under isoflurane anesthesia conditions (3% induction, 2.5% maintenance) by the intra-articular injection of MIA (Sigma-Aldrich, St. Louis, MO, USA). The right knee joint was shaved and flexed at a 90° angle, and 10 µL of MIA (15 mg/mL) dissolved in saline solution (NaCl 0.9%; SS) was injected. Control animals were injected with the same volume of SS.

2.3. Mechanical Allodynia

Mechanical allodynia was evaluated by measuring the hind paw withdrawal response after stimulation with von Frey filaments of different bending forces (0.008–3.5 g). The 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 [41]. The 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), which includes curve fitting of the data. The animals were habituated to the environment for 1 h before the experiment. Both ipsilateral and contralateral paws were tested.

2.4. Measurement of Grip Strength

We used a computerized grip strength meter (Model 47200, Ugo Basile, Varese, Italy) to measure grip strength according to the method reported by [42]. To measure grip strength in the hind paws, the experimenter held the animal by the base of the tail, allowing the mice to grasp the metal bar of the grip strength meter with both hind paws. The metal bar was connected to a force transducer that automatically recorded the peak force of each measurement in grams. For each mouse, the grip strength of the hind limbs was measured in triplicate. To prevent the mice from gripping the metal bar with their forepaws during the test, the animals were first allowed to grasp a wire mesh cylinder with their forepaws. Baseline grip strength values were recorded for each mouse as the average of three determinations before the administration of MIA or SS. This value was considered 100% of grip strength and was employed as a reference for the following determinations.

2.5. Cognitive Behavior

Object recognition memory was evaluated in a gray box (44 × 44 cm) with a non-reflecting base and four walls. This task consisted of 4 sessions of 10 min each (habituation, training, and test). On days 1 and 2, the mice were habituated to the empty box. On the 3rd day, for the training session, the mice were placed again in the box and 2 identical objects were presented. Twenty-four hours later, the mice were placed once again in the box, and one of the familiar objects was replaced by a novel object. The time exploring each of the 2 objects (novel and familiar) was filmed. The discrimination index ((time exploring the novel object–time exploring the familiar)/(time exploring novel + familiar) ∗ 100) was utilized as a measure of cognitive behavior, according to [43]. High values of discrimination represent good recognition memory. Mice were habituated to the testing room for 1 h before starting the evaluation, and the equipment was carefully cleaned between subjects.

2.6. Depressive Behavioral Tests

The evaluation of the depressive-like behaviors was performed by using the forced swimming test (FST) and tail suspension test (TST), in which the duration of immobility of the animals was quantified according to the methods described by [44,45], respectively.

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 the mice showed a sufficiently stable level of immobility.

In the TST, the 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 both tests, the mice were considered immobile when they remained completely quiet.

2.7. Anxiety Behavioral Tests

The anxiety-like behavior was assessed by using the elevated plus maze (EPM) [46], which consists of an X-shape apparatus with four arms, each 5 cm wide and 35 cm long, with two being open and two being closed, with walls 15 cm high. It was elevated off the ground by 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 they stayed in the open arms were calculated for each animal. The mice were habituated to the testing room for 1 h before starting the evaluation, and the equipment was carefully cleaned between subjects.

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

2.8. Western Blot Analysis

Twenty-nine days after MIA or SS injection, the animals were euthanized by cervical dislocation and the contralateral amygdala, periaqueductal gray matter, infralimbic cortex and anterior cingulate cortex were quickly extracted, frozen, and maintained at −80 °C until usage. In this study, we analyzed the expression of 4-HNE, PI3K, p-Akt, NOS2 and BAX. The sonication of tissues was made in cold lysis buffer RIPA Buffer (R0278; Sigma-Aldrich, MO, USA). 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 4 × 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, MO, USA) containing 5% nonfat dry milk, Tris-buffered saline with Tween 20 containing 5% bovine serum albumin (BSA; A-7906; Sigma-Aldrich, MO, USA) or 5% nonfat dry milk, and PBS with Tween 20 containing 5% BSA, for 1 h and 15 min. Then, the membranes were incubated with specific rabbit primary antibodies, the anti 4-HNE (1:200; ab46545, Abcam, Cambridge, UK), PI3K (1:150; ab109006, Abcam,

Cambridge, UK), phospho-Akt (1:150; 4060S, Cell Signaling Technology, Danvers, MA, USA), total Akt (1:250; 9272S, Cell Signaling Technology, Danvers, MA, USA), NOS2 (1:200; ab15323, Abcam, Cambridge, UK), BAX (1:250; 14796S, Cell Signaling Technology, Danvers, MA, USA) or β-actin (1:5000, ab8227, Abcam, Cambridge, UK) antibodies overnight at 4 °C. Afterward, blots were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, UK), and proteins were detected with chemiluminescence reagents (ECL kit; GE Healthcare, Little Chalfont, UK). Densitometric analysis was done using the Image-J program (National Institutes of Health, Bethesda, MD, USA).

2.9. Experimental Procedures

In the first experiments, we evaluated the effects of repetitive intraperitoneal administration, twice daily, of 200 μmol/kg of DADS and 32 μmol/kg of GYY4137, over 3 and 4 consecutive days, on the cognitive and grip strength losses and mechanical allodynia caused by osteoarthritis (n = 6–8 animals for each group). The doses of DADS and GYY4137 were chosen in conformity with another research [47]. The effects of the same treatments on associated depressive- and anxiety-like behaviors were further assessed (n = 8 animals for each group). In all experiments, SS plus vehicle-treated mice were used as controls.

MIA-injected animals treated with DADS, GYY4137 or vehicle (0.9% SS) were euthanized by cervical dislocation, and the protein levels of 4-HNE, PI3K, p-Akt, NOS2, and BAX in the amygdala, periaqueductal gray matter, infralimbic cortex and anterior cingulate cortex were evaluated by western blot. In these experiments, SS-injected mice treated with vehicle were used as controls (n = 3–4 samples per group).

2.10. Drugs

DADS and GYY4137 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in SS. Both drugs were intraperitoneally administered 1 h before testing, in a final volume of 10 mL/kg, in accordance with our previous pilot studies and other work [47]. Both drugs were prepared before use, and for each group treated with a drug, the respective control group received the same volume of 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. The effects of DADS and GYY4137 on the allodynia, anxiodepressive-like behaviors, grip strength, and memory deficits associated with osteoarthritis pain were analyzed by using a one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls test. The effects of both treatments on the expression of several proteins in different brain areas were also analyzed by using a one-way ANOVA and the post hoc Student–Newman–Keuls test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Treatment with DADS and/or GYY4137 Inhibits Osteoarthritis-Induced Cognitive Impairment, Hind Limb Grip Strength Deficits and Mechanical Allodynia

The effects of the repetitive intraperitoneal administration, twice daily, of 200 μmol/kg of DADS or 32 μmol/kg of GYY4137, over 3 and 4 consecutive days, on mechanical allodynia and the grip strength and cognitive impairments caused by osteoarthritis were evaluated.

Our data demonstrated the complete reversion of the mechanical allodynia ($p < 0.0001$; one-way ANOVA followed by the Student–Newman–Keuls test as compared with SS-vehicle treated mice) (Figure 1A) and grip strength impairments ($p < 0.0001$; one-way ANOVA followed by the Student–Newman–Keuls test as compared with SS-vehicle treated mice) (Figure 1B) provoked by the intra-articular injection of MIA in animals treated with

DADS or GYY4137. Both treatments did not have any significant impact on the mechanical allodynia in the contralateral paws of either MIA- or SS-injected mice (data not shown).

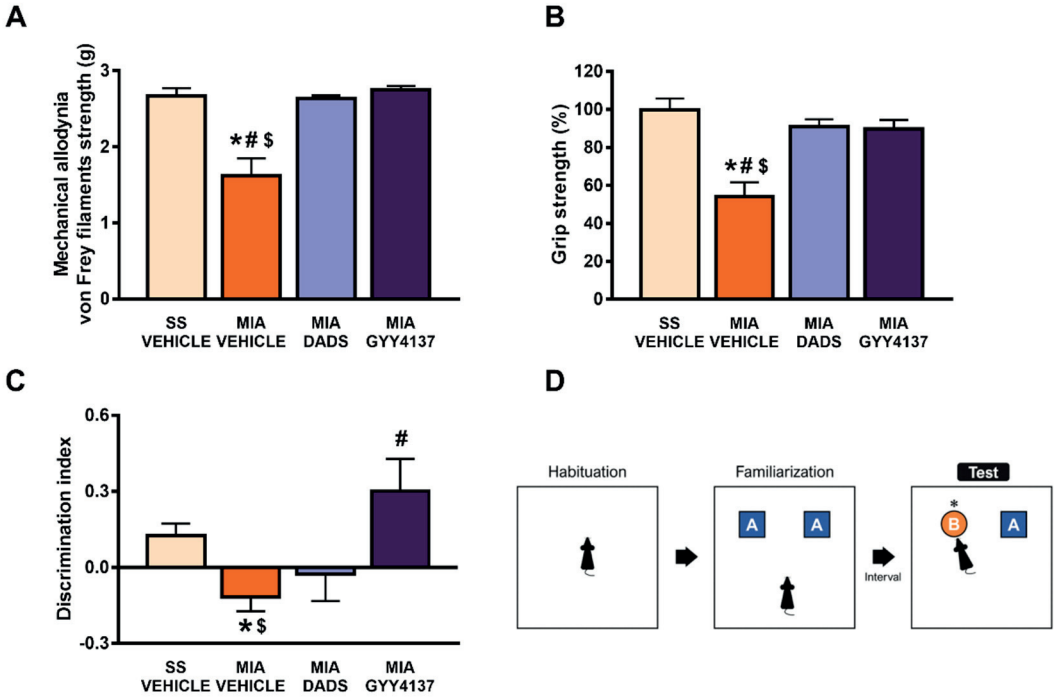

Figure 1. Treatment with DADS and/or GYY4137 inhibited the mechanical allodynia, grip strength, and cognitive deficits induced by monosodium acetate (MIA) injection. The mechanical allodynia, grip strength, and memory deficits induced by osteoarthritis were evaluated on day 29 after MIA or saline solution (SS) injection and treatment with DADS (200 μg/kg, twice daily for 3 days) or GYY4137 (32 μg/kg, twice daily for 4 days) in the von Frey filament strength (g) (**A**), grip strength (%) (**B**) and novel object recognition (discrimination index) (**C**) tests. A schematic representation of the novel object recognition test is shown (**D**). For each test, * denotes significant differences vs. SS-vehicle treated mice, # denotes significant differences vs. MIA-injected mice treated with DADS, and $ denotes significant differences vs. MIA-injected mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are shown as the mean values ± SEM; n = 6–8 animals per experimental group.

Osteoarthritis pain has frequently been associated with memory dysfunction. Our results confirmed these data by showing a decrease in the discrimination index in MIA-injected mice ($p < 0.0071$; one-way ANOVA followed by the Student–Newman–Keuls test vs. SS-vehicle treated mice; Figure 1C). This decrease in the discrimination index was reversed with the GYY4137, but not with the DADS treatment ($p < 0.001$; one-way ANOVA followed by the Student–Newman–Keuls test, as compared with MIA-vehicle treated mice), thus revealing that the impairment of recognition memory associated with chronic osteoarthritis was normalized by the GYY4137 treatment.

3.2. The Inhibition of the Anxiodepressive-Like Behaviors of DADS and GYY4137 in Animals with Chronic Osteoarthritis Pain

We evaluated whether the same administration pattern of both H_2S slow-releasing donors could normalize the emotive disorders accompanying osteoarthritis pain. At 29 days after MIA injection, depressive-like behaviors were analyzed in the FST and TST, while the anxiety-like behaviors were evaluated in the EPM test. Regarding the depressive-like comportment, both treatments reduced the high immobility time observed in their respective MIA-injected mice treated with vehicle in the FST ($p < 0.0054$; one-way ANOVA followed by the Student–Newman–Keuls test; Figure 2A,C) and TST ($p < 0.0016$; one-way ANOVA followed by the Student–Newman–Keuls test, Figure 2B,D), thus in-

dicating that both H_2S donors prevented the depressive-like behavior associated with osteoarthritic pain.

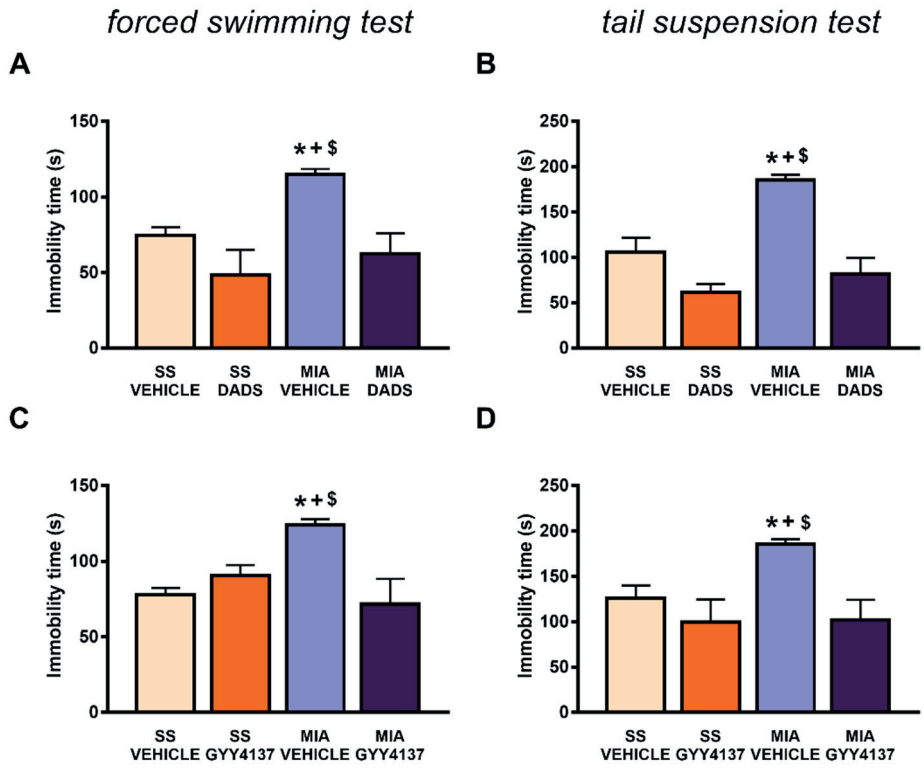

Figure 2. Treatment with DADS and GYY4137 inhibited the depressive-like behaviors linked with chronic osteoarthritis pain. The depressive-like behaviors were evaluated on day 29 after monosodium acetate (MIA) or saline solution (SS) knee injection in mice treated with DADS (200 μg/kg, twice daily for 3 days) or GYY4137 (32 μg/kg, twice daily for 4 days) in the forced swimming test (**A**,**C**) and tail suspension test (**B**,**D**). In the tail suspension and forced swimming tests the immobility time(s) is shown. For each test evaluated, * denotes significant differences vs. SS-injected mice treated with vehicle, + denotes significant differences vs. SS plus DADS or GYY4137, and $ denotes significant differences vs. MIA-injected mice treated with DADS or GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean values ± SEM; $n = 8$ animals per experimental group.

Osteoarthritis pain is also associated with anxiety-like behaviors, and this was reflected in the significant decrease in the number of entries ($p < 0.0306$ vs. SS-vehicle treated animals, one-way ANOVA followed by the Student–Newman–Keuls test) (Figure 3A,B) and in the time spent in the open arms ($p < 0.0226$ vs. SS-vehicle treated mice; one-way ANOVA followed by the Student–Newman–Keuls test) (Figure 3C,D) of the EPM. These anxiolytic-like responses were normalized through the repetitive administration of DADS (Figure 3A,C) and GYY4137 (Figure 3B,D). All groups of animals exhibited a similar number of entries into the closed arms, suggesting normal locomotor activity in this test independently of the treatments (Figure 3E,F). In summary, both treatments, DADS and GYY4137, inhibited the anxiodepressive-like behaviors accompanying chronic osteoarthritis pain.

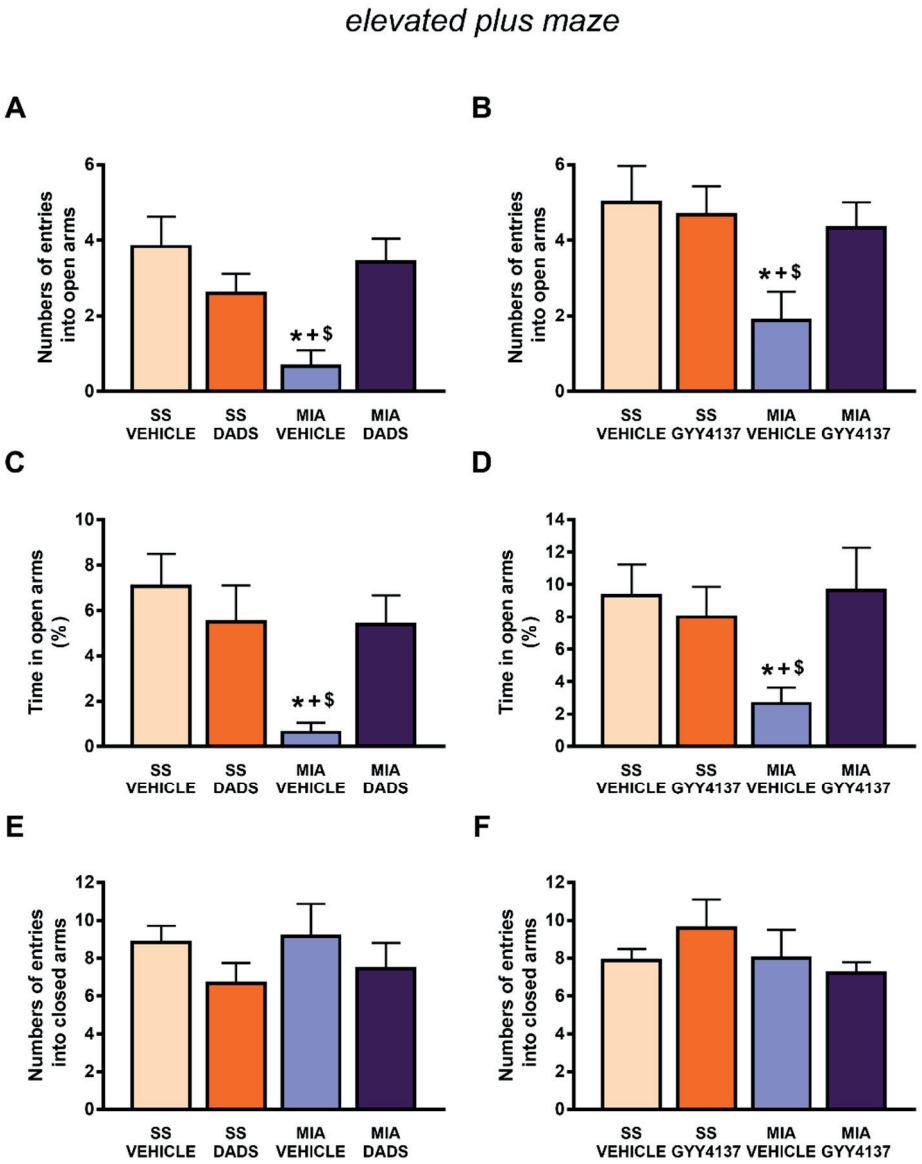

Figure 3. Treatment with DADS and GYY4137 inhibited the anxiety-like behaviors linked with chronic osteoarthritis pain. The anxiety-like behavior was evaluated on day 29 after monosodium acetate (MIA) or saline solution (SS) injection in mice treated with DADS (200 μg/kg, twice daily for 3 days) or GYY4137 (32 μg/kg, twice daily for 4 days) in the elevated plus maze. The number of entries into the open arms (**A**,**B**), percentage of time spent in the open arms (**C**,**D**), and the number of entries into the closed arms (**E**,**F**) are shown. For each response evaluated, * denotes significant differences vs. SS-injected mice treated with vehicle, + denotes significant differences vs. SS plus DADS or GYY4137, and $ denotes significant differences vs. MIA-injected mice treated with DADS or GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean values ± SEM; $n = 8$ animals per experimental group.

3.3. Effects of DADS and GYY4137 on the Protein Levels of 4-HNE, PI3K, p-Akt, NOS2 and BAX in the Amygdala of MIA-Injected Mice

In the amygdala, our results showed that the intra-articular injection of MIA caused a significant increase in the expression of 4-HNE ($p < 0.018$, one-way ANOVA; Figure 4A), PI3K ($p < 0.0118$, one-way ANOVA; Figure 4B), p-Akt/Akt ($p < 0.0263$, one-way ANOVA; Figure 4C), NOS2 ($p < 0.0236$, one-way ANOVA; Figure 4F), and BAX ($p < 0.0162$, one-way ANOVA; Figure 4G). Treatment with both DADS and GYY4137 normalized the up-regulated protein levels of 4-HNE, PI3K, p-AKT, NOS2, and BAX in this brain area.

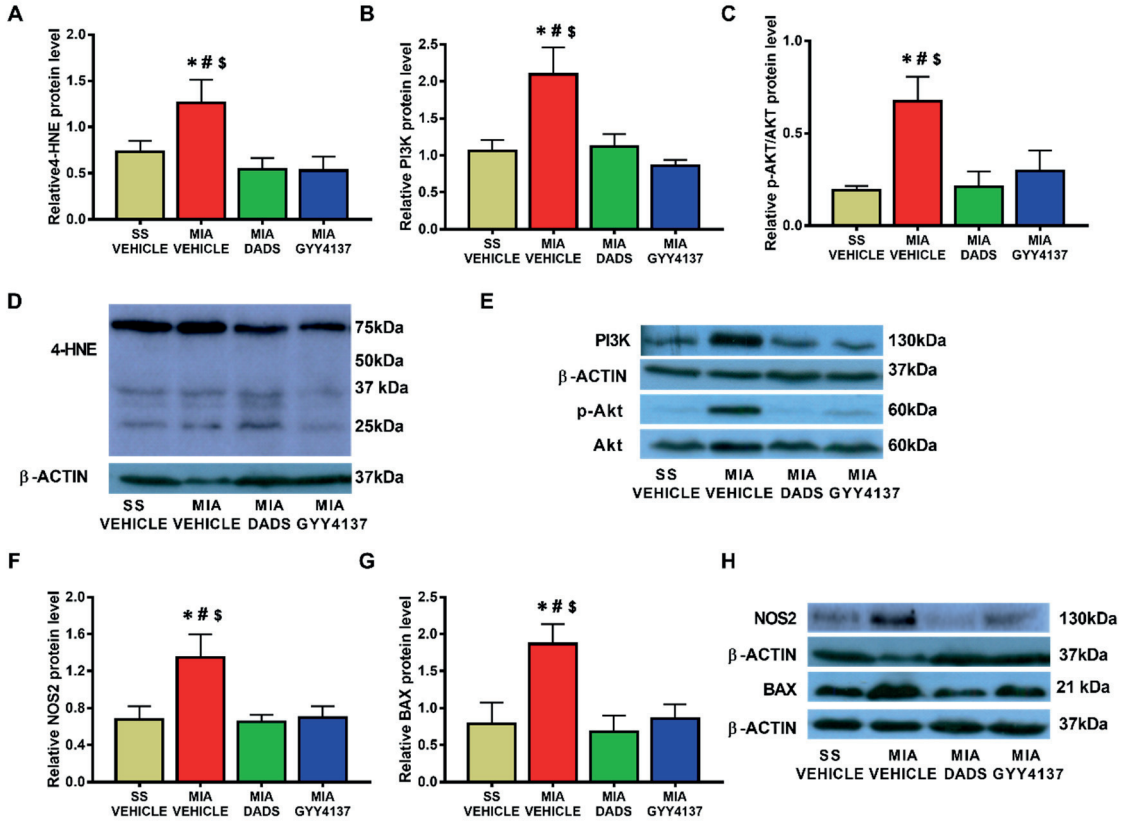

Figure 4. Effects of treatment with DADS and GYY4137 on the expression of 4-HNE, PI3K, p-Akt/Akt, NOS2, and BAX in the amygdala of monosodium acetate (MIA)-injected mice. DADS and GYY4137 treatments normalized the overexpression of 4-HNE (**A**), PI3K (**B**), p-Akt (**C**), NOS2 (**F**), and BAX (**G**) in the amygdala of mice with osteoarthritis pain. Saline solution (SS)-injected mice treated with vehicle were used as controls. Representative blots for 4-HNE (**D**), PI3K and p-Akt/Akt (**E**), NOS2 and BAX (**H**) are shown. All proteins are expressed relative to β-actin levels except P-Akt, which is expressed relative to total Akt. In all graphics, * denotes significant differences vs. SS-vehicle treated mice, # vs. MIA-injected mice treated with DADS, and $ vs. MIA-injected mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean ± SEM; n = 3–4 samples per experimental group.

3.4. Effects of DADS and GYY4137 on the Expression of 4-HNE, PI3K, p-Akt, NOS2 and BAX in the Periaqueductal Gray Matter of MIA-Injected Mice

In this brain area, MIA injection provoked a significant increase in the expression of PI3K ($p < 0.0269$, one-way ANOVA; Figure 5B) and NOS2 ($p < 0.0239$, one-way ANOVA; Figure 5F). Treatment with DADS or GYY4137 normalized the enhanced protein levels of PI3K and NOS2 in the periaqueductal gray matter. No changes in the expression of 4-HNE (Figure 5A), p-Akt (Figure 5C), and BAX (Figure 5G) were detected in any of the groups evaluated.

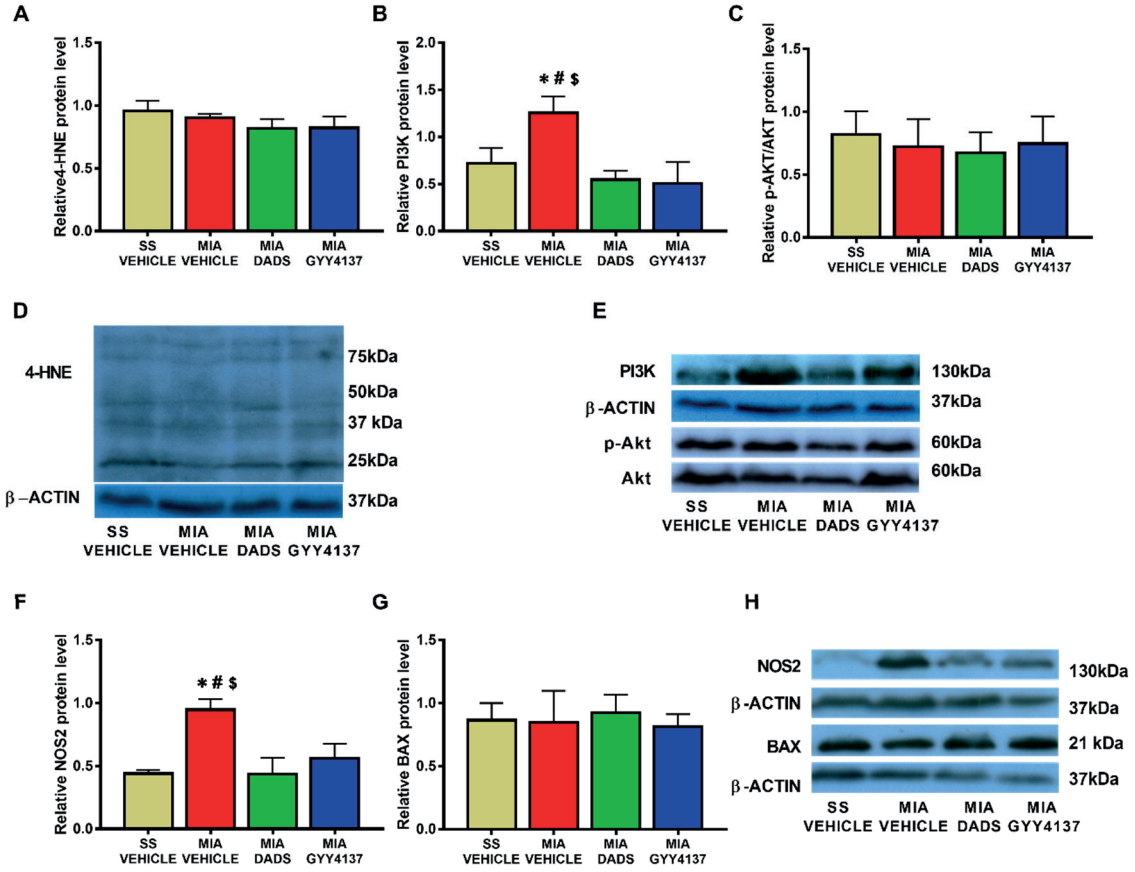

Figure 5. Effects of treatment with DADS and GYY4137 on the expression of 4-HNE, PI3K, p-Akt/Akt, NOS2, and BAX in the periaqueductal gray matter of monosodium acetate (MIA)-injected mice. DADS and GYY4137 treatments normalized the overexpression of PI3K (**B**) and NOS2 (**F**) in the periaqueductal gray matter of mice with osteoarthritis pain. Non-changes in the protein levels of 4-HNE (**A**), p-Akt (**C**), and BAX (**G**) were detected. Saline solution (SS)-injected mice treated with vehicle were used as controls. Representative blots for 4-HNE (**D**), PI3K and p-Akt/Akt (**E**), NOS2 and BAX (**H**) are shown. All proteins are expressed relative to β-actin levels except P-Akt, which is expressed relative to total Akt. In all graphics, * denotes significant differences vs. SS-vehicle treated mice, # vs. MIA-injected mice treated with DADS, and $ vs. MIA-injected mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean ± SEM; n = 3–4 samples per experimental group.

3.5. Effects of DADS and GYY4137 on the Protein Levels of 4-HNE, PI3K, p-Akt, NOS2 and BAX in the Infralimbic Cortex of Mice with Osteoarthritic Pain

A significant increase in the expression p-AKT (Figure 6C) and NOS2 (Figure 6F) was observed in the infralimbic cortex of MIA-injected mice ($p < 0.011$, one-way ANOVA; followed by the Student–Newman–Keuls test). While only the GYY4137 treatment normalized the enhanced protein levels of p-Akt, both H_2S donors prevented the increase in the protein levels of NOS2 in this brain area. No alterations in the expression of 4-HNE (Figure 6A), PI3K (Figure 6B), or BAX (Figure 6G) were observed.

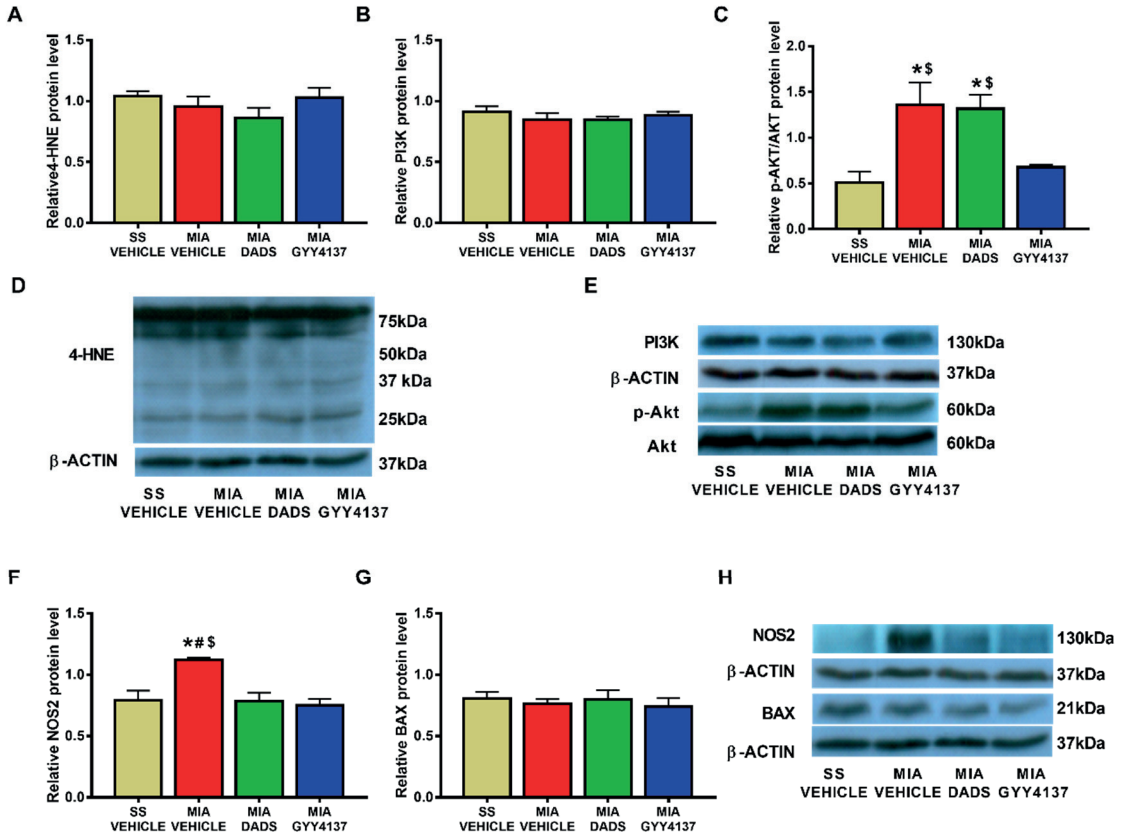

Figure 6. Effects of treatment with DADS and GYY4137 on the expression of 4-HNE, PI3K, p-Akt/Akt, NOS2, and BAX in the infralimbic cortex of monosodium acetate (MIA)-injected mice. DADS and/or GYY4137 treatments normalized the overexpression of p-AKT (**C**) and NOS2 (**F**) in the infralimbic cortex of mice with osteoarthritis pain. The protein levels of 4-HNE (**A**), PI3K (**B**), and BAX (**G**) remained unaltered. Saline solution (SS)-injected mice treated with vehicle were used as controls. Representative blots for 4-HNE (**D**), PI3K and p-Akt/Akt (**E**), NOS2 and BAX (**H**) are shown. All proteins are expressed relative to β-actin levels except P-Akt, which is expressed relative to total Akt. In all graphics, * denotes significant differences vs. SS-vehicle treated mice, # vs. MIA-injected mice treated with DADS, and $ vs. MIA-injected mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean ± SEM; n = 3–4 samples per experimental group.

3.6. Effects of Treatment with DADS or GYY4137 on the Protein Levels of 4-HNE, PI3K, p-Akt, NOS2 and BAX in the Anterior Cingulate Cortex of MIA-Injected Mice

Results showed that the proteins levels of PI3K are enhanced in MIA-injected mice ($p < 0.0033$, one-way ANOVA; followed by the Student–Newman–Keuls test), and only GYY4137 can reverse this increase (Figure 7B). Moreover, although MIA did not alter the proteins levels of 4-HNE (Figure 7A) or p-Akt (Figure 7C) in the anterior cingulate cortex, DADS decreased the expression of 4-HNE and GYY4137 down-regulated Akt activation in this brain area. The expression of NOS2 (Figure 7F) and BAX (Figure 7G) remained unaffected in the four groups.

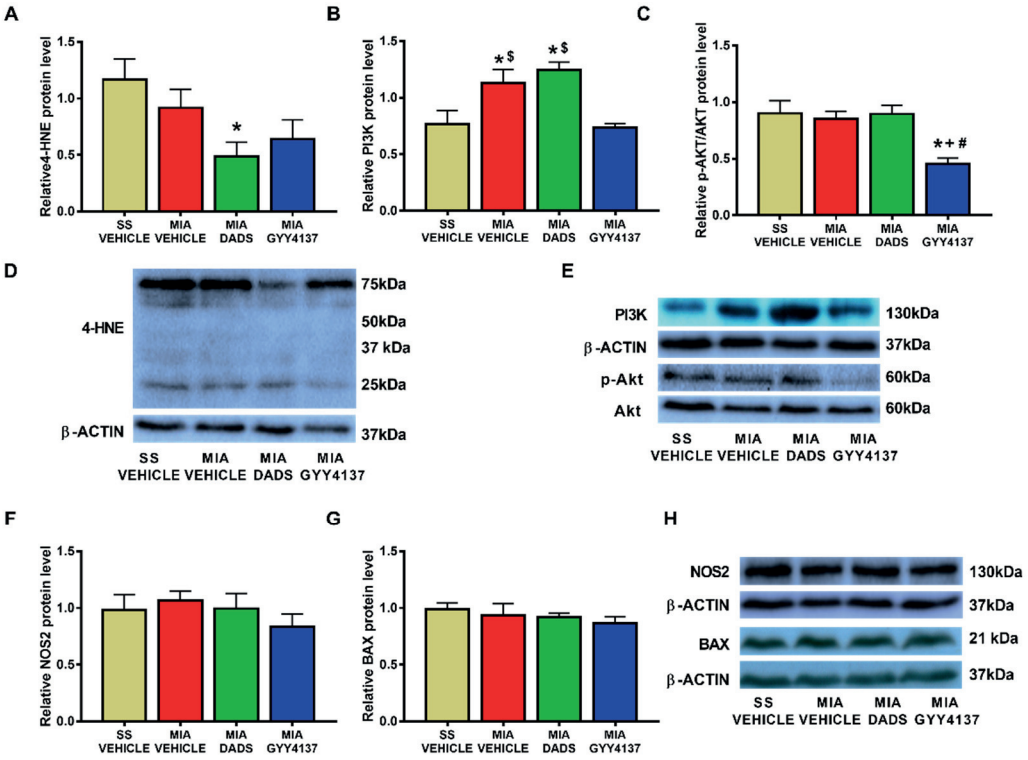

Figure 7. Effects of treatment with DADS and GYY4137 on the expression of 4-HNE, PI3K, p-Akt/Akt, NOS2, and BAX in the anterior cingulate cortex of monosodium acetate (MIA)-injected mice. Treatment with GYY4137 normalized the overexpression of PI3K (**B**). DADS reduced 4-HNE levels (**A**), and GYY4137 diminished the expression of p-Akt (**C**) in the anterior cingulate cortex of mice with osteoarthritis pain. The protein levels of NOS2 (**F**) and BAX (**G**) remained unaltered. Saline solution (SS)-injected mice treated with vehicle were used as controls. Representative blots for 4-HNE (**D**), PI3K and p-Akt/Akt (**E**), NOS2 and BAX (**H**) are shown. All proteins are expressed relative to β-actin levels except P-Akt, which is expressed relative to total Akt. In all graphics, * denotes significant differences vs. SS-vehicle treated mice, + denotes significant differences vs. MIA-injected mice treated with vehicle, # denotes significant differences vs. MIA-injected mice treated with DADS and $ denotes significant differences vs. MIA-injected mice treated with GYY4137 ($p < 0.05$; one-way ANOVA followed by the Student–Newman–Keuls test). The results are presented as the mean ± SEM; n = 3–4 samples per experimental group.

4. Discussion

Knee osteoarthritis is a chronic multifactorial disease that causes pain and serious disability problems, as the joints are affected during its development. Currently, there is no specific treatment for osteoarthritis; several pharmacological intervention strategies are available to only alleviate pain symptoms. Moreover, the existing therapies focused on knee regeneration show limited efficacy and, in some cases, significant side effects. Beside this, chronic osteoarthritis pain is known to be also accompanied by different comorbidities, such as alterations in working memory and emotional behaviors; therefore, it is important to palliate all these symptoms in order to achieve a successful treatment.

This study demonstrated the recovery of memory and hind limb grip strength deficits through repetitive treatment with GYY4137 and/or DADS, and further revealed the anxiolytic, antidepressant, and antinociceptive effects induced by both treatments in mice with chronic osteoarthritic pain. These actions seem mainly mediated via inhibiting oxidative stress, PI3K/p-Akt activation, NOS2, and/or BAX over-expression in different brain areas involved in the modulation of nociception and affective disorders, such as the amygdala, periaqueductal gray matter, infralimbic cortex, and/or anterior cingulate cortex.

In accordance with previous studies that have revealed impaired memory function in other chronic pain models [48,49], our study demonstrated a deficit in working memory in MIA-injected animals, as previously shown by [50,51]. Interestingly, the cognitive deficits

associated with osteoarthritis pain were completely reduced by the repetitive treatment with GYY4137, but not with DADS, thus highlighting the protective role of GYY4137 compared to DADS in osteoarthritic pain-associated memory impairment. The different effectiveness of both compounds might be a consequence of their different chemical structures, natural (DADS) vs. synthetic (GYY4137) compounds. These results showed the important role of GYY4137, a potent slow-releasing H_2S donor, in the recovery of memory deficits accompanying osteoarthritis pain.

The anterior cingulate cortex is a region involved in executive, attention, and decision-making processes [52]. Previous studies have reported a direct association between the presence of chronic pain and linked memory loss in this area [39,53,54]. In our pain model, an increased expression of PI3K was observed in the anterior cingulate cortex, which was only reversed by GYY4137 treatment. In addition, and although no changes in the protein levels of 4-HNE or p-Akt were observed in animals with osteoarthritis pain, treatment with DADS significantly decreased the expression of 4-HNE, while GYY4137 diminished that of p-Akt. Considering that common neuroplastic changes associated with chronic pain and emotional disorders have been proposed as important routes for the onset and reciprocal aggravation of both pathologies [55], the inhibition of PI3K/p-Akt induced by GYY4137 in the anterior cingulate cortex might have been responsible for the improvement in working memory observed in the MIA-injected mice treated with this synthetic slow-releasing H_2S donor. Although DADS decreased the 4-HNE levels, the lack of an effect of this compound on the expression of PI3K/p-AKT in this brain area might explain the non-effects of this treatment on the memory deficits accompanying osteoarthritic pain.

In this study, we also demonstrated the anxiolytic and antidepressant effects of DADS and GYY4137 in animals with chronic osteoarthritis pain. We thus confirmed the antidepressant effects induced by other slow-releasing compounds, such as allyl isothiocyanate and phenyl isothiocyanate, in animals with chronic osteoarthritic [36] or neuropathic pain [24], as well the anxiolytic and antidepressant actions performed by other H_2S donors, such as sodium hydrosulfide, in different animal models of anxiety or depression without pain [56,57], but in contrast to the non-anxiolytic properties of isothiocyanates during chronic pain [24,36]. The dissimilar chemical structure of isothiocyanates compared to GYY4137 or DADS and/or the different treatment guidelines used could be the most probable reason for these discrepant results. In this study, both treatments were administered twice daily, while isothiocyanates were only administer once daily [36].

The amygdala is closely correlated with the regulation of emotional disorders such as anxiety and depression [38], as well as memory disturbances [58,59]. Our data showed that the elevated levels of 4-HNE, PI3K, p-Akt, NOS2, and BAX in the amygdala of MIA-injected mice were completely normalized by both the DADS and GYY4137 treatments. These data revealed that the oxidative stress, plasticity changes, and inflammatory and apoptotic alterations provoked by knee osteoarthritis in this area were blocked by both H_2S donors. Thus, considering that one of the main causes of the pathogenesis of osteoarthritis and its associated comorbidities is generated by oxidative stress, PI3K/p-AKT activation, and pro-inflammatory and pro-apoptotic responses [11,25,27], their inhibition with DADS and GYY4137 might possibly explicate their anxiolytic and antidepressant actions in this pain model. These results correspond with other data showing that most of the therapeutic actions of H_2S may be carried out, at least in part, by reducing reactive oxygen species expression and PI3K/p-Akt/Bcl-2 pathway activation, thus preventing cell apoptosis [35,60,61]. The fact that both treatments inhibited 4-HNE overexpression and the anxiodepressive-like behaviors concurrent with chronic osteoarthritis pain supports the relationship between these disorders and oxidative stress in the amygdala [20,62].

The anterior cingulate cortex also regulates the emotional disturbances associated with pain, such as the anxiety- and depressive-like behaviors [63–66]. Consequently, and considering that oxidative stress and nociception are related to the development of emotional disorders [9], the fact that DADS and/or GYY4137 modulate the expression

of 4-HNE and PI3K/p-Akt in the anterior cingulate cortex might also contribute to the inhibition of anxiodepressive associated with osteoarthritic pain.

Previous studies have reported that the nociceptive, emotional, and cognitive components of pain are also processed in the medial prefrontal cortex, which includes the infralimbic cortex [40]. In this study, we proved that a MIA injection phosphorylated Akt and increased NOS2 expression in the infralimbic cortex, and that both treatments blocked NOS2 overexpression but only GYY4137 inhibited Akt activation, thus suggesting that in this region of the medial prefrontal cortex, DADS has more anti-inflammatory than anti-nociceptive actions; in contrast, GYY4137 diminished the inflammatory and nociceptive responses, thus explaining the higher effectiveness of GYY4137 compared to DADS in modulating the mechanical allodynia and grip strength deficits triggered by knee MIA injection.

The periaqueductal gray matter is an area related to pain modulation [67]. The high levels of PI3K and NOS2 displayed in the periaqueductal gray matter of MIA-injected mice support the fact that this brain area regulated the nociceptive and inflammatory processes implicated in the progression of osteoarthritis pain. Both treatments normalized their over-expression, establishing a causal relationship between the antiallodynic effects and the recovery of hind limb grip strength in DADS- and GYY4137-treated mice during osteoarthritis. In agreement with our results, previous studies have shown the anti-inflammatory effects induced by the knee injection of GYY4137 in another osteoarthritis pain model [35] and with the recovery of the mechanical allodynia and grip strength deficits produced by other slow-releasing H_2S donors in MIA and complete Freund's adjuvant-induced osteoarthritis pain [36,68], as well as in animals with nerve-injury- or chemotherapy-induced neuropathic pain [24,69].

5. Conclusions

In summary, our results revealed new properties of slow-releasing H_2S donors in memory impairment and anxiodepressive disorders linked with chronic osteoarthritis pain, as well as their effects on the central nervous system.

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The Interaction between Carbon Monoxide and Hydrogen Sulfide during Chronic Joint Pain in Young Female Mice

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Abstract: A relationship between carbon monoxide (CO) and hydrogen sulfide (H_2S) has been described in different pathological conditions, but their interaction in modulating joint pain has not yet been investigated. In young female mice with monosodium acetate-induced joint degeneration and pain, we assessed: (1) the effects of CORM-2 (tricarbonyldichlororuthenium(II)dimer), a CO-releasing molecule, and CoPP (cobalt protoporphyrin IX), an inducer of heme oxygenase 1 (HO-1), administered alone and combined with low doses of two slow-releasing H_2S donors, DADS (diallyl disulfide) and GYY4137 (morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate dichloromethane complex) on the mechanical allodynia and loss of grip strength provoked by joint degeneration; (2) the role of Nrf2, NAD(P)H: quinone oxidoreductase 1 (NQO1) and HO-1 in the antinociceptive actions of H_2S donors; (3) the impact of DADS and GYY4137 treatment on the expression of Nrf2 and several antioxidant proteins in dorsal root ganglia (DRG) and periaqueductal gray matter (PAG). Our data showed that treatment with H_2S donors inhibited allodynia and functional deficits, while CORM-2 and CoPP only prevented allodynia. The Nrf2 pathway is implicated in the analgesic actions of DADS and GYY4137 during joint degeneration. Moreover, the co-administration of low doses of CORM-2 or CoPP with DADS or GYY4137 produced higher antiallodynic effects and greater recovery of grip strength deficits than those produced by each of these compounds alone. The activation of the antioxidant system caused by H_2S donors in DRG and/or PAG might explain the enhancement of antinociceptive effects. These data reveal a positive interaction between H_2S and CO in modulating joint pain in female mice.

Keywords: analgesia; allodynia; carbon monoxide; grip strength; hydrogen sulfide; joint pain; oxidative stress

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1. Introduction

The gaseous neurotransmitters carbon monoxide (CO) and hydrogen sulfide (H_2S) exert multiple biological functions, and there are several similarities between both gases, including: (1) a similar pattern of distribution within the central (CNS) and peripheral nervous systems (PNS); (2) the ability to diffuse freely across intra- and intercellular compartments; (3) both mediate similar physiological functions such as the regulation of cardiovascular, neuronal, respiratory, digestive, and immune systems [1]. In addition, the crosstalk between CO and H_2S has been described, especially in the gastrointestinal digestive system [2]. For example, Magierowski et al. (2016) [3] demonstrated that H_2S requires the presence of CO to be able to carry out its protective effects on the gastric mucosa. Other studies also revealed an interaction between both neurotransmitters on the nervous system. Thus, the treatment with electrical acupuncture reduces hypoxic injury because of the improvement of CO levels induced by H_2S [4]. Moreover, in a rat model of recurrent febrile seizures, the administration of H_2S donors augmented the plasma levels of CO and the expression of heme oxygenase 1 (HO-1) enzyme in the hippocampus, while the

inhibition of H_2S decreased them [5]. These results suggest a positive interaction between CO and H_2S in regulating different pathological conditions.

Recent investigations further proved that the acute treatment with slow-releasing H_2S donors, for example, the natural garlic bioactive element diallyl disulfide (DADS) and the synthetic morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate dichloromethane complex (GYY4137), both inhibited neuropathic and inflammatory pain in a dose-dependent manner [6,7]. Other findings also show that the administration of the CO-releasing molecule tricarbonyldichlororuthenium(II)dimer (CORM-2) or the HO-1-inducer compound cobalt protoporphyrin IX (CoPP) reduces the inflammatory pain induced by the subplantar injection of complete Freund's adjuvant [8] and the neuropathic pain associated with type 2 diabetes (db/db mice) [9] or provoked by the administration of vincristine [10], a spared nerve injury [11] and the chronic constriction of the sciatic nerve [12]. Therefore, and considering that joint pain is difficult to treat with the current therapies [13], one of our objectives is to assess the effects of the acute administration of HO-1 inducers and CO or H_2S donors on the mechanical allodynia and functional disability associated with joint degeneration.

Other works have also showed that most of the anti-inflammatory and antinociceptive effects produced by H_2S donors during chronic inflammatory and neuropathic pain are mostly mediated by triggering the Nrf2 transcription factor signaling pathway [14,15]. This transcription factor, besides being involved in several protective mechanisms, is also implicated in the maintenance of homeostasis and nociception modulation by activating the transcription of several antioxidant enzymes, such as superoxide dismutase 1 (SOD-1), HO-1, glutathione S-transferase mu 1 (GSTM1), NAD(P)H: quinone oxidoreductase 1 (NQO1), etc. [16–18]. Other studies further revealed that most of the anti-inflammatory actions of H_2S donors were produced by increasing and/or maintaining high protein levels of several antioxidant enzymes in the paw and knee of rodents with chronic inflammation, highlighting the importance of the Nrf2 pathway in the anti-inflammatory properties of H_2S [7,14]. Nevertheless, the possible participation of Nrf2 signaling in the antiallodynic effects and in the recovery of grip strength induced by H_2S donors during joint pain has not yet been evaluated.

Finally, and given that most symptoms of pain and functional impairment associated with chronic joint degeneration are more common in women [19], this research was conducted in female mice.

Then, in a monosodium acetate (MIA)-induced model of joint degeneration and pain in female mice [20], we evaluated: (1) the impact of the acute systemic co-treatment of CORM-2 and CoPP with DADS and GYY4137 in the mechanical allodynia and grip strength deficits; (2) the reversion of the effects induced by H_2S donors with the administration of specific inhibitors of Nrf2, HO-1, and NQO1; and (3) the protein levels of Nrf2, HO-1, NQO1, SOD-1, and GSTM1 in the dorsal root ganglia (DRG) and periaqueductal gray matter (PAG) of animals treated with DADS and GYY4137. Both areas are involved in pain processing, DRG taking part in the transmission of pain from the periphery to the CNS [21] and PAG being a key brainstem nucleus that performs an essential role in the descending modulation of pain [22].

2. Materials and Methods

2.1. Animals

Female C57BL/6 mice (6–8 weeks old and between 21 and 26 g) obtained from Envigo Laboratories (Barcelona, Spain), which were kept under standard light/dark (12/12 h), temperature (22 °C), and humidity (66%) conditions with free access to food and water, were used. Experiments were performed after 7 days of acclimatization to the housing conditions and conducted between 9 a.m. and 5 p.m. The investigations were performed in conformity with the guidelines of the European Commission's directive (Directive 2010/63/EU) and Spanish law (RD 53/2013) regulating animal research, and were agreed by the Committee for the Use and Care of Animals of the Autonomous University of Barcelona (protocol

number: 9863). Every effort was made to reduce the amount of animals used and their suffering.

Based on the data obtained in a pilot test and accepting a risk of $\alpha = 0.05$ and $\beta = 0.2$ in a two-tailed test, 6 animals per group were needed to recognize as statistically significant the differences between groups in the behavioral experiments.

2.2. Induction of Joint Pain

Joint pain was generated by MIA (Sigma–Aldrich, St. Louis, MO, USA) intraarticularly injected under isoflurane anesthesia conditions (i.e., 3% induction and 2.5% maintenance). The right knee joint was shaved and flexed at a 90° angle, and 10 µL of MIA (15 mg/mL) dissolved in saline solution (NaCl 0.9%; SS) was administered. Control animals were injected with the equal amount of SS.

2.3. Mechanical Allodynia

Mechanical allodynia was evaluated by measuring the hind paw withdrawal response after stimulation with von Frey filaments. To do this, the animals were put in Plexiglas tubes 9 cm in diameter by 20 cm high placed on top of a wire mesh bottom where filaments of different strengths were introduced (North Coast Medical, Inc., San Jose, CA, USA) in accordance with the up–down paradigm [23]. We started the test with a filament of 0.4 g, and the strength of the following filament was enhanced or diminished in compliance with the animal's response. A filament of 3.0 g was utilized as a cutoff. We used the Excel program (Microsoft Iberia SRL, Barcelona, Spain), which includes curve fitting of the data, to determine the animal's threshold of the response. The animals were familiarized for 1 h to the environment prior to starting the test.

2.4. Measurement of Grip Strength

The grip strength was measured utilizing a computerized grip strength meter (Model 47200, Ugo Basile, Varese, Italy) according to the method depicted in [24]. To determine the grip strength of the hind legs, the investigator took the animal by the base of its tail, allowing the animal to grab the metal bar of the grip strength meter with both hind legs. The metal bar was coupled to a force transducer that automatically recorded the maximum force of each measurement (g). For each animal, the grip strength of the hind limbs was measured in triplicate. To prevent the mice from grabbing the metal bar with their front legs during the test, mice were first allowed to grab a wire mesh cylinder with their front legs. Baseline grip strength values were recorded for each animal as the mean value of three determinations performed prior to MIA or SS injection. This value was considered 100% grip strength and was utilized as a reference for subsequent determinations.

2.5. Western Blot Analysis

Mice injected with MIA or SS were euthanized by cervical dislocation at 29 days post-injection, and the ipsilateral DRG (L3–L5) and PAG were rapidly removed and kept at −80 °C until use. Samples from two animals were pooled into one experimental sample to obtain sufficient protein levels to perform Western blot analysis. We analyzed the protein levels of Nrf2, HO-1, NQO1, SOD-1, and GSTM1 in DRG and PAG. The sonication of tissues was made in cold lysis RIPA Buffer (Sigma–Aldrich, St. Louis, MO, USA). 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. After that, 60 µg of the supernatant (total protein) was mixed with 4× 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 for 75 min with phosphate-buffered saline (PBS; Sigma–Aldrich, St. Louis, MO, USA) containing nonfat dry milk at 5%, Tris-buffered saline with Tween 20 comprising bovine serum albumin at 5% (BSA; Sigma–Aldrich, St. Louis, MO, USA) or nonfat dry milk at 5%, and PBS with Tween 20 containing BSA at 5%. After that, membranes were incubated with specific rabbit

primary antibodies, anti Nrf2 (1:150) or HO-1 (1:150) from Abcam, Cambridge (UK), with NQO1 (1:200) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5000) from Sigma–Aldrich, St. Louis, MO (USA) and with SOD-1 (1:150) or GSTM1 (1:150) from Novus Biologic, Littleton, CO (USA), overnight at 4 °C. Afterwards, blots were incubated with a horseradish-peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chal-font, UK) for 1 h at room temperature. We used chemiluminescence reagents (ECL kit; GE Healthcare, Buckinghamshire, UK) to detect proteins and the ImageJ program (National Institutes of Health, Bethesda, MD, USA) to perform the densitometric analysis.

2.6. Experimental Procedures

First, we investigated the effects produced by various doses of DADS (3–30 mg/kg), GYY4137 (0.4–12 mg/kg), CORM-2 (5–10 mg/kg), and CoPP (0.5–10 mg/kg) or their respective vehicles intraperitoneally administered on the mechanical allodynia and grip strength deficits caused by the joint degeneration, at 29 days after MIA or SS injection (6 mice for group). Mice were tested at 1 h after DADS or GYY4137 injection and at 3 h after CORM-2 or CoPP injection in conformity with other works [6,25,26].

In other groups of experiments, we assessed the possible interaction between H_2S and CO pathways by evaluating the antiallodynic and the grip strength recovery effects induced by the co-injection of low doses of CORM-2 (5 mg/kg) or CoPP (0.5 mg/kg) with low doses of DADS (3 mg/kg) or GYY4137 (0.4 mg/kg), and the reversion of the antiallodynic and the grip strength recovery effects induced by high doses of DADS (30 mg/kg) or GYY4137 (6 mg/kg) with the administration of 25 mg/kg of ML385 (a specific Nrf2 inhibitor), 10 mg/kg of tin protoporphyrin IX, SnPP (a specific HO-1 inhibitor), or 10 mg/kg of dicoumarol (a specific NQO1 inhibitor).

The doses of ML385, SnPP, and dicoumarol used were in compliance with those used in another study [7], and the mice were tested 1 h after their administration. The doses of DADS, GYY4137, CORM-2, and CoPP that produced the maximal and minimal inhibitory effects were selected as of the dose–response curves completed in this research.

Finally, MIA-injected animals treated with DADS, GYY4137, or vehicle were euthanized by cervical dislocation, and the protein levels of the Nrf2 transcription factor and several antioxidant enzymes (HO-1, NQO1, SOD-1, and GSTM1) in the DRG and PAG were determined by Western blot. In this research, we employed SS-injected mice treated with vehicle as controls (3 samples for every group).

All experiments were performed by researchers blinded to the experimental conditions.

2.7. Drugs

DADS, GYY4137, CORM-2, and CoPP were purchased at Sigma–Aldrich (St. Louis, MO, USA). DADS and GYY4137 were dissolved in SS, whereas CORM-2 and CoPP were dissolved in dimethyl sulfoxide (1% in SS). Dicoumarol and ML-385 bought from Eurodiagnostico S.L. (Madrid, Spain) and SnPP purchased from Frontier Scientific (Livchem GmbH & Co., Frankfurt, Germany) were also dissolved in dimethyl sulfoxide (1% in SS). All drugs were administered in a final volume of 10 mL/kg.

All compounds were prepared just before injection. For each group treated with a drug, their control group was treated with equivalent volume of vehicle.

2.8. Statistical Analyses

Results are presented as the mean values ± standard error of the mean (SEM). The GraphPad software (version 8.0) was used to perform the statistical analysis.

In the behavioral tests, the analysis of the effects produced by different doses of DADS, GYY4137, CORM-2, CoPP, or vehicle, administered alone and combined, was conducted using one-way ANOVA followed by Student–Newman–Keuls test. The actions of DADS and GYY4137 co-administered with specific Nrf2, HO-1, and NQO1 antagonists were likewise examined with a one-way ANOVA and the Student–Newman–Keuls test.

In our experiments, the antiallodynic effects are shown as the percentage of the maximal possible effect in that test latencies pre- (baseline) and post-drug injection were compared and calculated in agreement with this equation:

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

The recovery of grip strength is shown as the percentage of the effect in that the grip strength pre- (baseline) and post-drug injection were compared and computed in agreement to this equation:

$$\text{Recovery of grip strength (\%)} = [(\text{drug} - \text{baseline})/(\text{baseline})] \times 100$$

Differences in Nrf2, HO-1, NQO1, SOD1, and/or GSTM1 levels in the DRG and PAG were also estimated applying the one-way ANOVA and the subsequent Student–Newman–Keuls test.

A $p < 0.05$ was considered significant.

3. Results

3.1. The Acute Administration of DADS and GYY4137 Inhibited the Mechanical Allodynia and Grip Strength Deficit Induced by MIA in a Dose-Dependent Manner

The effects of the acute intraperitoneal administration of diverse doses of DADS (3, 6, 9, 15, and 30 mg/kg) or GYY4137 (0.4, 0.8, 1.5, 6, and 12 mg/kg) on the mechanical allodynia and the loss of grip strength caused by joint degeneration at 29 days after MIA injection were evaluated. Our data showed that acute treatment with DADS dose-dependently reduced mechanical allodynia (Figure 1A) and loss of grip strength (Figure 1B) caused by MIA, achieving the maximum effect in both tests with 30 mg/kg. Treatment with GYY4137 also dose-dependently inhibited the mechanical allodynia (Figure 1C) and grip strength deficit provoked by MIA (Figure 1D) and reached the maximum effect with a dose of 6 mg/kg. In SS-injected animals, both drugs did not make any effect on the allodynia and the grip strength (data not displayed).

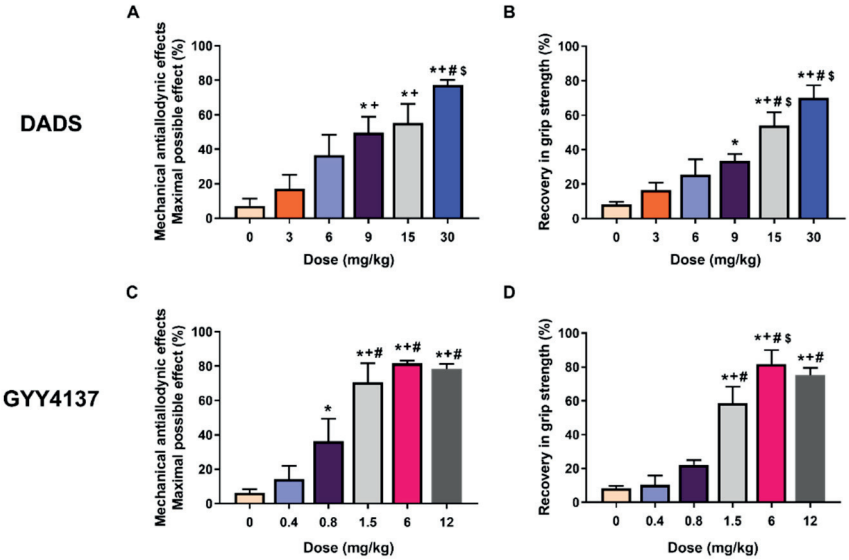

Figure 1. The administration of DADS and GYY4137 reduced the mechanical allodynia and grip strength deficit generated by MIA in a dose-dependent manner. The mechanical antiallodynic effects (**A**,**C**) and the recovery in grip strength (**B**,**D**) induced by different doses of DADS or GYY4137 (mg/kg) are shown. For each test and drug, * denotes significant differences compared to SS (0 mg/kg) treated mice; + compared with the effects of low doses of DADS (3 mg/kg) or GYY4137 (0.4 mg/kg); # compared with the effect of 6 mg/kg of DADS or 0.8 mg/kg of GYY4137 and $ compared with the

effects of DADS at 9 mg/kg ($p < 0.05$; one-way ANOVA, followed by the Student–Newman–Keuls test). Data are represented as the mean values of maximal possible effect (%) for the mechanical allodynia and the recovery of grip strength (%) ± SEM; $n = 6$ animals per dose and treatment.

3.2. Acute Treatment with CORM-2 and CoPP Inhibited the Mechanical Allodynia but Not the Loss of Grip Strength Generated by MIA

The actions of the acute intraperitoneal injection of CORM-2 at 5, 7, and 10 mg/kg or CoPP at 0.5, 2.5, 5, and 10 mg/kg on the tactile allodynia and the loss of grip strength caused by joint degeneration at 29 days after MIA injection were also assessed.

Our data showed that CORM-2 treatment dose-dependently reduced the mechanical allodynia (Figure 2A), but not the grip strength deficit (Figure 2B), achieving the top effect with a dose of 10 mg/kg. Treatment with CoPP likewise reduced the MIA-induced mechanical allodynia in a dose-dependent way, achieving the greatest effect with 10 mg/kg (Figure 2C) but without effects on the grip strength deficit (Figure 2D).

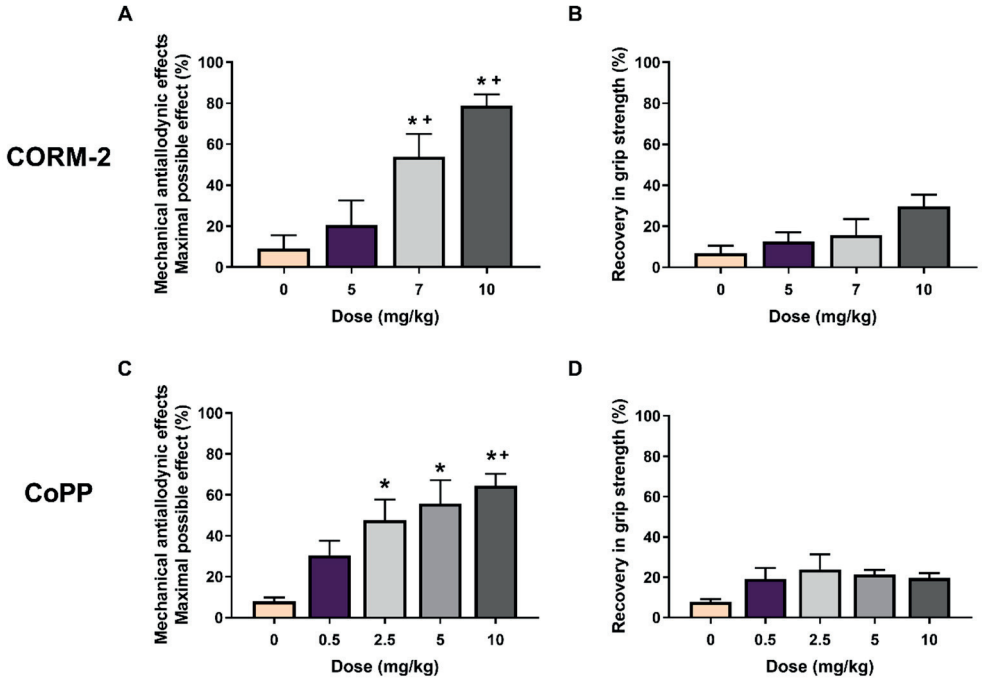

Figure 2. CORM-2 or CoPP treatments inhibited the mechanical allodynia but not the grip strength deficit induced by MIA in a dose-dependent manner. Mechanical antiallodynic (**A**,**C**) and the recovery in grip strength (**B**,**D**) effects induced by different doses of CORM-2 or CoPP (mg/kg) are represented. For each test and treatment, * indicates significant differences vs. vehicle (0 mg/kg)-treated mice and + vs. the effect produced by low doses of CORM-2 (5 mg/kg) or CoPP (0.5 mg/kg) ($p < 0.05$; one-way ANOVA, followed by the Student–Newman–Keuls test). Data are expressed as the mean values of the maximal possible effect (%) for the mechanical allodynia and the recovery in grip strength (%) ± SEM; $n = 6$ animals per dose and treatment.

Both treatments did not show any significant effect on the allodynia and the grip strength of SS-injected animals (data not shown).

3.3. The Mechanical Antiallodynic and the Recovery of Grip Strength Effects Induced by the Co-Treatment of Low Doses of CORM-2 or CoPP with DADS or GYY4137 during Joint Pain

The antiallodynic effects (Figure 3A) and the recovery of grip strength (Figure 3B), induced by low doses of the CO-releaser agent CORM-2 (5 mg/kg) or the HO-1 inducer CoPP (0.5 mg/kg), administered alone and in combination with low doses of DADS (3 mg/kg) or GYY4137 (0.4 mg/kg) were evaluated.

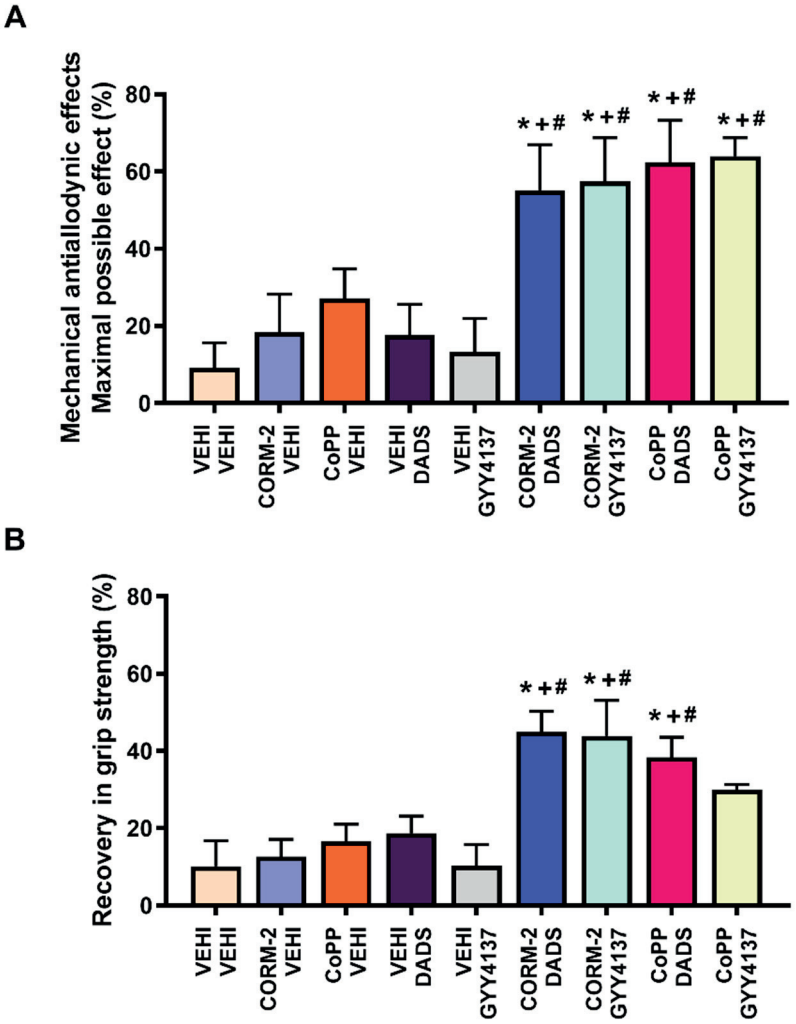

Figure 3. Effects of the co-administration of a CO-releasing molecule and an HO-1 inducer with two H_2S donors on MIA-induced mechanical allodynia and loss of grip strength. The mechanical antiallodynic effects (**A**) and the recovery of grip strength (**B**) induced by the acute intraperitoneal administration of 5 mg/kg of CORM-2, 0.5 mg/kg of CoPP, 3 mg/kg of DADS, or 0.4 mg/kg of GYY4137 administered alone or combined are displayed. For each test, * denotes significant differences vs. vehicle plus vehicle-treated mice; + vs. CORM-2 or CoPP plus vehicle-treated mice and # vs. vehicle plus DADS- or GYY4137-treated mice ($p < 0.05$; one-way ANOVA, followed by the Student–Newman–Keuls test). Data are expressed as the mean values of the maximal possible effect (%) for the mechanical allodynia and the recovery in grip strength (%) ± SEM; $n = 6$ animals per dose and treatment.

The results showed that the intraperitoneal co-administration of CORM-2 or CoPP with DADS or GYY4137 significantly enhanced the mechanical antiallodynic actions generated by each of them administered alone ($p < 0.001$, one-way ANOVA and Student–Newman–Keuls test; in comparison with their equivalent control groups injected with CORM-2, CoPP or vehicle plus vehicle, and vehicle plus DADS or GYY4137.

Similar findings were detected concerning the recovery of grip strength in which the co-administration of CORM-2 or CoPP with DADS or GYY4137 also displayed significantly greater effects than their corresponding control groups treated with CORM-2 and/or CoPP plus vehicle, as well as versus animals administered with vehicle plus DADS or GYY4137 ($p < 0.001$, one-way ANOVA, Student–Newman–Keuls test). However, although the recovery of grip strength induced by the co-administration of CoPP plus GYY4137

increased, it does not reach statistical significance as compared to those produced by both treatments administered alone.

Finally, neither vehicle injection nor any of the tested combinations exerted any action on allodynia and grip strength in SS-injected animals (data not shown).

3.4. Reversal of the Mechanical Antiallodynic Actions and Recovery of Grip Strength Produced by DADS or GYY4137 by Their Co-Treatment with Specific Nrf2, HO-1, and NQO1 Inhibitors

To study the possible contribution of Nrf2, HO-1, and NQO1 in the modulatory effects of H_2S donors, we assessed the effects produced by high doses of DADS (30 mg/kg) or GYY4137 (6 mg/kg) co-administered with 25 mg/kg of ML-385 (Nrf2 inhibitor) and 10 mg/kg of SnPP (HO-1 inhibitor), or dicoumarol (NQO1 inhibitor) on the mechanical allodynia and the loss of grip strength caused by MIA.

The results showed that the antiallodynic effects (Figure 4A,C) and the recovery of grip strength (Figure 4B,D) induced by DADS (Figure 4A,B) or GYY4137 (Figure 4C,D) in MIA-injected animals were completely reversed with the administration of ML-385, SnPP, or dicoumarol ($p < 0.0001$, one-way ANOVA, Student–Newman–Keuls). The administration of ML-385, SnPP, or dicoumarol alone did not alter the mechanical allodynia and grip strength deficit provoked by MIA.

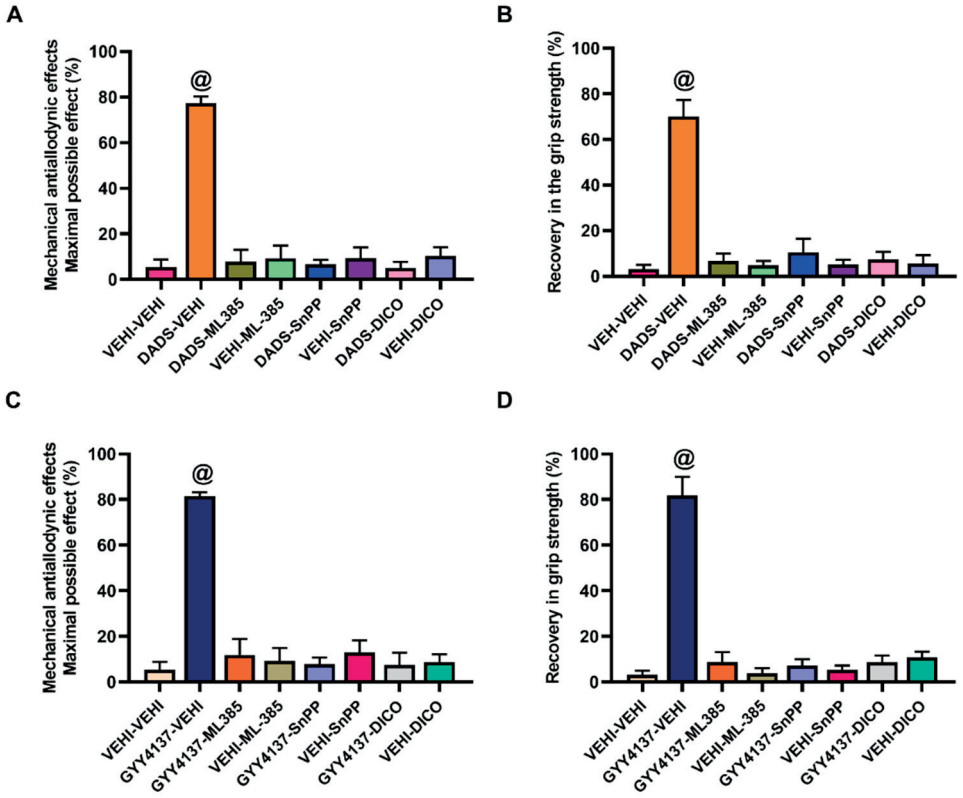

Figure 4. Effects of the co-administration of DADS and GYY4137 with vehicle, ML-385, SnPP or dicoumarol in the mechanical allodynia and loss of grip strength caused by MIA. The antiallodynic (**A**,**C**) and the recovery of grip strength (**B**,**D**) induced by DADS (30 mg/kg) or GYY4137 (6 mg/kg) combined with 25 mg/kg of ML-385 or 10 mg/kg of SnPP or dicoumarol (DICO) are shown. The effects produced by each of these treatments administered alone are also represented. For each treatment and test assessed, @ denotes significant differences vs. the other groups ($p < 0.05$, one-way ANOVA; Student–Newman–Keuls test). Data are expressed as mean values of the maximal possible effect (%) for the mechanical allodynia and the recovery of grip strength (%) ± SEM; $n = 6$ animals per dose and treatment.

Moreover, treatment with DADS or GYY4137 alone or combined with ML-385, SnPP, or dicoumarol did not exert any effect in SS-injected mice (data not shown).

3.5. Effects of DADS or GYY4137 on the Expression of Nrf2, HO-1, NQO1, SOD-1, and GSTM1 on the DRG of MIA-Injected Animals

To evaluate the plausible involvement of the Nrf2 signaling pathway in the inhibitory actions produced by H_2S donors at the biochemical level, the Nrf2, HO-1, NQO1, SOD-1, and GSTM1 protein levels in the DRG of MIA-injected animals administered with DADS or GYY4137 were assessed.

Our data showed that MIA injection did not alter the protein levels of Nrf2 (Figure 5A), HO-1 (Figure 5B), NQO1 (Figure 5C), or GSTM1 (Figure 5E), but it significantly increased the expression of SOD-1 ($p < 0.013$, one-way ANOVA vs. SS plus vehicle-injected mice; Figure 5D). Nevertheless, both DADS and GYY4137 increased the protein levels of Nrf2 ($p < 0.017$), HO-1 ($p < 0.006$), NQO1 ($p < 0.013$), and GSTM1 ($p < 0.002$) (one-way ANOVA vs. their respective SS or MIA plus vehicle-treated mice) and retained the upregulation of SOD-1 generated by MIA in the DRG.

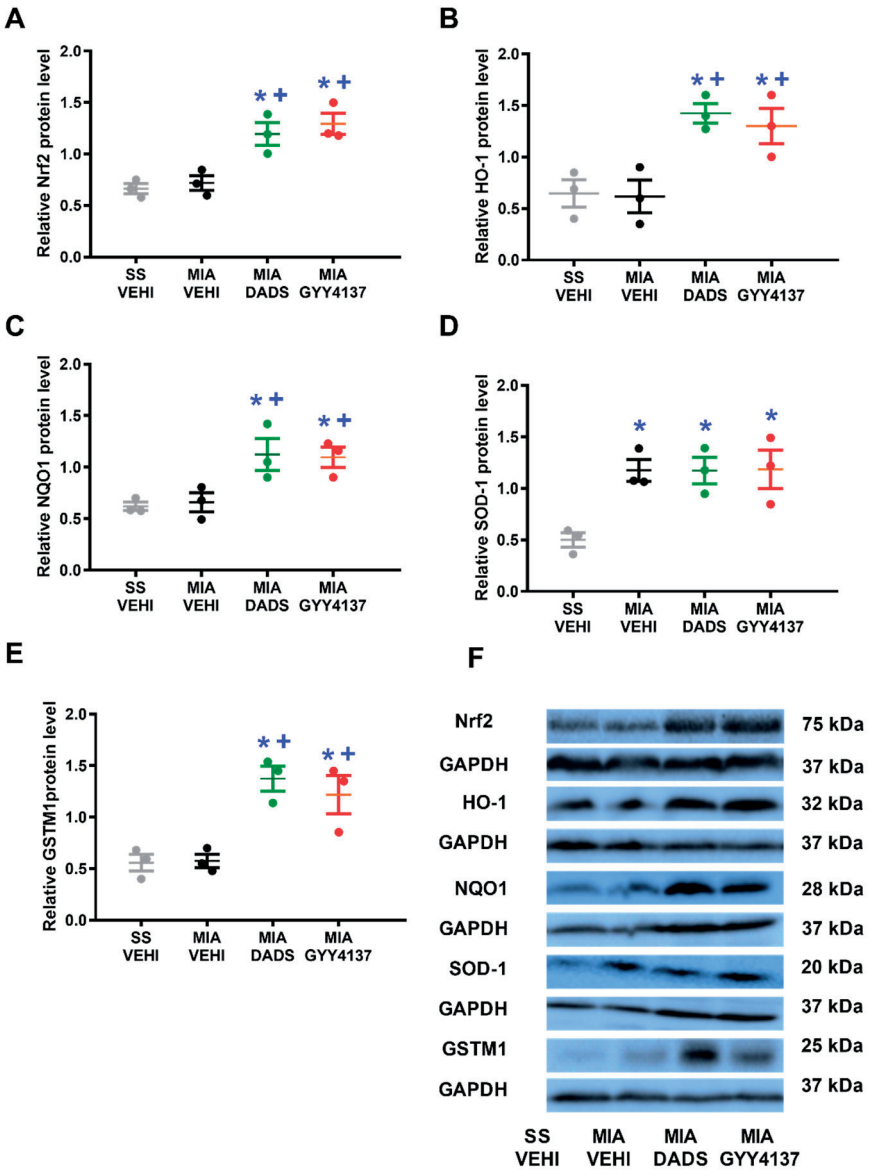

Figure 5. Effects of treatment with DADS and GYY4137 on the Nrf2, HO-1, NQO1, SOD-1, and GSTM1 protein levels in the ipsilateral DRG of MIA-injected mice. The expression of Nrf2 (**A**), HO-1 (**B**), NQO1 (**C**), SOD-1 (**D**), and GSTM1 (**E**) in DRG of MIA-injected animals treated with vehicle (MIA-VEHI), DADS (MIA-DADS) or GYY4137 (MIA-GYY4137) and of SS-injected animals administered with vehicle (SS-VEHI) are represented. All proteins are expressed relative to GAPDH

levels. For each protein, * denotes significant differences vs. SS plus vehicle-treated mice and + vs. MIA plus vehicle-treated animals ($p < 0.05$, one-way ANOVA; Student–Newman–Keuls test). Examples of blots for Nrf2, HO-1, NQO1, SOD-1, GSTM1, and GAPDH are shown (**F**). Results are represented as the mean ± SEM ($n = 3$ samples).

3.6. Effects of DADS or GYY4137 on the Expression of Nrf2, HO-1, NQO1, SOD-1, and GSTM1 in the PAG of MIA-Injected Animals

To evaluate the effects of DADS and GYY4137 in the expression of Nrf2, HO-1, NQO1, SOD-1, and GSTM1 in CNS of animals with joint pain, the protein levels of these antioxidant enzymes were also assessed in the PAG.

Our data showed that the protein levels of Nrf2 ($p < 0.036$; Figure 6A), HO-1 ($p < 0.003$; Figure 6B), and NQO1 ($p < 0.024$; Figure 6C) diminished in MIA-injected mice (one-way ANOVA; vs. their corresponding SS plus vehicle-treated animals), and both DADS and GYY4137 treatments avoided this downregulation. Furthermore, although MIA injection did not modify the expression of GSTM1 (Figure 6E) in the PAG, both treatments enhanced its expression ($p < 0.002$, one-way ANOVA, as compared with SS- and MIA-injected mice treated with vehicle). Lastly, the expression of SOD-1 (Figure 6D) remained unchanged in the four groups.

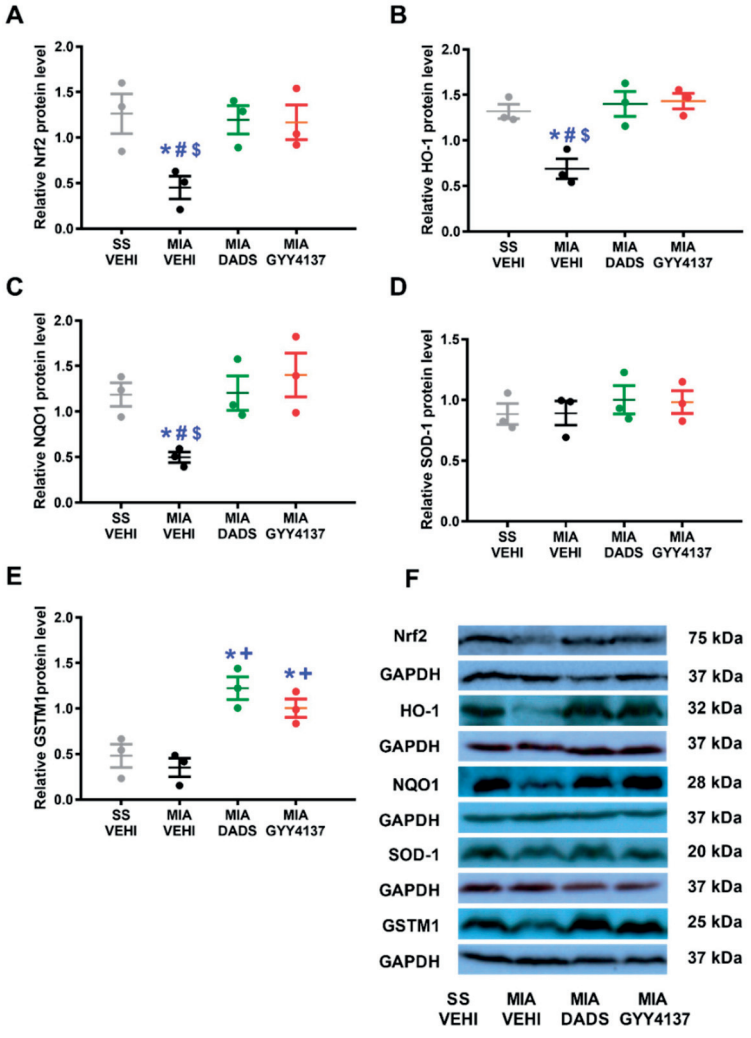

Figure 6. Effects of treatment with DADS and GYY4137 on the Nrf2, HO-1, NQO1, SOD-1, and GSTM1 protein levels in the PAG of MIA-injected mice. The expression of Nrf2 (**A**), HO-1 (**B**), NQO1 (**C**), SOD-1 (**D**)

and GSTM1 (**E**) in PAG of MIA-injected animals treated with vehicle (MIA-VEHI), DADS (MIA-DADS) or GYY4137 (MIA-GYY4137) and of SS-injected mice treated with vehicle (SS-VEHI) are represented. All proteins are expressed relative to GAPDH levels. For each protein, * indicates significant differences vs. SS plus vehicle-treated mice; + vs. MIA plus vehicle-treated animals; # vs. MIA plus DADS-treated mice and $ vs. MIA plus GYY4137-injected mice ($p < 0.05$, one-way ANOVA; Student–Newman–Keuls test). Examples of blots for Nrf2, HO-1, NQO1, SOD-1, GSTM1, and GAPDH are shown (**F**). Results are displayed as the mean ± SEM ($n = 3$ samples).

4. Discussion

This study revealed that the acute administration of an HO-1 inducer, a CO releasing molecule or H_2S donors inhibited the mechanical allodynia in a dose-dependent manner, but only H_2S donors inhibited the grip strength deficits caused by joint degeneration. Moreover, the co-administration of low dose of CORM-2 or CoPP with DADS or GYY4137 potentiated the antiallodynic actions as well as the recovery of grip strength produced by each of these compounds. Thus, this suggests a positive interaction between H_2S and CO on the modulation of joint pain. Our data also revealed the involvement of the Nrf2/HO-1-NQO1 signaling path in the antiallodynic effects and recovery of grip strength induced by both H_2S donors during joint pain at the pharmacological and biochemical levels.

In accordance with previous studies showing the antinociceptive effects induced by the acute administration of substances capable of releasing H_2S slowly during neuropathic pain [6], our findings further demonstrated that the acute treatment with DADS and GYY4137 also reduced the mechanical allodynia and functional deficits provoked by joint degeneration in a dose-dependent manner. Furthermore, and considering that the maximum inhibition of mechanical allodynia and grip strength deficit was obtained with 30 mg/kg of DADS, while only 6 mg/kg of GYY4137 was required, these results suggested a greater effectiveness of GYY4137 vs. DADS in terms of inhibition of the mechanical allodynia and grip strength deficits induced by MIA, as previously demonstrated in neuropathic pain [6].

Our data additionally showed that the activation of HO-1/CO signaling with CoPP and CORM-2 treatments induced opposite effects in tactile allodynia and grip strength. That is, both compounds dose-dependently inhibited the mechanical allodynia but did not ameliorate the grip strength deficits provoked by MIA. Thereby, the maximal antiallodynic effects of both compounds were obtained with a dose of 10 mg/kg, but only a 20% recovery of grip strength was observed in all doses tested. In conformity with our results, other works also established the antiallodynic properties of CoPP and CORM-2 during inflammatory and neuropathic pain [25,26], as well as the lack of effects produced by CO inhalation on the grip strength deficits caused by arthritis during the first days of treatment, although a reduction in the loss of grip strength was observed from day 49 of CO inhalation [27]. It is possible that chronic treatment with CORM-2 and CoPP is required for inhibiting the grip strength deficit induced by MIA. In summary, these data showed that under joint pain conditions H_2S-releasing agents are more effective than HO-1 activators or CO-releasing molecules in inhibiting the grip strength deficits induced by MIA.

The interaction between H_2S and CO has previously been evaluated in different disorders [4,5], especially in inflammatory processes [3]. In this study, we showed that the co-treatment of CoPP or CORM-2 with two H_2S donors produced greater antiallodynic effects and the recovery of grip strength than those produced by each of them separately. This revealed a positive interaction between both CO and H_2S, not only in the antiallodynic effects but in the rescue of the physical disfunction provoked by joint degeneration as well. Thus, this study proposes a new strategy for the management of the allodynia and the loss of grip strength associated with joint degeneration. Moreover, joint pain is difficult to treat with the current therapies [13,28], mainly due to their low efficacy and side effects, especially those affecting the gastrointestinal system [13]. The fact that the co-administration of two gaseous neurotransmitters with important gastroprotective effects [3]

produces an increase in their antinociceptive effects is of great relevance for the treatment of joint pain and associated functional disabilities in humans.

To study the possible paths involved in the interaction between these gaseous neurotransmitters, we assessed the role played by the Nrf2 signaling pathway in the effects of H_2S donors during joint pain. In agreement with other pain models [14], our data further revealed that the antiallodynic effect and recovery of grip strength induced by DADS or GYY4137 were blocked with the administration of specific inhibitors of Nrf2 (ML-385), HO-1 (SnPP), and NQO1 (dicoumarol). This indicates that the activation of the antioxidant system triggered by Nrf2 might be involved in the modulatory role played by both H_2S donors during joint pain. These findings also suggested that H_2S effects, including the joint pain modulation, may be dependent on the endogenous CO biosynthesis initiated with the Nrf2/HO-1 pathway activation as occurs with the gastroprotective actions of H_2S donors [3]. These results agreed with the involvement of the Nrf2/HO-1 system in the painkiller actions of these and other H_2S donors during inflammatory and neuropathic pain [6,7]. In addition, similar results were obtained in regarding the interaction between CO and other gaseous neurotransmitters such as nitric oxide, where CO needed the presence of nitric oxide to inhibit chronic pain [29].

To evaluate the biochemical interaction between H_2S and CO, the effects of treatment with DADS or GYY4137 in the expression of the Nrf2 transcription factor and several antioxidant enzymes activated by it (e.g., SOD-1, NQO1, HO-1, and GSTM1) in two areas, one in PNS (DRG) and other in the CNS (PAG), both involved in pain modulation, were evaluated [21,22]. Our data showed that both H_2S donors increased the Nrf2, HO-1, NQO1, and GSTM1 expression in the DRG, thus revealing that the antioxidative effects of DADS and GYY4137 in joint pain were mainly mediated through triggering the Nrf2 antioxidant signaling pathway activation. These results agree with those obtained in animals with paw inflammation, which also proved the antioxidant capacity of DADS through maintaining elevated levels of HO-1 and NQO1 and normalizing the downregulation of GSTM1 induced by peripheral inflammation in the paw [7]. Our findings further showed a positive interaction between H_2S and CO in the PNS of animals with joint pain.

It is well known that during chronic pain, several oxidative responses also take place in specific areas of the CNS [30], especially in the PAG, which plays an essential role in modulating descending pain [31,32]. Then, the effects of DADS and GYY4137 systemically administered on the oxidative responses induced by MIA injection in the PAG, as proved with the diminished levels of Nrf2, HO-1, and NQO1 proteins in this brain area, were also evaluated. The antioxidant properties of DADS or GYY4137 in the CNS were demonstrated with the normalization of the downregulation of the Nrf2, HO-1, and NQO1 caused by MIA in the PAG. In addition, both treatments increased the expression of the antioxidant enzyme GSTM1 in this brain area, thus revealing that a positive interaction between H_2S and CO also occurs in the CNS of animals with joint pain. Our findings were supported by the antioxidant actions caused by garlic consumption in patients with rheumatoid arthritis [33] as well as with the reversion of the oxidative stress produced by treatments with DADS and GYY4137 in the PAG of animals with neuropathic pain [6]. Finally, our results suggested that the activation of the peripheral and central endogenous antioxidant system induced by H_2S donors may contribute to the increased antiallodynic effects and the recovery of grip strength induced by their co-administration with CORM-2 or CoPP during joint pain.

This study has some limitations such as the fact that it was performed with immature female mice, in a specific model of joint pain, and by the absence of confirmation of the pathology during pain evaluation and its grade during the evaluation of the effects of treatments.

5. Conclusions

In summary, this study demonstrated that acute treatment with the H_2S donors DADS and GYY4137 inhibited the tactile allodynia and the functional deficits provoked by joint degeneration, while CORM-2 and CoPP only inhibited the mechanical allodynia. Moreover,

a positive interaction between CO and H_2S was demonstrated with the enhancement of the antiallodynic effects, and the recovery of grip strength induced by the co-administration of CORM-2 or CoPP with DADS or GYY4137 and with the fact that treatment with H_2S donors also activated the Nrf2 signaling pathway at the pharmacological and biochemical levels. Therefore, this study suggests that the systemic co-administration of CO and H_2S activators might be considered as a new strategy for the management of joint pain and the physical disabilities associated.

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5. Discussion

OA pain is a multifaceted phenomenon that involves changes in physical functioning and is associated with several emotional and cognitive disorders, which have a negative effect on patients' daily lives (Axford et al., 2010; Moriarty et al., 2011). OA pain and its comorbidities associated are difficult to treat. Several studies have found different treatments to reduce OA pain and only some of the affective disorders associated, and most of them carried important side effects. Therefore, it is important to find a global and effective treatment for OA pain and the associated affective and memory deficiencies.

The role of H_2S on pain modulation is controversial due to its nociceptive and antinociceptive roles depending on the type of H_2S-releasing compound and the doses used (Szabo and Papapetropoulos, 2017). Previous reports have shown that fast H_2S-releasing donors, such as NaSH, have no effects on pain modulation, even reaching increasing it (Cunha et al., 2008; Ekundi-Valentim et al., 2010). In contraposition, slow H_2S-releasing agents exerted potent anti-inflammatory actions (Huang et al., 2016) and antinociceptive effects in neuropathic pain models (Di Cesare Mannelli et al., 2017; Wang, et al., 2020). Moreover, and due to the potent antioxidant, anti-inflammatory, anti-apoptotic, anxiolytic and antidepressant properties of these compounds demonstrated in multiple diseases with low side effects (Sun et al., 2017; Faller et al., 2018; Powell et al., 2018) we evaluated the possible effects produced by slow-releasing H_2S donors as a possible global therapy for OA pain.

Furthermore, and considering that women are more likely to suffer chronic pain than men and since most of the symptoms and disorders linked with OA pain are more prevalent in women (Heidari, 2011), this study was carried out in female mice.

In the first study, besides the allodynia, we also evaluated the impact of treatment with two slow releasing H_2S donors (A-ITC and P-ITC) on the physical dysfunction provoked by OA and the anxiety- and depressive-like behaviors associated with MIA-provoked OA pain. Our results demonstrated that the repeated treatment with two isocyanates, A-ITC or P-ITC, administered at one time per day, both inhibited the mechanical allodynia and grip strength deficits provoked by MIA in a time-dependent manner. Moreover, while the treatment with A-ITC needed four days to completely reversed the allodynia and functional disabilities, P-ITC required ten days, showing the major effectiveness of A-ITC versus P-ITC. These results revealed, for the first time, the inhibitory effects produced by the repeated administration of these isocyanates on the allodynia and functional incapabilities generated by OA. Thus, supporting the demonstrated antiallodynic properties of these compounds in animals with neuropathic pain (Cabarga et al., 2020) and revealing their capacity to reduce the decreased grip strength detected in animals with OA pain.

In addition, and seeing the negative mental effects associated with chronic pain, we wanted to analyze the actions of A-ITC and P-ITC on the depressive- and anxiety-like behaviors related to OA pain. Our data showed that the repeated administration of these donors inhibited depressive-like behaviors, but not the associated anxious-like behaviors. Thus, demonstrating the antidepressant properties of these treatments during OA pain, as had been previously proved in animals' models of depression (Chen et al, 2013c), but did not support the anxiolytic actions of H_2S in animal models of anxiety or in diabetic animals with anxiety-like behaviors (Chen et al., 2013c; Tang et al., 2015). It's possible that higher doses or different treatment guidelines with A-ITC and P-ITC may be required for inhibiting the anxiety-like behaviors related to OA pain.

Several works have revealed that the antinociceptive actions of H_2S during neuropathic pain generated by chemotherapy or sciatic nerve injury were mediated via opening the Kv7 potassium channels (Lucarini et al., 2018; Bai et al., 2021). The reversion of the antiallodynic, grip strength

recovery and antidepressant actions of A-ITC and P-ITC with the administration of a Kv7 potassium channel blocker, XE-991, revealed that these channels are involved, not only in the antiallodynic effects, but also in the inhibition of the functional disabilities and in the antidepressant actions produced by these compounds in mice with OA pain.

To analyzing the possible other pathways participating in the inhibitory actions of A-ITC and P-ITC during OA pain, we analyzed the impact of these treatments in the nociceptive and inflammatory responses triggered by MIA in the HIP, a brain area highly immersed in regulating depressive-like behaviors associated with chronic pain (Mokhtari et al., 2019). Our results supported these data by demonstrating increased levels of CD11b/c (microglial activation), NOS2 (inflammation) and PI3K/p-AKT (nociception) in the HIP of animals with OA pain. Moreover, we demonstrated that both treatments normalized all these responses. Thus, considering that the administration of microglia, NOS2 and/or PI3K/p-AKT inhibitors can alleviate the allodynia and/or the depressive-like behaviors in different animal models (LaBuda et al., 2006; Tomaz et al., 2014; Dai et al., 2019) we postulated that the inhibitory actions of A-ITC and P-ITC in the allodynia, functional incapacity and/or the depressive-like behaviors accompanying OA pain might be in part mediated via regulating these pathways in the HIP. The lack of changes in the expression of GFAP, an astroglial marker, in the HIP of animals with OA pain, with and without treatment with A-ITC or P-ITC, suggested that this type of glial cell does not play a relevant part in the modulation of OA pain in this brain area.

The endogenous antioxidant system, activated by the Nrf2 transcription factor and the consequent synthesis of the antioxidant and detoxicant enzymes, for instance HO-1, NQO1 and GSTM, participate in preserving the body homeostasis (Nguyen et al., 2009; Pol, 2021; Basu et al., 2022). In our study, both H_2S slow releasers kept the endogenous antioxidant system activated in the HIP, thus sustaining a compensatory mechanism to the oxidative stress promulgated by OA in this brain area and confirming the antioxidant properties of these compounds. These findings agreed with the effects of these or others slow-releasing H_2S donors in animals with neuropathic or inflammatory pain (Cabarga et al., 2020; Porta et al., 2021). Moreover, contemplating the antidepressant actions of several antioxidants such as dimethyl fumarate (De Souza et al., 2022) and its painkiller activities during neuropathic pain (Singh et al., 2022), we postulated that the antioxidative effects produced by A-ITC and P-ITC might also contribute to its antidepressant and antinociceptive activity during OA pain.

These data suggest that treatment with A-ITC and P-ITC might be an interesting strategy for the management of the allodynia, functional disability, and depressive-like conducts related to OA pain by modulating inflammatory and nociceptive paths, and/or by preserving the antioxidant system activated in the HIP.

Nevertheless, and in accordance with the idea of finding a more complete treatment for OA pain that also avoided the anxiety-like behaviors and the memory loss linked with this type of pain, we studied the actions of two other slow releasing H_2S donors: DADS (an organosulfur compound) and GYY4137 (a synthetic water-soluble molecule). Interestingly, the repeated administration of DADS and GYY4137, injected twice daily for three and four consecutive days, reduced not only the allodynia, grip strength deficits and the linked-depressive behaviors to OA pain, but also remedied the anxiety-like behaviors associated with OA pain. Contrasting with the non-anxiolytic effects of A-ITC and P-ITC. A plausible justification for these divergent findings could be concerned with the chemical structure differences among isothiocyanates and GYY4137 or DADS, and/or with the different treatment guidelines used in both studies (one and two administrations per day).

Another important enigma in patients suffering from OA pain is the memory loss (Innes and Sambamoorthi, 2018), what is also manifested in our preclinical pain model with a negative discrimination index on the novel object recognition test. In this case, of the two treatments evaluated (DADS and GYY4137), only GYY4137 allowed the recovery of memory deficits

associated with OA pain in mice. These differences could be related to the different nature of both compounds, while DADS is a natural bioactive component of garlic, GYY4137 is a synthetic donor, and/or the duration of treatment, three and four days, respectively. In summary, the capacity of GYY4137 to palliate the allodynia, functional disabilities, anxiodepressive-like behaviors, and cognitive deficits accompanying OA pain, suggests that this synthetic slow-releasing H_2S donor can be a possible candidate for the global treatment of this type of pain.

The oxidative, inflammatory, and apoptotic responses activated by knee OA in different brain areas, such as the AMG, PAG, IF-PFC and ACC are in part responsible for the development of OA pain and the associated comorbidities (Lee et al., 2011; Fan et al., 2018). Therefore, to study the main biochemical mechanisms involved in the inhibitory actions of DADS and GYY4137 during OA pain, we assessed the effects of these treatments on the expression of several proteins: 4-HNE (oxidative stress), PI3K/Akt (nociceptive pathway), NOS2 (inflammation) and BAX (apoptosis) in the above-mentioned brain areas.

Interestingly, GYY4137 and/or DADS normalized the oxidative and apoptotic responses in the AMG, the up-regulation of PI3K/p-AKT in the AMG, PAG, IF-PFC and/or ACC, and the NOS2 over expression in the AMG, PAG and IF-PFC of animals with OA pain. Thus, revealing the antioxidant, anti-nociceptive, anti-inflammatory, and anti-apoptotic actions of these two slow-releasing H_2S donors in different brain areas involved in the modulation of pain, anxiety, depression, and cognitive behaviors (Nestler et al., 2002; Bremner 2004; Ossipov et al., 2014; Rolls, 2019) and suggesting that all the biochemical changes induced by GYY4137 and DADS might contribute to the inhibitory effects produced by these compounds during OA pain.

The interaction between H_2S and CO has been described in several systems, remarkably in the gastrointestinal (Glowacka et al., 2020). Both neurotransmitters share several properties, such as a similar distribution in the CNS and PNS and the high capacity to cross membranes (Vijay et al., 2009; Mancuso et al., 2010; Farrugia and Szurszewski, 2014). We analyzed the potential interaction between H_2S and CO in the modulation of OA pain at pharmacological and biochemical level. Interestingly, whereas the acute administration of CORM-2 and CoPP, as well as of DADS and GYY4137 diminished the mechanical allodynia in a dose-dependent manner, only the H_2S donors recovered the grip strength deficits induced by OA in a dose-dependent manner. Thus, revealing the major effectiveness of slow-releasing H_2S donors vs the CO-releaser molecule or HO-1 inducer in relieving the functional disabilities accompanying OA. This study further proved that the co-administration of low doses of CORM-2 or CoPP with DADS or GYY4137 potentiated the antiallodynic actions obtained with these treatments given separately and substantially improved the low recovery of grip strength deficits produced by CORM-2 or CoPP administered alone. Thus, showing a positive interaction between both gaseous neurotransmitters in the modulation of the allodynia and physical dysfunctions provoked by OA.

Regarding the possible ways implicated in this interaction, the reversion of the antiallodynic and grip strength recovery effects of DADS and GYY4137 with the administration of specific inhibitors of Nrf2 (ML-385), HO-1 (SnPP), and NQO1 (dicoumarol), point in out the involvement of the endogenous antioxidant system triggered by Nrf2 in the effects played by H_2S during OA pain. These data were supported by the normalization of the low levels of these antioxidant proteins in the PAG and by its activation in the DRG of MIA-injected mice treated with DADS or GYY4137. Thus, the activation of the antioxidant system induced by H_2S donors might explain the improvement of the inhibitory effects produced by CO/HO-1 activators after its co-treatment with DADS and GYY4137. These results agree with other studies showing a positive interaction between CO and H_2S in regulating the gastrointestinal system (Magierowski et al., 2016; Glowacka et al., 2020) and propose the combined treatment of CO and H_2S activators as a new stratagem for treating the allodynia and functional impairments induced by OA.

A summary of the results of this work are represented in Figure 11.

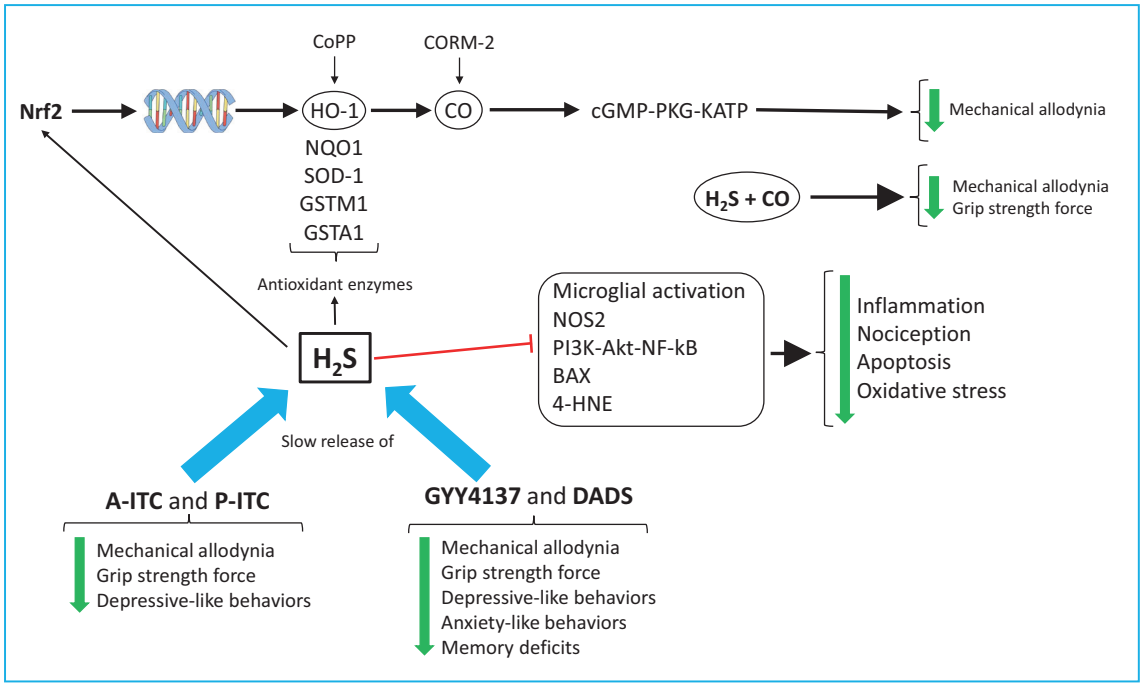

Figure 11. Summary of the effects of slow H_2S-releasing donors and of the interaction between H_2S and CO signaling pathways in the modulation of OA pain and/or comorbidities associated.

6. Conclusions

In female mice with OA pain, this study demonstrated that:

a) The repeated administration of low doses of A-ITC and P-ITC inhibits the mechanical allodynia and the grip strength deficits caused by OA as well as the depressive-like behaviors associated by attenuating the inflammatory and nociceptive responses and maintaining activated the endogenous antioxidant system in the HIP.

b) GYY4137 and/or DADS, repeatedly administered, exert potent antiallodynic, anxiolytic and antidepressant effects and recover the memory and grip strength deficits by inhibiting the oxidative, nociceptive, inflammatory and/or apoptotic replies provoked by the OA in the AMG, PAG, IF-PFC and/or ACC.

c) The involvement of Kv7 channels and the Nrf2/HO-1/NQO1 signaling pathway in the antiallodynic actions and of functional disability recovers produced by slow-releasing H_2S donors during OA.

d) The acute administration of HO-1/CO activators and H_2S donors, all inhibit the mechanical allodynia in a dose-dependent manner, but only H_2S improves the OA-provoked grip strength deficits, thus revealing the major effectiveness of H_2S donors vs CO activators.

e) The enhancement of the antiallodynic effects and of the recovery of grip strength produced by the co-administration of CORM-2 or CoPP with DADS or GYY4137, and the activation of the endogenous antioxidant system induced by H_2S in the CNS and PNS, demonstrate a positive interaction between CO and H_2S in modulating OA pain.

In summary, this study reveals a positive interaction between H_2S and CO in the modulation of OA pain and suggests that treatment with GYY4137 could be an interesting approach to treat not only allodynia and functional disabilities caused by OA, but also associated emotional and memory disorders.

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