

Treatment with sulforaphane produces antinociception and improves morphine effects during inflammatory pain in mice

Alejandro Redondo, Pablo Aníbal Ferreira Chamorro, Gabriela Riego, Sergi Leáñez and Olga Pol

Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau & Institut de Neurociències, Universitat Autònoma de Barcelona, Barcelona, Spain (A.R., P.A.F.C., G.R., S.L., O.P.)

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Corresponding author: Dr. Olga Pol. Grup de Neurofarmacologia Molecular, Institut d'Investigació Biomèdica Sant Pau & Institut de Neurociències, Facultat de Medicina. Edifici M2-115. Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain. Tel: 34 619 757 054; Fax: 34 935 811 573; E-mail: opol@santpau.es

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Abbreviations: ANOVA, analysis of variance; ARE, antioxidant response element; CFA, complete Freund's adjuvant; CoPP, cobalt protoporphyrin IX; ERK, extracellular signal regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, heme oxygenase 1; IL, interleukin; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; MOR, μ -opioid receptor; NOS2, inducible nitric oxide synthase; NQO1, NAD(P)H: quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; TNF α , tumor necrosis factor- α ; SEM, error of the mean; SFN, sulforaphane.

ABSTRACT

The activation of nuclear factor erythroid 2-related factor 2 (Nrf2) exerts potent anti-oxidative and anti-inflammatory effects, however its participation in the modulation of chronic inflammatory pain and on the antinociceptive effects of μ opioid receptor (MOR) agonists has not been evaluated. We investigated whether the induction of Nrf2 could alleviate chronic inflammatory pain, augments the analgesic effects of morphine and mechanisms implicated. In male C57BL/6 mice with inflammatory pain induced by complete Freund's adjuvant (CFA) subplantarily administered, we assessed: 1) antinociceptive actions of the administration of 5 and 10 mg/kg of a Nrf2 activator, sulforaphane (SFN); 2) effects of SFN on the antinociceptive actions of morphine and on protein levels of Nrf2, heme oxygenase 1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) enzymes, microglial activation and inducible nitric oxide synthase (NOS2) over-expression, as well as on mitogen-activated protein kinase (MAPK) and MOR expression in the spinal cord and paw of animals with inflammatory pain. Results showed that treatment with SFN inhibited allodynia and hyperalgesia induced by CFA and increased the local antinociceptive actions of morphine. This treatment also augmented the expression of Nrf2, HO-1, NQO1 and MOR, inhibited NOS2 and CD11b/c overexpression, and MAPK phosphorylation induced by inflammation. Thus, this study shows that the induction of Nrf2 might inhibit inflammatory pain and enhances the analgesic effects of morphine by inhibiting oxidative stress and inflammatory responses induced by peripheral inflammation. This study suggests the administration of SFN alone and in combination with morphine as new ways of treating chronic inflammatory pain.

Introduction

Chronic inflammatory pain is a recurrent disorder, affecting approximately at 20% of world population, and the actual therapies (nonsteroidal anti-inflammatory drugs, opioids, etc) may be limited in scope, have dose-limiting side effects. That reflects the need to search new strategies to treat more effectively this type of pain.

The transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates two important cytoprotective pathways: the anti-oxidative and the anti-inflammatory (Kim et al., 2010). A crucial factor for the antioxidant activity of Nrf2 is the regulation of the antioxidant response element (ARE) found in the promoter regions of numerous genes that encode antioxidant and detoxification enzymes, for example heme oxygenase 1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), superoxide dismutase 1, etc. which maintain the homeostatic equilibrium by regulating the reactive oxygen species (ROS) cumulating. Recent investigations also demonstrated that Nrf2 activation, in addition to modulating oxidative stress, it's also able to directly inhibit the transcription of cytokines (interleukin (IL)-1 β , IL-6) by an independent mechanism of ARE and ROS levels (Kobayashi et al., 2016). Therefore, while the lack of Nrf2 augmented the inflammatory response in the joints and the expression of the tumor necrosis factor- α (TNF α) and IL-6 accelerating the arthritis signs (Maicas et al., 2011), its activation reduced inflammation by decreasing the synthesis of several pro-inflammatory mediators, like the inducible isoform of nitric oxide synthase (NOS2), cyclooxygenase-2, IL-1 β , IL-6, and the mitogen-activated protein kinase (MAPK) in the cartilage from osteoarthritic animals (Kim et al., 2009; Davidson et al., 2013). More interesting is the result that the activation of Nrf2 also reduced acute pain provoked by nitroglycerin and neuropathic pain associated to diabetes or generated by nerve-injury in rodents (Negi et al, 2011; Di et al. 2016; McDonnell et al., 2017; Wang and Wang, 2017). Nevertheless, the role played by Nrf2 on the allodynia and hyperalgesia induced by chronic peripheral inflammation has not been studied.

We and other authors have demonstrated that treatment with HO-1 inducer compounds, like the cobalt protoporphyrin IX (CoPP), besides to inhibit chronic pain also potentiated the

antinociceptive properties of μ -opioid receptor (MOR) agonists during neuropathic and inflammatory pain (Hervera et al., 2013a; 2013b; Carcolé et al., 2014). On the basis of these results, we postulated that Nrf2 induction could also play an important role on the modulation of the antinociceptive effects of morphine (a MOR agonist) in animals with chronic inflammatory pain.

It is well known that activated microglia is implicated in the progress of inflammatory and neuropathic pain (Martini et al., 2016) by mediating the synthesis of abundant inflammatory mediators (TNF α , IL, NOS2, etc) and its inhibition reduced allodynia and hyperalgesia in animals with chronic pain (Pol, 2007; Hervera et al., 2012; Mika et al., 2013). In addition, MAPK also exercises a crucial role in the regulation of inflammatory reactions. That is, during inflammatory pain c-Jun NH2-terminal kinase (JNK), extracellular signal regulated kinase (ERK1/2) and p38 MAPK are differentially activated in neurons and glial cells and its inhibition attenuates pain (Gao and Ji, 2008; Ji et al., 2009). Nevertheless, the role played by sulforaphane (SFN), a Nrf2 inducer compound, in the modulation of these inflammatory mediators in mice with chronic inflammatory pain is still unknown.

In a complete Freund's adjuvant (CFA) induced chronic inflammatory pain in mice we assessed the: 1) antiallodynic and antihyperalgesic effects of the repetitive intraperitoneal administration of 5 and 10 mg/kg of SFN; 2) antinociceptive effects of the administration of SFN in combination with a subanalgesic dose of morphine; 3) effects of SFN on the expression of Nrf2, HO-1, NQO1, NOS2, CD11b/c (microglial marker), MOR and MAPK (JNK, ERK1/2 and p38) activation in the spinal cord and paw of CFA-injected mice.

Materials and Methods

Experimental animals. For experimental procedures, male C57BL/6 mice acquired in Envigo (Barcelona, Spain) were employed. The weight of all mice was between 21 to 25 g. Mice were accommodated under 12-h/12-h light/ dark conditions in a room with controlled temperature of 22° C and humidity of 66 %. Animals with free access to food and water were used after a minimum of 7 days acclimatization to the housing conditions. All experiments were executed according with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health and approved by the local Committee of Animal Use and Care of the Autonomous University of Barcelona. Maximal exertions to reduce animal suffering and number of animals employed were made.

Induction of chronic inflammatory pain. We induced chronic inflammatory pain with the subplantar injection of CFA (30 µl; Sigma-Aldrich, St. Louis, MO) into the right hind paw under brief anesthetic conditions with isoflurane according to our other studies (Leáñez et al., 2009; Hervera et al., 2009). Mechanical allodynia was measured by means of the von Frey filaments and thermal hyperalgesia with the plantar test. Animals were checked in both behavioral tests before and at 3, 4, 7, 10 and 14 days after CFA-injection.

Nociceptive behavioral tests. Mechanical allodynia was measured by evaluating the hind paw withdrawal response to von Frey filament stimulation. Mice were sited in methacrylate cylinders of 20 cm (high) x 9 cm (diameter) (Servei Estació, Barcelona, Spain) on a wire grid bottom across which the von Frey filaments (North Coast Medical, Inc., San Jose, CA) with a bending force in the range of 0.008-3.5 g were applied by employing an adapted version of the up-down paradigm described by Chaplan et al. (1994). We start the test with the filament of 0.4 g and the filament of 3.0 g was utilized as a cut-off. The strength of next filament was increased or decreased in accordance to the answer. Threshold of response was calculated from the sequence of filament strength used during the up-down procedure using an Excel program (Microsoft Iberia SRL, Barcelona, Spain) which includes curve fitting of the data. A clear paw

withdrawal, lick or shaking the paw was considered to be a nociceptive-like response. Both ipsilateral and contralateral hind paws were tested.

Thermal hyperalgesia was evaluated by evaluating paw withdrawal latency in response to radiant heat by using the plantar test apparatus (Ugo Basile, Varese, Italy) and in accordance to the method proposed by Hargreaves et al. (1988). Mice were placed in methacrylate cylinders of 20 cm (high) x 9 cm (diameter) situated on a glass surface. The heat source was positioned under the plantar surface of each of both hind paws (contralateral and ipsilateral) and activated with a light beam intensity selected in previous works with baseline latencies from 8 to 10 s under control conditions. To avoid tissue damage in lack of response, a cut-off time of 12 s was established. The mean paw withdrawal latencies were calculated from the average of 3 distinct trials, taken at 5 min pauses to avoid thermal sensitization and behavioral disturbances.

In all of these nociceptive behavioral assays, mice were habituated to the test for 1 h before testing to obtain a suitable behavioral immobility and they were performed by a male experimenter blinded to the treatments applied.

Western blot analysis. Animals were sacrificed by cervical dislocation at 0 (naïve) and 14 days after CFA injection from SFN or vehicle treated mice. Tissues from the ipsilateral side of the lumbar section of the spinal cord or hindpaw were extracted quickly after killing, frozen in liquid nitrogen and maintained at 80°C until use. For all tissues, samples from four animals were pooled together to have the adequate levels of proteins for doing Western blot assay. The protein levels of Nrf2, HO-1, NQO1, NOS2, CD11/bc, MOR and MAPK (pJNK/JNK, pERK1/2/ERK1/2, pp38/p38) were analyzed. The homogenization of tissues was performed in ice-cold lysis buffer (50 mM Tris·Base, 150 mM 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, and 1% phosphatase inhibitor cocktail). Except NP-40 that was purchased from Calbiochem (Darmstadt, Germany), all other reagents were acquired at Sigma (St. Louis, MO). After solubilisation of crude homogenate for 1 h at 4°C, it was sonicated during 10 s and centrifuged at 4°C for 15 min at 700 g. Then, the supernatant (60

µg of total protein) was mixed with 4 x laemmli loading buffer and loaded onto 4% stacking/10% separating sodium dodecyl sulfate polyacrylamide gels. After that, proteins were electrophoretically transferred onto PVDF membrane for 120 min and then blocked with phosphate buffered saline plus 5% nonfat dry milk or with Tris-buffered saline with Tween 20 plus 5% nonfat dry milk or 5% bovine serum albumin for 1h 15 min, and then incubated with specific rabbit primary antibody anti Nrf2 (1:160, Abcam, Cambridge, United Kingdom); HO-1 (1:300, Abcam, Cambridge, United Kingdom), NQO1 (1:333, Sigma-Aldrich, St. Louis, MO, USA), NOS2 (1:200, Abcam, Cambridge, United Kingdom), CD11b/c (1:200, Novus Biological, Littleton, CO, USA), MOR (1:333, Merck, Billerica, MA, USA), phospho JNK, total JNK, phospho ERK1/2, total ERK1/2 and total p38 (1:250, Cell Signaling Technology, Danvers, MA) and phospho p38 (1:200, Cell Signaling Technology, Danvers, MA) overnight at 4°C. A horseradish peroxidase-conjugated anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was used to detect proteins which were visualized with chemiluminescence reagents (ECL kit; GE Healthcare) and by exposure onto hyperfilm (GE Healthcare). Blots intensity was quantified by densitometry. Stripped membranes were reprobed with a rabbit anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH) (1:5000, Merck, Billerica, MA, USA) utilized as a loading control.

Experimental procedure. Baseline responses were established after the habituation period, beginning with the von Frey filaments followed by plantar test. After paw inflammation induction, animals were once again tested in each test at days 3, 4, 7, 10 and 14 after CFA injection as for baseline measures by using the same sequence. We used contralateral paws as controls (n = 6 animals for group). CFA-injected mice were administered with 5 and 10 mg/kg of SFN, an Nrf2 transcription factor activator, or vehicle (DMSO 1% solution in saline) during 11 consecutive days, from day 4 to 14 after CFA-injection.

In a second set of studies, we evaluated the mechanical antiallodynic and thermal antihyperalgesic effects produced by 10 mg/kg of SFN intraperitoneally administered alone and in combination with a subanalgesic dose of morphine (50 µg) subplantarily injected in the right

hindpaw. Both, contralateral and ipsilateral paws of CFA injected animals were tested (n = 6 animals for group). The dose of SFN used for the combination was obtained from this study and the dose of morphine was selected from a previous work (Carcolé et al., 2014), as the one that displayed a minimal antinociceptive effect. Researcher performing these experiments was blinded to the treatments applied.

The protein levels of Nrf2, HO-1, NQO1, NOS2, CD11b/c, MOR and MAPK (p-JNK/total JNK, pERK1/2/total ERK1/2, pp38/total p38) in the ipsilateral site of spinal cord and paw from mice with peripheral inflammation and treated with SFN or vehicle, were also evaluated by Western blot assay. Naïve mice treated with vehicle were utilized as controls (n = 4 samples for group).

Drugs. While SFN was purchased from Merck Chemicals and Life Science S.A.U. (Madrid, Spain), morphine hydrochloride was acquired in Alcaiber S.A. (Madrid, Spain). SFN was dissolved in DMSO (1% in saline solution 0.9%) and administered at a 5 and 10 mg/kg via intraperitoneal, in a final volume of 10 ml/kg, at 3-4 h earlier behavioral testing. Morphine hydrochloride was dissolved in saline solution (0.9 %) and subplantarily injected at 50 µg into the right paw, in a final volume of 30 µl, at 30 min earlier behavioral testing. All drugs were prepared daily before its administration. For each group treated with a drug, the respective control group received the same volume of the corresponding vehicle.

Statistical analysis. Data are expressed as mean ± SEM. The statistical analysis was accomplished by utilizing the SPSS program (version 17 for Windows, IBM España, Madrid, Spain). In each behavioral test assessed, the effects of the administration of 5 and 10 mg/kg of SFN versus those produced by their corresponding vehicle in the contra and ipsilateral paw of CFA-injected mice were compared through a three-way ANOVA repeated measures (paw, treatment and time as between factors of variation) followed by the appropriate one-way ANOVA and the Student Newman–Keuls test whenever suitable.

In each behavioral test, the effects produced by SFN administered alone and in combination with morphine were compared through a one way ANOVA and the Student Newman Keuls test. In these studies, antinociception in the von Frey filaments and plantar tests is expressed as the percentage of maximal possible effect, where the test latencies pre (baseline) and postdrug administration are compared and calculated in accordance to the next equation:

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

Finally, changes in protein levels of Nrf2, HO-1, NQO1, NOS2, CD11b/c, MOR and MAPK in spinal cord and paw tissues from CFA-injected mice treated with SFN or vehicle and in naïve receiving vehicle were examined with a one-way ANOVA and the corresponding Student-Newman-Keuls test. $P < 0.05$ was considered statistically significant.

Results

Effects of repeated treatment with SFN on the mechanical allodynia and thermal hyperalgesia induced by CFA. Animals were intraperitoneally administered with 5 and 10 mg/kg of SFN or vehicle during 11 consecutive days, starting the treatment at 4 days after CFA injection. At 1, 4, 7 and 11 days of SFN or vehicle treatment, mechanical allodynia and thermal hyperalgesia were sequentially assessed.

For mechanical allodynia, the subplantar administration of CFA significantly decreased the threshold for evoking hind paw withdrawal to a mechanical stimulus. This effect was clearly observed after 3 days of CFA injection, and it is maintained until day 14 in the ipsilateral paw of vehicle treated animals when compared to its respective contralateral paw ($P < 0.001$; one way ANOVA; Fig. 1A). The three way ANOVA repeated measure showed a significant effect of the paw, treatment and time ($P < 0.001$) and the interactions between paw and treatment ($P < 0.001$), treatment and time ($P < 0.001$), paw and time ($P < 0.001$) and between paw, treatment and time ($P < 0.001$). Consequently, mechanical allodynia was significantly attenuated in mice daily treated with 5 and 10 mg/kg of SFN during 11 days ($P < 0.001$, one-way ANOVA, compared to the ipsilateral paw of CFA-injected mice treated with vehicle; Fig. 1A). But while mechanical allodynia was equally reduced with the administration of 5 and 10 mg/kg of SFN on day 1 of treatment, the antiallodynic effects produced by 10 mg/kg of SFN after 7 days of treatment were higher than those obtained by the small dose ($P < 0.001$, one-way ANOVA). Moreover, although the antiallodynic effects produced by both doses increased progressively from days 1 to 7 of treatment ($P < 0.001$, one-way ANOVA, vs to the ipsilateral paw of CFA-injected mice treated with vehicle), while mechanical allodynia was completely reversed at 7 days of treatment (10 days after CFA injection) with 10 mg/kg of SFN, 11 days of treatment with 5 mg/kg are needed to obtain this effect.

Peripheral inflammation also produced a significant decrease of the threshold for evoking ipsilateral paw withdrawal to a thermal stimulus in vehicle treated mice from days 3 to 14 after CFA injection as compared to their corresponding contralateral paws ($P < 0.001$, one-way

ANOVA; Fig 1B). The effects of the intraperitoneal administration of SFN at 5 and 10 mg/kg have been also evaluated. The three-way ANOVA repeated measures showed a significant effect of the paw, treatment and time ($P < 0.001$) as well as the interaction between paw and treatment ($P < 0.001$), treatment and time ($P < 0.001$), paw and time ($p < 0.001$) and among paw, treatment and time ($P < 0.001$) (Fig 1B). Our results showed that thermal hyperalgesia caused by CFA was progressively attenuated after the daily treatment with SFN (5 and 10 mg/kg), but similar to that occurs with the mechanical allodynia, the complete reversion of thermal hyperalgesia was achieved at 7 and 11 days of treatment with 10 or 5 mg/kg of SFN, respectively ($P < 0.001$, one-way ANOVA, in comparison to the ipsilateral paw of mice with peripheral inflammation treated with vehicle).

Effects of acute treatment with SFN on the antinociceptive effects produced by the subplantar administration of morphine in animals with peripheral inflammation. The effects of the acute intraperitoneal administration of 10 mg/kg of SFN alone and in combination with 50 μ g of morphine on the mechanical allodynia and thermal hyperalgesia caused by peripheral inflammation were evaluated.

Data demonstrated that whereas the intraperitoneal administration of SFN or morphine administered alone did not produce significant antiallodynic (Fig. 2A) and antihyperalgesic (Fig. 2B) effects as compared to those produced by vehicle. SFN treatment significantly improved the mechanical antiallodynic (Fig. 2A) and thermal antihyperalgesic effects (Fig. 2B) produced by morphine locally administered in the ipsilateral paw of CFA-injected mice ($P < 0.001$, one-way ANOVA, versus vehicle group treated with saline or morphine, or with SFN administered alone). The local administration of morphine alone or combined with SFN did not have any effect on the contralateral paw of CFA-injected animals, neither for mechanical allodynia (Fig. 2C) nor for thermal hyperalgesia (Fig. 2D).

Effects of repeated treatment with SFN on the Nrf2, HO-1, NQO1, NOS2, MOR and CD11b/c expression in the spinal cord and paw from animals with peripheral

inflammation. The Nrf2 levels and the expression of the antioxidant (HO-1 and NQO1) and inflammatory (NOS2) enzymes as well as of MOR and/or of microglial marker (Cd11b/c) in the spinal cord (Fig. 3) and paw (Fig. 4) from CFA-injected mice treated during 11 days with 10 mg/kg of SFN or vehicle, and from naïve mice receiving vehicle were evaluated. Indeed, while the diminished expression of Nrf2 in the spinal cord from CFA-injected mice ($P < 0.01$, one-way ANOVA when compared to naïve mice treated with vehicle) was normalized by treatment with SFN ($P < 0.01$, one-way ANOVA versus CFA-injected mice treated with vehicle) (Fig. 3A). A significant increased expression of Nrf2 induced by SFN was demonstrated in the paw from mice with peripheral inflammation ($P < 0.01$, one-way ANOVA versus naïve and CFA-injected mice treated with vehicle) (Fig. 4A). Regarding HO-1, while peripheral inflammation increased the spinal cord levels of this enzyme ($P < 0.001$, one-way ANOVA versus naïve vehicle treated mice) (Fig. 3B), non-statistically significant alterations in its expression in the paw from CFA-injected mice was detected (Fig. 4B). Nevertheless, SFN treatment increased HO-1 protein levels in both tissues ($P < 0.001$, one-way ANOVA versus naïve and CFA-injected mice treated with vehicle). Our findings also showed that SFN treatment enhanced the expression of NQO1 enzyme in the spinal cord (Fig. 3C) and paw (Fig. 4C) as compared to naïve and CFA-injected mice treated with vehicle ($P < 0.01$, one-way ANOVA). Moreover, while peripheral inflammation did not modify the spinal cord levels of NOS2 (Fig 3E), a significant overexpression of this enzyme was observed in the paw ($P < 0.004$, one-way ANOVA versus naïve treated with vehicle) (Fig 4E), which was significantly reversed by SFN administration. In addition, whereas the unaltered MOR levels observed in the spinal cord (Fig. 3F) and paw (Fig. 4F) from CFA injected mice remain intact with SFN treatment in the spinal cord, a significant augmented expression of MOR was revealed in the paw tissues from SFN treated animals ($P < 0.028$ one-way ANOVA versus naïve and CFA-injected mice treated with vehicle). Finally, the spinal microglial activation caused by peripheral inflammation ($P < 0.002$ one-way ANOVA versus naïve mice treated with vehicle) was totally reversed with SFN treatment ($P < 0.002$ one-way ANOVA versus CFA-injected mice treated with vehicle) (Fig. 3G).

Effects of repeated treatment with SFN on the expression of JNK, ERK1/2 and p38 in the spinal cord and paw from animals with peripheral inflammation. Results revealed that the increased levels of phosphorylated JNK in the spinal cord (Fig. 5A) and paw (Fig. 6A) from CFA-injected mice ($P < 0.05$ one-way ANOVA, versus naïve mice treated with vehicle) were significantly diminished by SFN treatment in both tissues ($P < 0.05$ one-way ANOVA versus CFA-injected mice treated with vehicle). Regarding ERK1/2, while non-changes in its expression was observed in the spinal cord (Fig. 5B), treatment with SFN prevented its activation in the paw from CFA-injected mice ($P < 0.005$ one-way ANOVA, versus CFA-injected mice treated with vehicle) (Fig. 6B). Lastly, the spinal cord activation of p38 induced by inflammation (Fig. 5C) was as well significantly reduced in SFN treated mice ($P < 0.028$ one-way ANOVA versus CFA-injected mice treated with vehicle), while non-significant changes in the expression of pp38 were observed in the paw from CFA-injected mice (Fig. 6C).

Discussion

This study revealed that the repeated intraperitoneal administration of SFN, an activator of the transcription factor Nrf2, completely reversed the mechanical allodynia and thermal hyperalgesia caused by chronic peripheral inflammation in mice. This treatment activated the Nrf2/HO-1/NQO1 signaling, inhibited NOS2 over-expression and microglial activation as well as MAPK phosphorylation in the spinal cord and/or paw tissues from CFA-injected animals. Our data also showed that SFN treatment improved the antiallodynic and antihyperalgesic effects produced by the subplantar injection of morphine by enhancing the local expression of MOR in animals with peripheral inflammation.

Several studies demonstrated that the transcription factor Nrf2, furthermore to its antioxidant and neuroprotective effects, also plays an important role on the modulation of inflammation (Kobayashi et al., 2016). Therefore, loss or disruption of Nrf2 signaling causes an increased susceptibility to oxidative stress as well as to inflammatory tissue injuries (Kim et al., 2010). Other works also shown the antinociceptive effects elicited by substances capable to activate the transcription factor Nrf2 during acute inflammatory pain (Rosa et al., 2008) and in neuropathic pain associated to type 1 and 2 diabetes (Negi et al., 2011; McDonnell et al., 2017). In this work we demonstrated that treatment with SFN (a Nrf2 activator) also decreased the mechanical and thermal hypersensitivity induced by chronic peripheral inflammation, in a dose-dependent manner. That is, whereas 7 days of SFN treatment at 10 mg/kg are needed to inhibit inflammatory pain, 11 days of the continuous administration of this drug at 5 mg/kg are required. These data supported the preceding reports and furthermore demonstrated the antiallodynic and antihyperalgesic actions of SFN in chronic inflammatory pain in mice.

Our findings also proved that the administration of SFN avoided the reduced levels of Nrf2 in the spinal cord and enhanced its expression in the paw tissues from animals with inflammatory pain. This treatment also enhanced the down-stream enzymes activated by Nrf2, such as HO-1 and NQO1 in both tissues suggesting that the antiallodynic and antihyperalgesic effects produced by SFN during inflammatory pain might be in part produced by its antioxidant activity mediated by the induction of these antioxidant enzymes. Supporting our results, the

antinociceptive role played by the activation of HO-1 in inflammatory and neuropathic pain conditions have been also described (Hervera et al., 2012, 2013b; Carcolé et al., 2014). SFN treatment reduced the increased expression of NOS2 in the paw from CFA-injected mice. These data agree with the diminished NOS2 expression identified in the sciatic nerve from animals with diabetic neuropathy or nerve-injury induced neuropathic pain treated with SFN (Negi et al., 2011; Wang and Wang, 2017), as well as with HO-1 inducers (Castany et al., 2016) and further demonstrated the negative regulatory role played by Nrf2 on the expression of NOS2 under inflammatory pain conditions.

It is also well known the involvement of microglia in the development of chronic pain. Using the expression of CD11b/c, as a microglial marker, our data reveals significant microglial activation in the spinal cord of animals with peripheral inflammation, which was reversed by SFN treatment. Therefore and considering the antinociceptive effects produced by specific microglial inhibitors in animals with chronic pain (Mika et al., 2009; 2013), the alleviation of inflammatory pain induced by SFN might have also in part due by inhibiting spinal microglial activation.

In order to evaluate if the inhibition of MAPK activated by inflammatory pain is also involved in the effects produced by SFN, we investigated the expression of phosphorylated JNK, ERK1/2 and p38 in the spinal cord and paw tissues from mice treated with SFN. It is well known that the activation of JNK executes an important role in the development and maintenance of chronic pain (Middlemas et al., 2006; Gao and Ji, 2008). Our findings confirmed these results with the increased levels of phosphorylated JNK in the spinal cord as well as in the paw from mice with chronic inflammatory pain and furthermore revealed that treatment with SFN completely reduced the JNK activation in both tissues. Agreeing to our results, Gao et al. (2010) also demonstrated an increased expression of JNK phosphorylated in the spinal cord from CFA-injected rats at 14 days after induction. Moreover, because treatments with specific JNK inhibitors reduced the mechanical allodynia caused by inflammation (Gao et al., 2010), the decreased activation of JNK performed by SFN might also contribute to explain the antinociceptive effects produced by this Nrf2 inductor under inflammatory pain conditions.

ERK1/2 is another member of MAPK which phosphorylated form also increased in response to several stimulus (inflammation, nerve injury, diabetes) at different levels of pain processing pathway (Borges et al., 2015). Our data demonstrated that the augmented expression of pERK1/2 in the paw from mice with chronic inflammatory pain was significantly reduced by SFN treatment. In accordance to us, a significant activation of pERK1/2 was also identified in the dorsal root ganglia from animal with acute and chronic peripheral inflammation (Cheng et al., 2008; Cheng and Keast, 2009; Gao and Ji, 2010). Since the administration of specific ERK1/2 inhibitors diminished the nociceptive response induced by inflammatory pain, the fact that SFN inhibited ERK phosphorylation in the paw also supports the hypothesis that the antinociceptive effects of this Nrf2 inductor during chronic inflammatory pain might also be mediated via inhibition pERK1/2 in the paw. Finally, p38 activation is also responsible for the peripheral inflammation induced hyperalgesia (Ji et al., 2002; Hudmon, et al., 2008) and of the up-regulation of numerous inflammatory mediators in the spinal cord (Svensson et al., 2003). Accordingly, a significant increased levels of pp38 was detected in the spinal cord from CFA-injected mice, that was completely blocked with the SFN treatment revealing that the effectivity of this treatment against chronic inflammatory pain also include the modulation of p38 phosphorylation in the spinal cord of these animals. Finally, although we only assessed the effects of the systemic administration of SFN on the spinal cord and paw from animals with peripheral inflammation, it's probable that this treatment also produces relevant effects in other areas, such as the dorsal root ganglia, as occurs with other related treatments, for example with the administration of HO-1 activators in mice with chronic pain (Hervera et al., 2012; Carcolé et al., 2014; Castany et al., 2016).

Our study demonstrated that the small antinociceptive effects produced by the local administration of a low dose of morphine during peripheral inflammation were significantly improved by SFN treatment. These results expanded our previous data indicating that the activation of HO-1 with CoPP potentiated the peripheral antinociceptive actions of morphine in animals with inflammatory and neuropathic pain caused by nerve-injury or associated to diabetes (Hervera et al., 2013a; Carcolé et al., 2014; Castany et al., 2016) and further revealed

that the activation of Nrf2 might also potentiates the analgesic effects of morphine under inflammatory pain conditions. Moreover, the enhancement of MOR expression induced by SFN in the paw might explain the improvement of the local antiallodynic and antihyperalgesic effects of morphine elicited by SFN in animals with inflammatory pain. Due to that SFN induced the synthesis of HO-1, which also up-regulated the expression of MOR in the dorsal root ganglia from CFA-injected mice (Carcolé et al., 2014), we hypothesized that the activation of Nrf2/HO-1 signaling pathway might be the main responsible for the enhanced expression of MOR observed in the paw from SFN treated animals. Nonetheless, the antioxidant and antiinflammatory effects triggered by SFN administration might also contribute in the improvement of the local antinociceptive effects of morphine produced by this treatment.

In summary, this study indicated that the induction of Nrf2 might inhibit inflammatory pain and enhance the analgesic effects of morphine by inhibiting oxidative stress and inflammatory responses caused by peripheral inflammation. This study suggests the administration of SFN alone and in combination with morphine as new ways of treating chronic inflammatory pain.

Authorship Contributions

Participated in research design: Pol

Conducted experiments: Redondo, Ferreira, Riego and Leánez

Performed data analysis: Redondo and Pol

Wrote the manuscript: Pol

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Footnotes:

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Figure Legends

Fig. 1. Effects of repetitive treatment with SFN on the mechanical allodynia and thermal hyperalgesia induced by CFA. The development of the mechanical allodynia (A) and thermal hyperalgesia (B) in the ipsilateral paw (continuous lines) and contralateral paw (discontinuous lines) from mice treated during 11 consecutive days with vehicle or SFN at 5 mg/kg or 10 mg/kg are shown. Results of the evaluations at 3, 4, 7, 10 and 14 days after CFA injections are also shown. For each day and treatment evaluated, * indicates significant differences versus their respective contralateral paw, + indicates significant differences versus ipsilateral paws of animals treated with SFN at 5 mg/kg and # indicates significant differences versus ipsilateral paws of animals treated with SFN at 10 mg/kg ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test). Results are represented as mean values \pm SEM; n=6 animals per experimental group.

Fig. 2. Effects of acute treatment with SFN on the antiallodynic and antihyperalgesic responses to morphine. Effects of the acute administration of 10 mg/kg of SFN or vehicle, alone and in combination with 50 μ g of morphine, on the inhibition of the mechanical allodynia (A) and thermal hyperalgesia (B) induced by CFA in the ipsilateral paw are shown. The effects of the administration of SFN and vehicle alone and in combination with morphine on the contralateral paw for mechanical allodynia (C) and thermal hyperalgesia (D) are also shown. In all panels,* indicates significant differences versus vehicle plus saline treated mice, + indicates significant differences versus vehicle plus morphine treated mice and # indicates significant differences versus SFN plus saline treated mice ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test). Data are expressed as mean values of the maximal possible effect (%) \pm SEM; n=6 animals per experimental group.

Fig. 3. Effects of treatment with SFN on the expression of Nrf2, HO-1, NQO1, NOS2, MOR and CD11b/c in the spinal cord from animals with peripheral inflammation. The protein levels of Nrf2 (A), HO-1 (B), NQO1 (C), NOS2 (E), MOR (F) and CD11b/c (G) in the

ipsilateral site of the spinal cord from CFA-injected mice treated with vehicle (CFA-vehicle) or SFN (CFA-SFN) are represented. The expression of these proteins in the spinal cord from naïve mice treated with vehicle (naïve-vehicle) is also represented as controls. For each protein, * indicates significant differences when compared versus naïve vehicle treated mice, + indicates significant differences when compared to CFA-injected mice treated with vehicle and # indicates significant differences when compared to CFA-injected mice treated with SFN ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test). Representative examples of Western blots for Nrf2, HO-1 and NQO1 proteins (D) and for NOS2, MOR and CD11b/c (H) in which GAPDH was used as a loading control, are also shown. Data are expressed as mean values \pm SEM; n = 4 samples per group.

Fig. 4. Effects of treatment with SFN on the expression of Nrf2, HO-1, NQO1, NOS2 and MOR in the paw from animals with peripheral inflammation. The protein levels of Nrf2 (A), HO-1 (B), NQO1 (C), NOS2 (E) and MOR (F) in the ipsilateral paw from CFA-injected mice treated with vehicle (CFA-vehicle) or SFN (CFA-SFN) are represented. The expression of these proteins in the paw from naïve mice treated with vehicle (naïve-vehicle) is also represented as controls. For each protein, * indicates significant differences when compared versus naïve vehicle treated mice, + indicates significant differences when compared to CFA-injected mice treated with vehicle and # indicates significant differences when compared to CFA-injected mice treated with SFN ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test). Representative examples of Western blots for Nrf2, HO-1 and NQO1 proteins (D) and for NOS2 and MOR (G) in which GAPDH was used as a loading control, are also shown. Data are expressed as mean values \pm SEM; n = 4 samples per group.

Fig. 5. Effects of treatment with SFN on the expression of pJNK, pERK1/2 and pp38 in the spinal cord from animals with peripheral inflammation. The protein levels of pJNK/total JNK (A), pERK1/2/total ERK1/2(B), pp38/total p38 (C) in the ipsilateral site of the spinal cord from CFA-injected mice treated with vehicle (CFA-vehicle) or SFN (CFA-SFN) are

represented. The expression of these proteins in the spinal cord from naïve mice treated with vehicle (naïve-vehicle) is also represented as controls. For each protein, * indicates significant differences when compared versus naïve vehicle treated mice and # indicates significant differences when compared to CFA-injected mice treated with SFN ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test). Representative examples of Western blots for pJNK/total JNK (A), pERK1/2/total ERK1/2 (B) and pp38/total p38 (C) proteins are also shown. Data are expressed as mean values \pm SEM; n = 4 samples per group.

Fig. 6. Effects of treatment with SFN on the expression of pJNK, pERK1/2 and pp38 in the paw from animals with peripheral inflammation. The protein levels of pJNK/total JNK (A), pERK1/2/total ERK1/2 (B), pp38/total p38 (C) in the ipsilateral paw from CFA-injected mice treated with vehicle (CFA-vehicle) or SFN (CFA-SFN) are represented. The expression of these proteins in the paw from naïve mice treated with vehicle (naïve-vehicle) is also represented as controls. For each protein, * indicates significant differences when compared versus naïve vehicle treated mice and # indicates significant differences when compared to CFA-injected mice treated with SFN ($P < 0.05$, one-way ANOVA followed by Student-Newman-Keuls test). Representative examples of Western blots for pJNK/total JNK (A), pERK1/2/total ERK1/2 (B) and pp38/total p38 (C) proteins are also shown. Data are expressed as mean values \pm SEM; n = 4 samples per group.

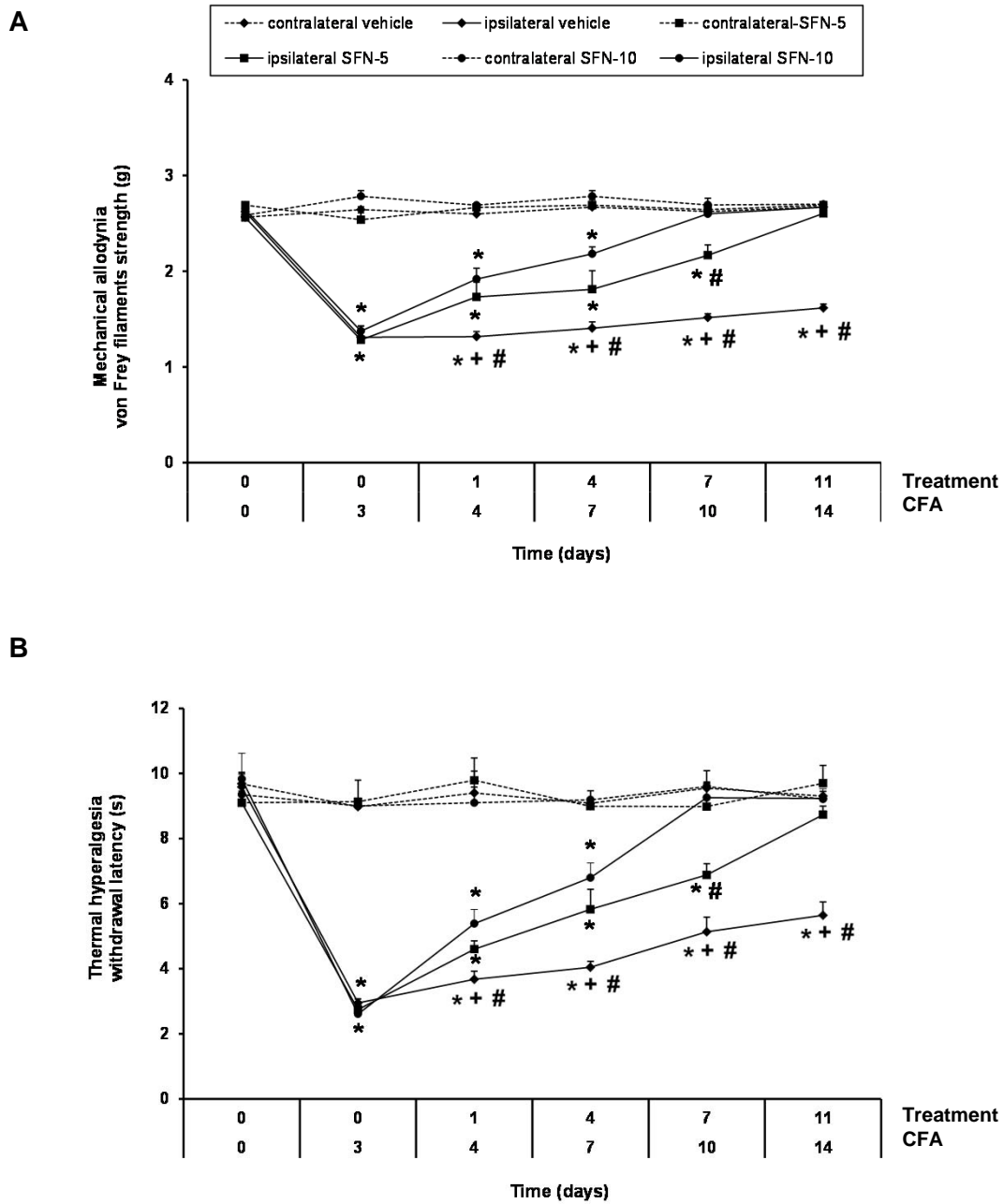


Figure 1

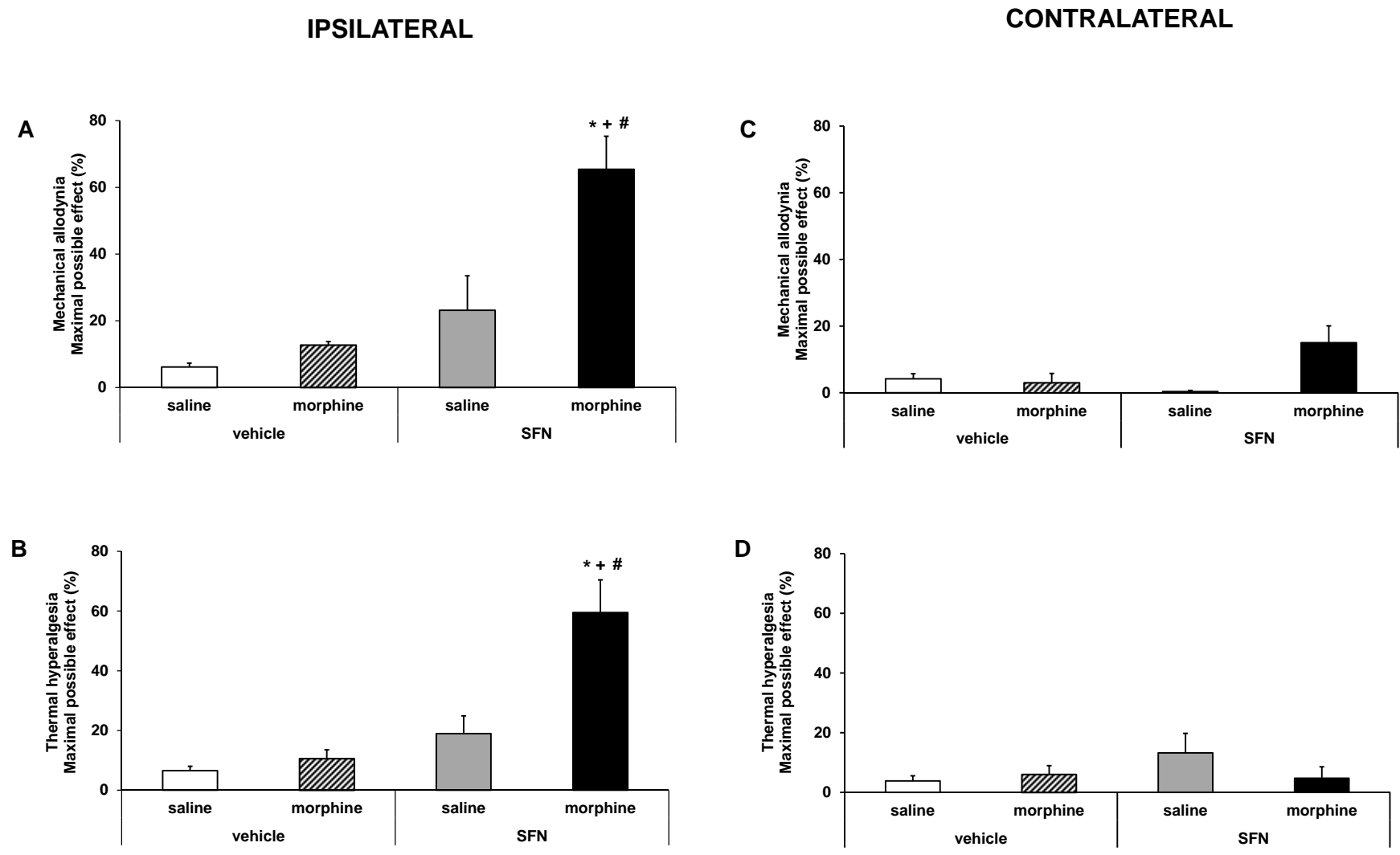


Figure 2

SPINAL CORD

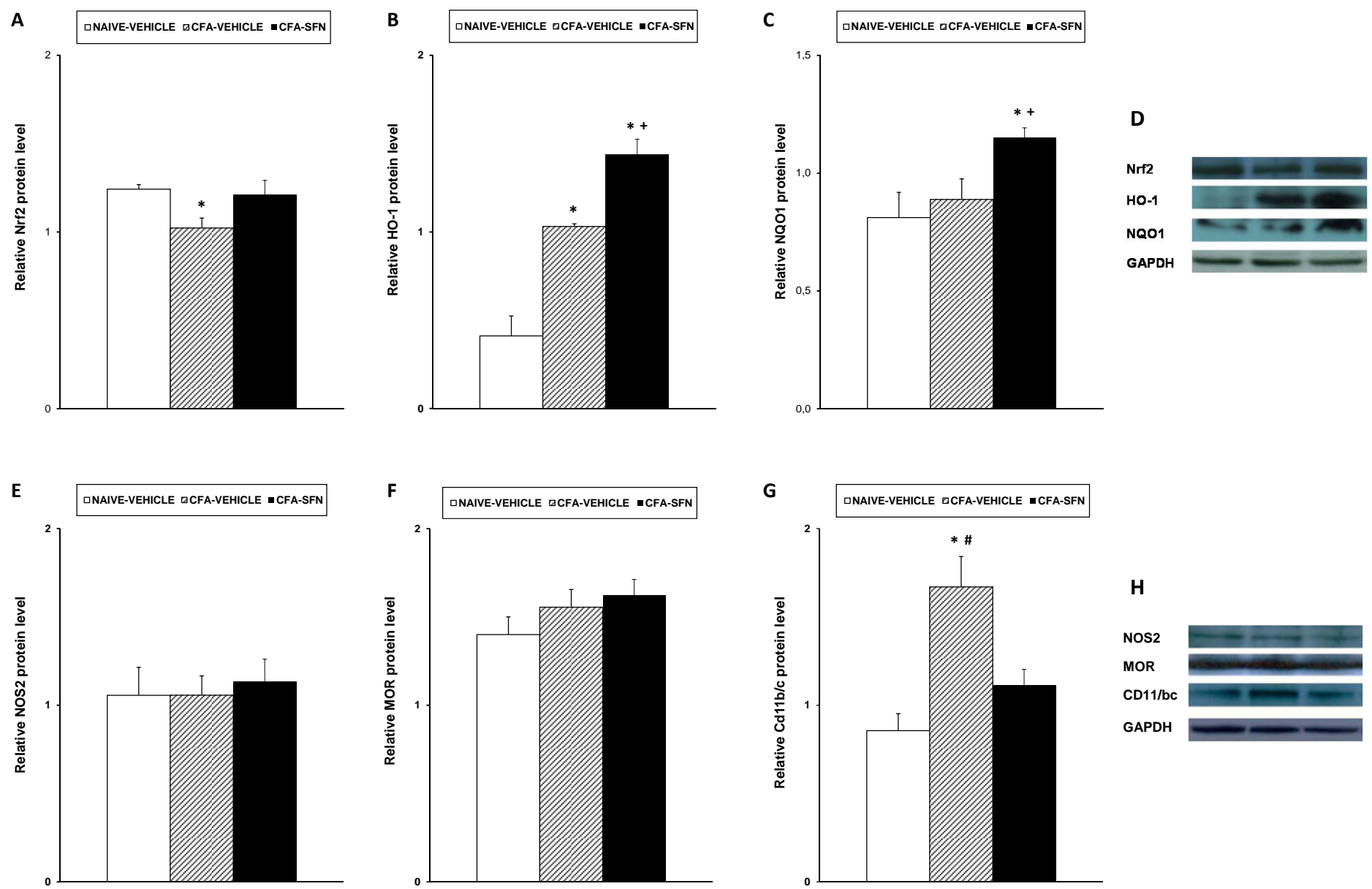


Figure 3

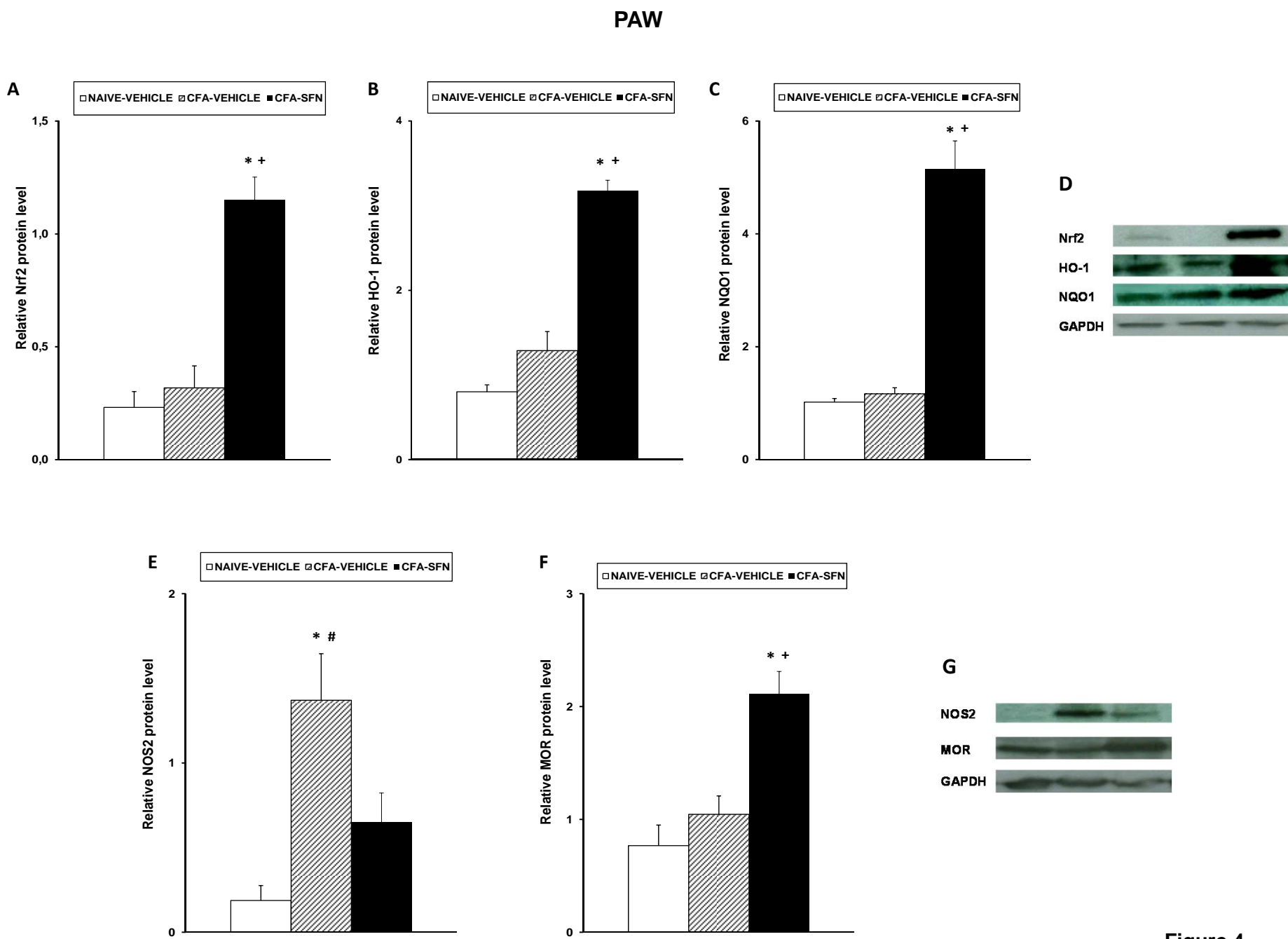
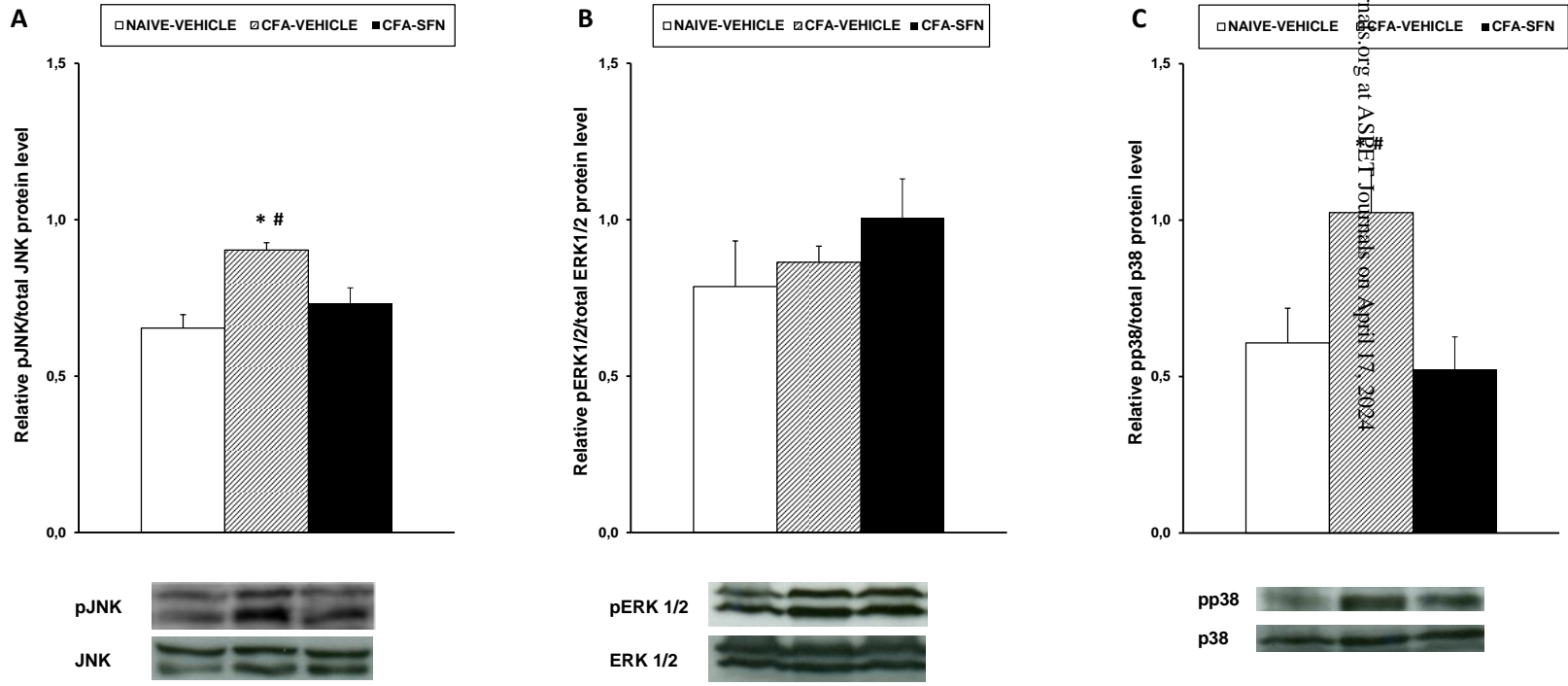


Figure 4

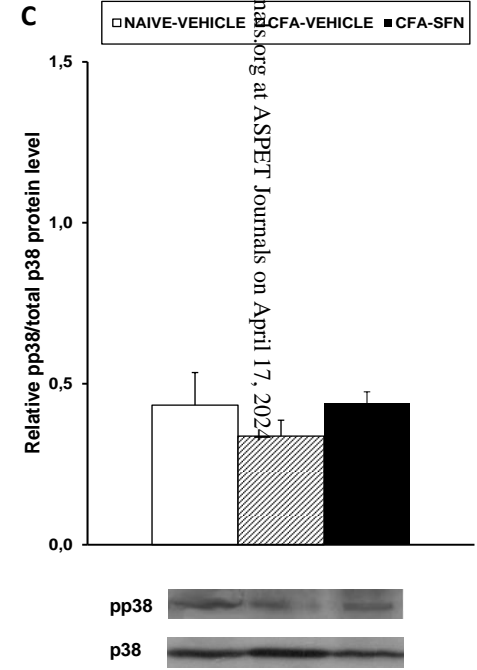
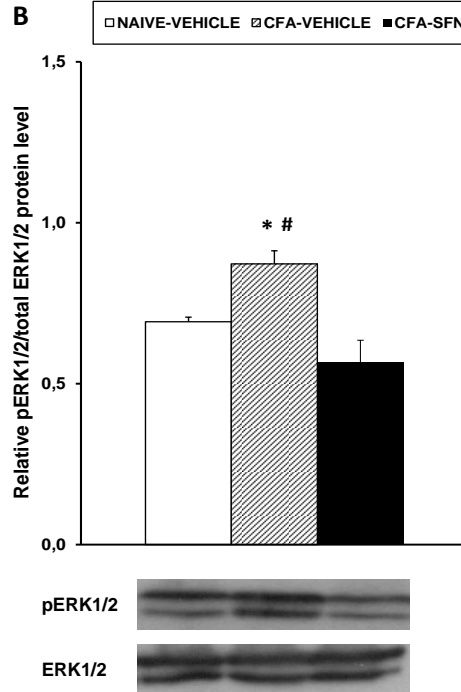
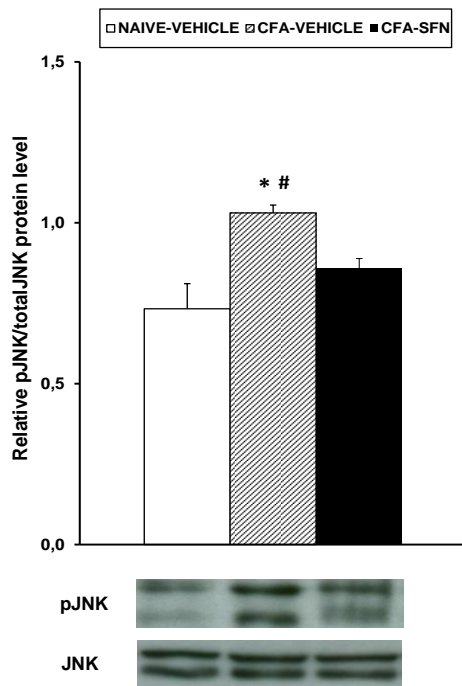
SPINAL CORD



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Figure 5

PAW



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Figure 6