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Nrf2 participates in depressive disorders through an anti-inflammatory mechanism

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Summary A causative relationship between inflammation and depression is gradually gaining consistency. Because Nrf2 participates in inflammation, we hypothesized that Nrf2 could play a role in depressive disorders. In this study, we have observed that Nrf2 deletion in mice results in: (i) a depressive-like behavior evaluated as an increase in the immobility time in the tail-suspension test and by a decrease in the grooming time in the splash test, (ii) reduced levels of dopamine and serotonin and increased levels of glutamate in the prefrontal cortex, (iii) altered levels of proteins associated to depression such as VEGF and synaptophysin and (iv) microgliosis. Furthermore, treatment of Nrf2 knockout mice with the anti-inflammatory drug rofecoxib reversed their depressive-like behavior, while induction of Nrf2 by sulforaphane, in an inflammatory model of depression elicited by LPS, afforded antidepressant-like effects. In conclusion, our results indicate that chronic inflammation due to a deletion of Nrf2 can lead to a depressive-like phenotype while induction of Nrf2 could become a new and interesting target to develop novel antidepressive drugs.

1. Introduction

A relationship between depression and immunity has been described for almost 25 years (Irwin and Miller, 2007). In 1991 Smith proposed the macrophage theory of depression

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(Smith, 1991) which postulates that excessive secretion of activated macrophage monokines is implicated in the pathophysiology of depression. Almost at the same time, the group of Maes described a relationship between inflammation and depression (Maes et al., 1992, 1993). Supporting these observations, (i) immune activation with lipopolysaccharide (LPS) is known to produce a set of behavioral and cognitive alterations (anhedonia, anorexia, memory deficits, among others) that resemble depression both in animals and in humans (Yirmiya et al., 2000; Reichenberg et al., 2010); (ii) depression is commonly accompanied by an inflammatory response expressed as an increase in serum levels of TNF-α, IFN-γ, IL-6, IL-1β and C-reactive protein (Maes et al., 1997; Mikova et al., 2001; Howren et al., 2009). These cytokines are known to cause behavioral changes (Asnis and De La Garza, 2006; Kaster et al., 2012), affect neurotransmitter metabolism (Guillemin et al., 2001; Sakash et al., 2002; Barrientos et al., 2004) and decrease neuroplasticity (Barrientos et al., 2004; Ben Menachem-Zidon et al., 2008; Goshen et al., 2008; Koo and Duman, 2008). Of note, patients who suffer from refractory depression (30—40%) show higher levels of acute phase response markers (IL-6, reactive C protein, *etc.*) (Sluzewska et al., 1995; Maes et al., 1997), therefore inflammation could become a new therapeutic target for this particular subset of patients.

Nuclear factor (erythroid 2-derived)-like 2 (Nrf2) is a transcription factor that plays a central role in cellular defense against oxidative and electrophilic insults. Nrf2 binds to antioxidant response elements (ARE) located in the promoter region of genes encoding many phase II detoxifying or antioxidant enzymes and related stress-responsive proteins. It has been recently demonstrated that Nrf2-ARE signaling is also involved in attenuating inflammation-associated pathogenesis, such as autoimmune diseases, rheumatoid arthritis, asthma, emphysema, gastritis, colitis and atherosclerosis (Lee and Johnson, 2004). Thus, disruption or loss of Nrf2 signaling causes enhanced susceptibility not only to oxidative and electrophilic stresses but also to inflammatory injuries. Furthermore, a relationship between Nrf2 and protective effects have also been described in the central nervous system (CNS) (Innamorato et al., 2008).

Since Nrf2 has been recently described to play a crucial role in regulating inflammation, and inflammation has been related to depression, this study was designed to determine if Nrf2 could participate in the pathogenesis of depression through an inflammatory mechanism and/or whether it could be considered a pharmacological target against depression. Our results show for the first time that Nrf2 deficient mice exhibit depressive-like behavior and that this phenotype is reversed by an anti-inflammatory drug such as rofecoxib. Furthermore, Nrf2 knockout (KO) mice showed significant changes in serotonin, glutamate and dopamine levels in the prefrontal cortex, compared to Nrf2 (+/+) mice. We have also demonstrated that the Nrf2 activator sulforaphane can reverse the depressive phenotype in an inflammatory model of depression elicited by lipopolysaccharide (LPS) administration. Taken together, our results support the hypothesis that Nrf2 deletion can lead to depressive-like behavior and that activation of Nrf2 could become a new therapeutical target against depression.

2. Methods and materials

2.1. Animals

Male wild-type C57BL/6 mice (Nrf2 (+/+)) and Nrf2 KO (Nrf2 −/−)) and male Swiss mice (3—4 months) were housed at room temperature under a 12 h light—dark cycle. Food and water were provided *ad libitum*. All experimental procedures with animals were approved by the Ethical Committee of the *Hospital La Paz Health Research Institute (IdiPAZ)*, for the care and use of animals in research, in accordance with the European Community Council Directive of November 24 1986 (86/609/EEC) and with the Spanish Real Decreto (RD-1201/2005).

Nrf2 KO mice and their wild-type littermates were obtained thanks to the courtesy of Dr. Masayuki Yamamoto (Tohoku University, Graduate School of Medicine, Sendai, Japan) (Itoh et al., 1997).

2.2. Drugs and treatments

The non-steroidal anti-inflammatory drug (NSAID), rofecoxib (Toronto Research Chemicals, Canada), was dissolved in saline and administered by the intraperitoneal (IP) route; it was administered once daily during 7 days at a dose of 2 mg/kg based on previous studies (Jain et al., 2001; Singal et al., 2004). Twenty-four hours after the last administration, the tail-suspension test (TST), the splash test (ST) or the open-field test (OFT) were carried out. After this, animals were sacrificed under deep anesthesia with isoflourane.

R,S-sulforaphane (SFN) was from LKT Laboratories (Minnesota, USA) and LPS (Serotype O26:B6) from Sigma (St. Louis, USA); they were prepared in saline solution. Mice received SFN (1 mg/kg, IP) for 7 consecutive days and the day after, LPS (0.1 mg/kg, IP) was injected. Once the experimental schedule was completed, animals were deeply anesthetized with isofluorane and sacrificed.

2.3. Behavioral tests

The behavioral tests were performed by blind experimented researchers under faint light conditions and were scored manually. The open field test was performed after the tail suspension test or after the splash test (4—5 min interval).

2.4. Tail-suspension test (TST)

The total duration of immobility induced by tail-suspension was measured according to the method described elsewhere (Steru et al., 1985). Briefly, mice, both acoustically and visually isolated, were suspended 50 cm above the floor by an adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6 min period. Mice were considered immobile only when they hung passively and completely motionless.

2.5. Splash test (ST)

The ST consisted in squirting a 1% sucrose solution on the dorsal coat of a mouse placed in a 14 cm glass cylinder. The

solution dirties and sweetens the mouse fur and animals initiate grooming behavior. The time spent grooming was recorded for a period of 6 min as an index of self-care, motivational behavior and anhedonia.

2.6. Open-field test (OFT)

The ambulatory behavior was assessed with the OFT as previously described (Kaster et al., 2007). Briefly, the apparatus consisted of a plastic box measuring 60 cm × 60 cm × 60 cm with the floor of the arena divided into 9 equal squares. The number of squares crossed with all paws (crossings) and the number of times the front paws were raised (rearings) were counted in a 6-min session.

2.7. Determination of glutamate

Glutamate concentrations in prefrontal cortex and hippocampus from C57BL6 wild type animals and their Nrf2 (−/−) littermates were determined by high performance liquid chromatography with electrochemical detection (HPLC-EC) (DECADE, Antec Leyden, The Netherlands). Briefly, tissues were dissected and washed with cold phosphate-buffered saline, homogenized by sonication in an extraction buffer (0.1 M sodium tetraborate, pH 9.1) and, finally, centrifuged at 12,000 rpm at 4 °C for 20 min. Supernatants were injected using an automatic sample injector (Waters 717 plus). A high-sensitivity analytic flow cell (VT-03) was used and the working electrode was set at 0.7 V. A column (biophase ODS 5 μm, 4.6 mm × 150 mm) including pre-column derivatization with ophthaldehyde and β-mercapthoethanol (Sigma–Aldrich, Seelze, Germany) was used.

2.8. Determination of dopamine (DA) and serotonin (5-HT)

Concentrations of serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), homovanillic acid (HVA) and 3,4-dihydroxyphenilacetic acid (DOPAC) were determined by HPLC-EC as previously described (Goni-Allo et al., 2006) in the prefrontal cortex and the hippocampus of Nrf2 (+/+) and Nrf2 (−/−) mice. Briefly, tissues were dissected and washed with cold phosphate-buffered saline, homogenized by sonication in an extraction buffer (0.4 N perchloric acid, 1 mM EDTA and 0.1% sodium metabisulfite) and, finally, centrifuged at 12,000 rpm 4 °C for 20 min. Supernatants were injected using an automatic sample injector (Waters 717 plus) onto a Spherisorb ODS-2 reverse phase C18 column (5 μm, 150 × 4.6 mm; Teknokroma, San Cugat del Valles, Spain) connected to a DECADE amperometric detector (Antec Leyden, Zoeterwoude, The Netherlands), with a glassy carbon electrode maintained at 0.7 V with respect to a Ag/AgCl reference electrode.

2.9. Immunoblotting

Hippocampi and prefrontal cortices were washed once with cold phosphate-buffered saline and lysed in 100 μl ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate and 1 mM Na_3VO_4). Protein (30 μg) from these cell lysates were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes (Amersham). Membranes were incubated with anti-β-actin at 1:100,000, anti-VEGF (vascular endothelial growth factor) at 1:1000 (Sigma, Madrid, Spain), anti-BDNF (brain-derived neurotrophic factor) at 1:1000, anti-HO-1 at 1:1000 (Chemicon, MA, USA), anti-iNOS at 1:1000 or anti-synaptophysin at 1:1000 (Milipore, Madrid, Spain). Appropriate peroxidase-conjugated secondary antibodies (1:10,000) were used to detect proteins by enhanced chemiluminescence. Optical density was quantified using the program Scion Image® Alpha 4.0.3.2. Control conditions were taken as 100% and experimental variables were normalized with respect to this value.

2.10. Immunofluorescence of GFAP and Iba1

Mice were anesthetized with isoflurane and transcardially perfused: first with a saline solution, followed by a fixative solution (freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Then, the brains were extracted and immersed in the fixative solution at 4 °C, embedded in paraffin and cut into 10-μm sections on a rotary microtome (RM2255; Leica, Wetzlar, Germany). After deparaffination and rehydration, sections were immersed in 0.01 M citrate buffer, at pH 6.0, and boiled for 20 min. Sections were then blocked with 10% goat serum and incubated overnight with the primary antibody mouse anti-GFAP (1:500; Chemicon International, Temecula, CA) or anti-Iba1 (1:200; Wako, Richmond, VA). Sections were then incubated with fluorochrome-conjugated secondary antibody Alexa 488-anti-mouse, Invitrogen, Carlsbad, CA and counterstained with Hoechst 33342 (Invitrogen, Carlsbad, CA). Negative control sections were incubated without the primary antibodies. Sections were mounted and analyzed in a confocal microscope (TCS SPE; Leica, Wetzlar, Germany).

2.11. Immunofluorescence analysis of glia

Sholl analysis was used to measure the number of processes that intersected with concentric circles starting at the center of the soma (Gensel et al., 2010); the sholl analysis plugin for Image J program was used. Sholl analysis was corroborated by analyzing the length of the processes by NeuronJ plugin for ImageJ in immunostained preparations. Briefly, the length of all processes was measured; the longest process was selected in each cell for every experiment and condition (Nrf2 wild type or KO mice).

Area of the microglial soma was analyzed as described previously (Gwak et al., 2008). Data are represented as % changes of microglia area; the area of the soma of Nrf2 (+/+) mice was considered 100%. All images were quantified by an experimenter blind to condition.

2.12. Analysis of mRNA levels by quantitative real-time PCR (qPCR)

Total RNA from tissues was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen). One microgram of RNA from the different samples were treated with DNase (Invitrogen) and reverse-transcribed

Table 1 Genes and primers for quantitative PCR amplification.

Gene product	Forward primer	Reverse primer
F4/80	5′-TGCCTTACAACTATGAAGCTCCAC-3′	5′-ACACCAGAAGAAAGTGCATAGGAA-3′
β-Actin	5′-TCCTTCCTGGGCATGGAG-3′	5′-AGGAGGAGCAATGATCTTGATCTT-3′

for 30 min at 42 °C using 4 μl of High Capacity RNA-to-cDNA Master Mix (Applied Biosystem, Foster City, CA). For Real-Time PCR analysis, the reaction was performed in 25 μl using the fluorescent dye Power SYBER Green PCR Master Mix (Applied Biosystem, Foster City, CA) and a mixture of 5 pmol of reverse and forward primers. Primer sequences are shown in Table 1. Quantification was performed on a StepOne detection system (Applied Biosystem, Foster City, CA). PCR cycles proceeded as follows: initial denaturation for 10 min at 95 °C, then 40 cycles of denaturation (15 s, 95 °C), annealing (30 s, 60 °C), and extension (30 s, 60 °C). The melting-curve analysis showed the specificity of the amplications. Threshold cycle, which inversely correlates with the target mRNA level, was measured as the cycle number at which the reporter fluorescent emission appears above the background threshold (data not shown). To ensure that equal amounts of cDNA were added to the PCR, the β-actin housekeeping gene was amplified. Data analysis is based on the ΔΔCt method with normalization of the raw data to housekeeping genes.

2.13. Statistical analysis

All experimental results are given as the mean ± SEM. Comparisons between groups were performed using the Student's *t*-test, the Mann—Whitney test, the one-way ANOVA followed by Newman—Keuls test or the two-way ANOVA followed by the Bonferroni test, when appropriate. A value of $P < 0.05$ was considered to be significant. All statistical procedures were carried out using GraphPad Prism 5 for Windows.

3. Results

3.1. Behavioral outcome in Nrf2 knockout mice

We initially performed three behavioral tests to evaluate the putative depressive behavior of Nrf2 KO mice in comparison to their control littermates. As shown in Fig. 1A, the duration of immobility in the tail-suspension test (TST) was increased by 60% in Nrf2 (−/−) *vs* Nrf2 (+/+) mice (Fig. 1A) [$U = 4$, $P = 0.0140$]. In the open-field test (OFT), no detectable difference between Nrf2 (+/+) *vs* Nrf2 (−/−) mice were found in the number of crossings (Fig. 1B) or in the number of rearings (data not shown). Finally, in Fig. 1C it is shown that Nrf2 KO mice exhibited 30% decrease in the grooming time in the splash test (ST) [$t_5 = 2.449$, $P = 0.0255$]. Taken together, these results indicated that Nrf2 KO mouse present a depressive-like behavior.

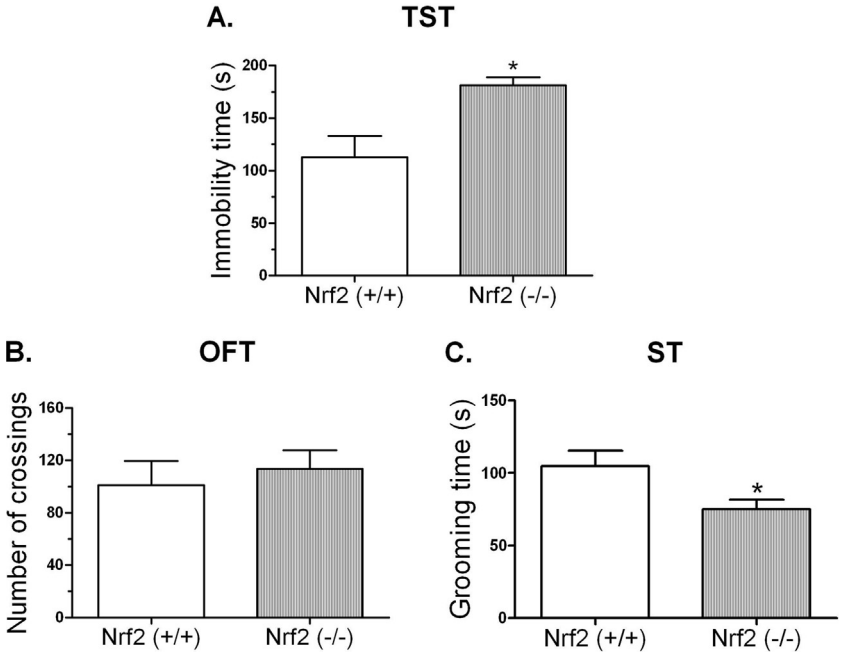

Figure 1 Nrf2 (−/−) mice show a depressive-like phenotype. Nrf2 gene deletion leads to alterations in the immobility time in the TST (tail-suspension test) and the grooming time in the ST (splash test) but not in the number of crossings in the OFT (open-field test). Male wild-type C57BL/6 mice and Nrf2 knockout littermates weighing 22—30 g were subjected to the TST (A), OFT (B) and the ST (C). Bars represent the mean ± SEM ($n = 7$). (A) *$P < 0.05$ *vs* Nrf2 (+/+), Mann—Whitney test (B and C) *$P < 0.05$ *vs* Nrf2 (+/+), Student's *t*-test.

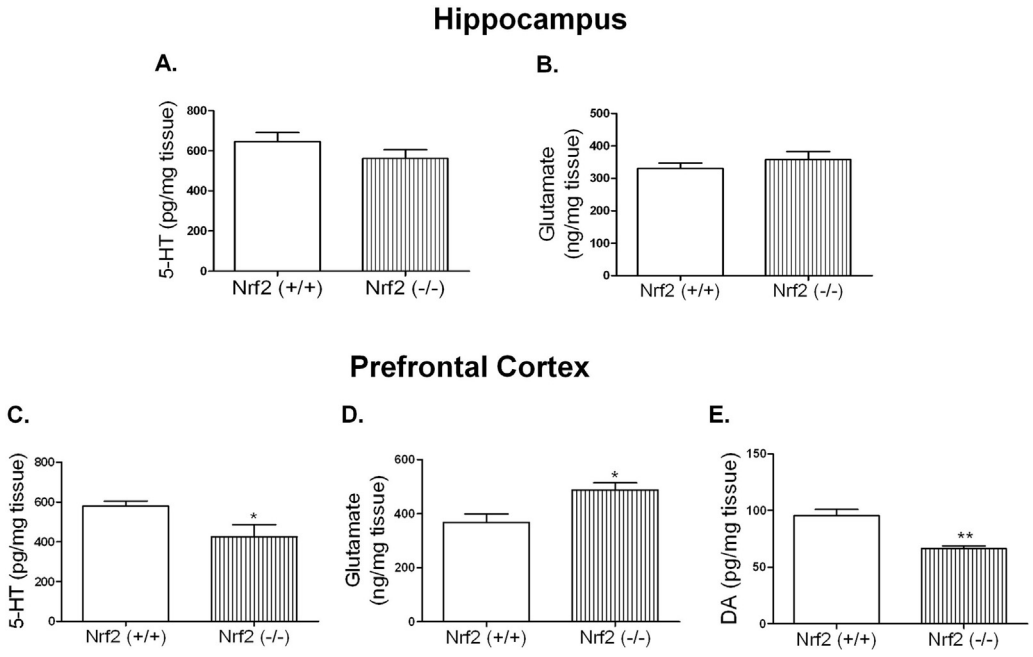

Figure 2 Neurotransmitter levels are altered in the prefrontal cortex but not in the hippocampus of Nrf2 (−/−) mice. Neurotransmitters were determined by HPLC in the prefrontal cortex and the hippocampus of wild type (white bars) and Nrf2 KO (hatched bars) mice. (A) and (B) represent serotonin (5-HT) and glutamate levels in the hippocampus. (C)−(E) represent 5-HT, glutamate and dopamine (DA), respectively, in the prefrontal cortex. Bars indicate the mean ± SEM (n = 6−8). *$P < 0.05$; **$P < 0.01$ *vs* Nrf2 (+/+), Student's t-test.

3.2. Dopamine, serotonin and glutamate levels in Nrf2 knockout mice

As neurotransmitter levels of serotonin, dopamine and glutamate are altered in major depression, we determined their concentrations in Nrf2 (+/+) and Nrf2 (−/−) mice. In extracts from the hippocampus, dopamine levels were undetectable; as to serotonin and glutamate, no differences were found between Nrf2 (+/+) and Nrf2 (−/−) mice (Fig. 2A and B). However, in extracts from the prefrontal cortex, serotonin and dopamine levels were significantly reduced (27% and 31%, respectively) [5-HT: $t_{11} = 2.448$, $P = 0.0324$; DA: $t_{12} = 4.040$, $P = 0.0037$] (Fig. 2C and D) while glutamate levels were significantly increased (32%) in Nrf2 (−/−) mice *vs* Nrf2 (+/+) mice (Fig. 2E) [$t_{13} = 2.838$, $P = 0.0149$].

The DA metabolites, HVA and DOPAC, and the 5-HT metabolite, 5-HIAA, were not augmented in Nrf2 KO mice (Table 2). Therefore, these results suggest that the reduction of 5-HT and DA levels found in the prefrontal cortex are basically related to a reduction in their synthesis, rather than to an increase in their degradation.

Table 2 5-HT and DA metabolite levels in prefrontal cortex (pg/mg tissue).

	5-HIAA	HVA	DOPAC
Nrf2 (+/+)	682.4 ± 49.52	335.6 ± 30.42	116 ± 8.19
Nrf2 (−/−)	723.7 ± 60.59	268.6 ± 5.08	95 ± 2.254

3.3. Alterations in neuroplasticity in Nrf2 knockout mice

A classical theory linked to depression is that there is an alteration in trophic factor levels in the CNS. As shown in Fig. 3, BDNF expression was reduced by 30% in the hippocampus of Nrf2 (−/−) mice (Fig. 3A) although it did not reach statistical significance [$t_{15} = 1.101$, $P = 0.2883$]. In the prefrontal cortex, there were no differences (Fig. 3D). VEGF was diminished in the hippocampus by 20% (Fig. 3B) [$t_{12} = 4.237$, $P = 0.001$] and increased by 60% (Fig. 3E) in the prefrontal cortex of Nrf2 (−/−) mice [$t_{12} = 2.274$, $P = 0.0421$]. Synaptophysin, a marker of synaptogenesis, was diminished by 30% in the prefrontal cortex [$t_{22} = 2.270$, $P = 0.0317$] but remained unaltered in the hippocampus of Nrf2 (−/−) mice.

3.4. Astroglial in the hippocampus of Nrf2 knockout mice

As we observed an alteration in neuroplasticity in the hippocampus of Nrf2 (−/−) mice, we performed immunostaining against GFAP, an astroglial marker (Fig. 4A). Sholl analysis showed a decrease in the number of intersections with concentric circles starting at the center of the soma of around a 38% (Fig. 4B) [$t_{126} = 3.230$, $P = 0.0016$] in Nrf2 (−/−) mice *vs* Nrf2 (+/+) mice. These results are indicative of significant retraction of astroglial processes in Nrf2 KO mice.

3.5. Effect of Nrf2 gene deletion on microglia

To evaluate if microglia was activated in Nrf2 KO mice, immunofluorescence against Iba1 was performed in Nrf2

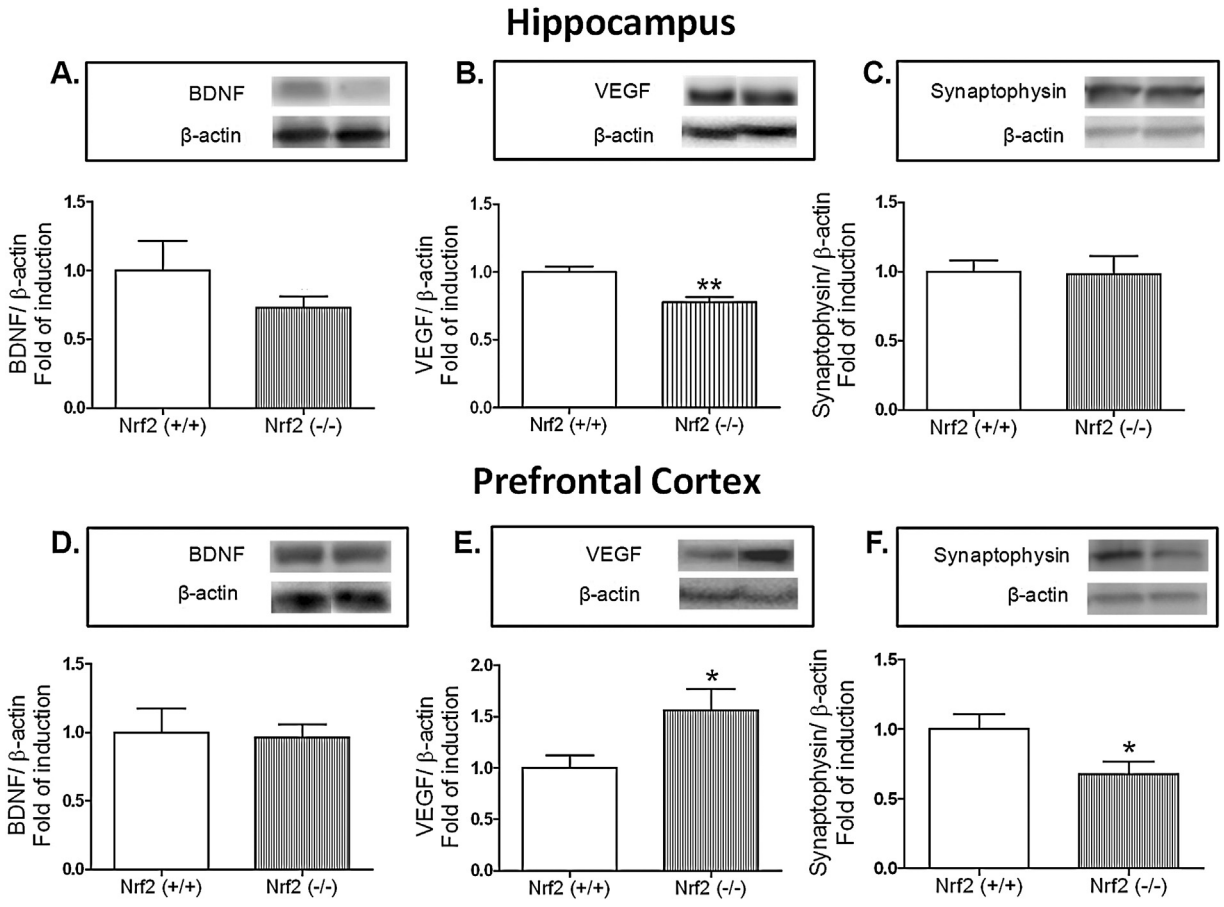

Figure 3 Nrf2 (−/−) mice present modified levels of VEGF and synaptophysin. Trophic factor expression and synaptophysin were studied by western blot in Nrf2 (+/+) and Nrf2 (−/−) mice. (A)−(C) represent the levels of BDNF, VEGF and synaptophysin in the hippocampus. (D)−(F) represent BDNF, VEGF and synaptophysin in the prefrontal cortex. The image accompanying each bar graph is a representative immunoblot showing levels of the three different proteins on the top of the image and β-actin at the bottom as a loading control. Bars represent the mean ± SEM (n = 7−17). *$P < 0.05$; **$P < 0.01$ *vs* Nrf2 (+/+), Student's t-test.

(+/+) and Nrf2 (−/−) mice (Fig. 5A). The maximal length of the microglial process was decreased by 30% in Nrf2 (−/−) *vs* Nrf2 (+/+) mice [$t_6 = 4.973$, $P = 0.0076$] (Fig. 5B). Sholl analysis demonstrated that Nrf2 KO mice showed significant retraction of microglial processes (Fig. 5C) [$t_{222} = 2.277$, $P = 0.0241$]. The microglial cell body area of Nrf2 (−/−) mice was significantly increased by 28% compared to Nrf2 (+/+) mice (Fig. 5D) [$t_6 = 2.541$, $P = 0.04$].

To reinforce the existence of an inflammatory response, mRNA of F4/80 was measured by qPCR in samples taken from the hippocampus and prefrontal cortex of Nrf2 (−/−) and Nrf2 (+/+) mice. F4/80 showed a remarkable 12-fold increase in hippocampus [$U = 0$, $P = 0.0095$] and over 4-fold increase in prefrontal cortex in Nrf2 KO mice in comparison to their control littermates [$U = 0$, $P = 0.0159$] (Fig. 5E and F).

3.6. Effect of the anti-inflammatory drug rofecoxib on the depressive-like behavior of Nrf2 knockout mice

Once shown the presence of microgliosis in Nrf2 (−/−) mice, we intended to corroborate the participation of inflammation in the depressive-like behavior of Nrf2 (−/−) mice. We therefore treated the animals with rofecoxib, a selective COX-2 inhibitor (COX-2/-1; IC_{50} ratio: 0.013), during 7 days at a dose of 2 mg/kg IP. One day after the last administration of rofecoxib, different tests were performed in Nrf2 (+/+) and Nrf2 (−/−) mice. As shown in Fig. 6A, rofecoxib significantly diminished the immobility time by 75% in Nrf2 (−/−) mice in comparison to the non-treated KO mice. The two-way ANOVA revealed a significant effect of rofecoxib [$F_{1,35} = 14.30$, $P < 0.001$], a significant effect of genotype [$F_{1,35} = 10.03$, $P < 0.01$] and treatment × genotype interaction [$F_{1,35} = 5.92$, $P < 0.05$]. However, in the ST there was a lack of statistical difference in the grooming time in Nrf2 (−/−) *vs* Nrf2 (+/+) mice. The two-way ANOVA revealed a non-significant effect of rofecoxib [$F_{1,35} = 1.979$, $P > 0.05$], a significant effect of genotype [$F_{1,35} = 4.332$, $P < 0.05$] and no treatment × genotype interaction [$F_{1,35} = 1.334$, $P > 0.05$]. As for the locomotor activity, no differences were found in the horizontal or in the vertical exploratory behavior (Fig. 6C and D). Taken together, these results indicate that the anti-inflammatory drug rofecoxib can reverse some depression-like behavior in Nrf2 (−/−) mice without altering the locomotor activity.

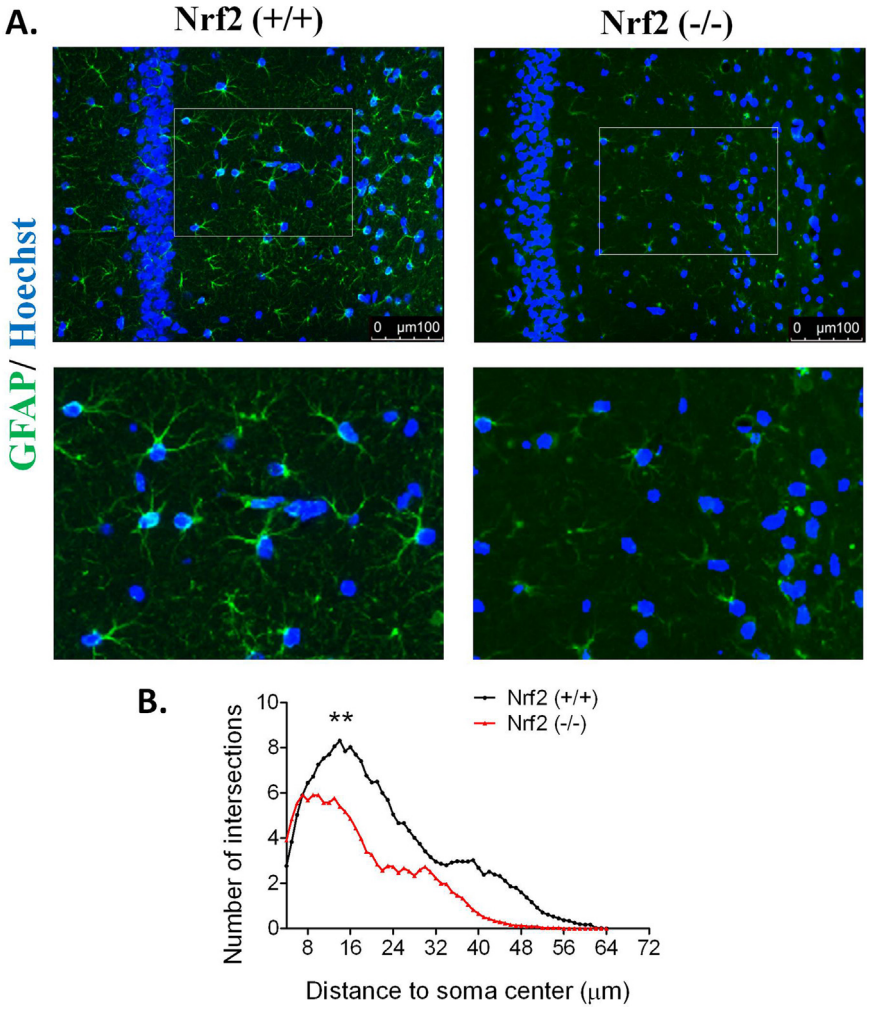

Figure 4 Nrf2 gene deletion causes retraction of astroglial processes in the hippocampus. GFAP immunofluorescence was performed in CA1 region of the hippocampus. (A) Upper images represent microphotographs at ×200 magnification: wild-type animals on the left and Nrf2 KO mice on the right. The lower images show a magnification of the images above. (B) Sholl analysis of astroglial processes in Nrf2 (+/+) and Nrf2 (−/−) mice. Data correspond to the mean ± SEM (N = 4 mice/group, n = 5–12 astrocytes/slice), Student's t-test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

3.7. Effect of Nrf2 induction by sulforaphane on the depressive-like behavior induced by LPS

To analyze the potential antidepressive effects of Nrf2 activation, we treated Swiss male mice with sulforaphane (SFN), 1 mg/kg/day *via* IP for 7 consecutive days, to induce Nrf2-dependent gene expression (Zhao et al., 2007a,b). The day after, LPS was injected to the animals (0.1 mg/kg *via* IP) to induce an inflammatory-dependent depressive behavior. The behavioral tests (TST and ST) were performed 2 h after LPS injection. As illustrated in Fig. 7A, sulforaphane was able to prevent the increase in the immobility time caused by LPS, reaching almost control levels in the TST [$F_{2,41}$ = 5.241, P = 0.0094]. LPS administration decreased the grooming time in the ST and sulforaphane treatment prevented its effects in a significant manner [$F_{2,41}$ = 3.335, P = 0.045] (Fig. 7B).

To determine if SFN was activating Nrf2 under our experimental conditions, we measured two proteins encoded by Nrf2, *i.e.* hemeoxygenase-1 (HO-1) and glutamate cysteine ligase modulatory subunit (GCLm). The day after the last administration of sulforaphane (day 8), animals were sacrificed and hippocampi were dissected to determine HO-1 and GCLm expression by western blot. Our sulforaphane treatment regime increased HO-1 expression by 30% [t_{17} = 2.422, P = 0.0269] and GCLm by 50% [t_8 = 2.460, P = 0.0393], indicating that Nrf2 was being activated. Furthermore, SFN treatment increased BDNF expression by 45% (Fig. 7E) [U = 6, P = 0.0047].

Finally, to confirm the anti-inflammatory action of SFN treatment, we measured iNOS expression in the hippocampus of the mice. As expected, LPS induced iNOS by 60% and SFN, administered sub-chronically, significantly reduced its induction (Fig. 7F) [$F_{2,9}$ = 5.910, P = 0.0230].

4. Discussion

In this study we show that Nrf2, a transcription factor recently described to be a therapeutic target against brain

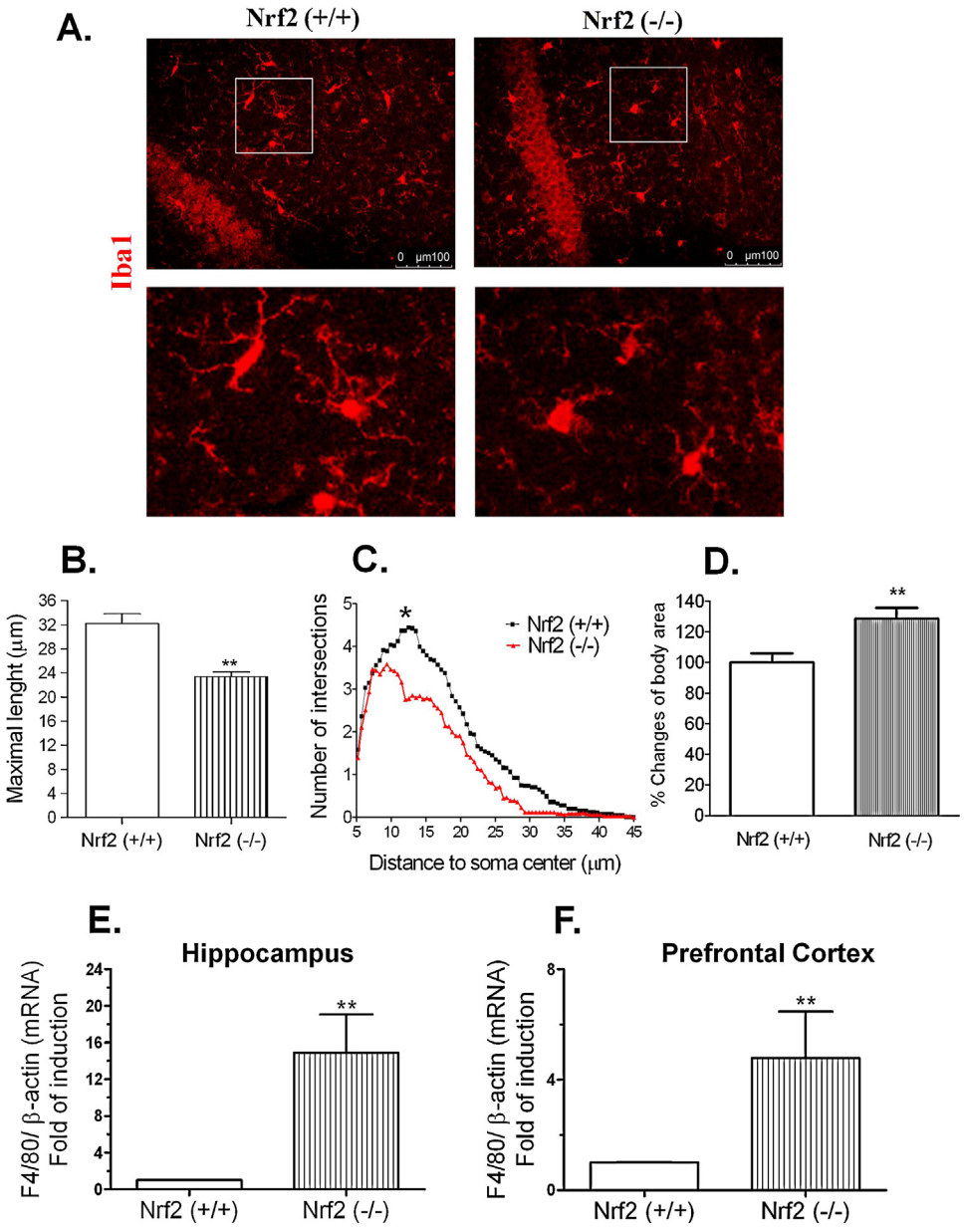

Figure 5 Nrf2 (−/−) mice exhibit microgliosis in the hippocampus. (A) Iba1 immunofluorescence in the hippocampus of Nrf2 (+/+) and Nrf2 (−/−) mice. Top images show microphotographs taken at ×200 magnification of Nrf2 (+/+) mice (on the left) and Nrf2 (−/−) mice (on the right). The lower images show a magnification of the area indicated by the white square on the top image; they are representative of others performed in 4 independent animals. (B) Represents the maximum length of the microglial prolongations measured in Nrf2 (+/+) and Nrf2 (−/−) mice. $^{**}P < 0.01$ *vs* Nrf2 (+/+). Data correspond to the mean ± SEM ($N = 4$ mice/group, $n = 8$–15 microglia/slice), Student's *t*-test. (C) Sholl analysis of microglia of Nrf2 (+/+) and Nrf2 (−/−) mice. $^{*}P < 0.05$ *vs* Nrf2 (+/+). $^{*}P < 0.05$ *vs* Nrf2 (+/+). Data correspond to the mean ± SEM ($N = 4$ mice/group, $n = 5$–11 microglia/slice), Student's *t*-test. (D) Analysis of the area of the microglial soma of Nrf2 (+/+) and Nrf2 (−/−) mice. $^{**}P < 0.01$ *vs* Nrf2 (+/+). Data correspond to the mean ± SEM ($N = 4$ mice/group, $n = 12$–21 microglia/slice), Student's *t*-test. (E) and (F) represent F4/80 mRNA levels measured by qPCR in the hippocampus and in the prefrontal cortex, respectively. Bars represent the mean ± SEM ($n = 4$–6). $^{**}P < 0.01$ *vs* Nrf2 (+/+), Mann–Whitney test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

inflammation (Innamorato et al., 2008), is implicated in depression. This assumption is based on the facts that Nrf2 (−/−) mice exhibited depressive-like behavior, altered neurotransmitter levels, altered neuroplasticity markers and microgliosis. Furthermore, anti-inflammatory treatment of Nrf2 (−/−) mice with rofecoxib prevented their depressive-like behavior while induction of Nrf2 by sulforaphane treatment prevented the depressive-like behavior induced by the pro-inflammatory drug LPS.

The effect of Nrf2 deletion on the depressive behavior was corroborated in two independent tests. In the TST we found an increase in the immobility time (Fig. 1A) while a decrease

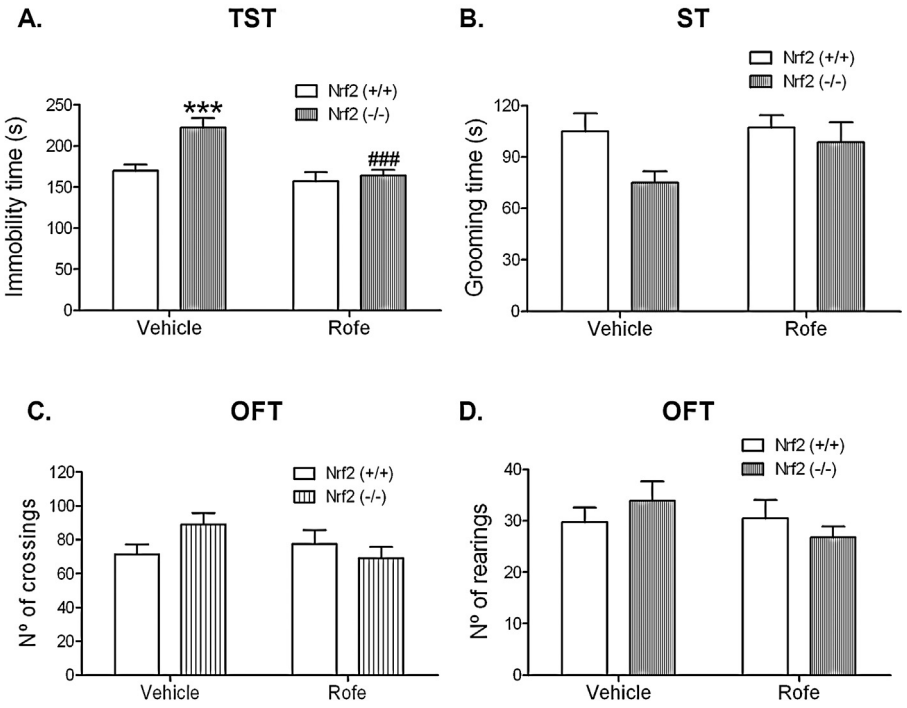

Figure 6 Rofecoxib reverts the depressive like behavior exhibited in Nrf2 KO mice. Nrf2 (+/+) and Nrf2 (−/−) mice were treated with a single dose per day of rofecoxib (2 mg/kg, IP) for 7 days. The day after the end of the treatment, the TST (A), the ST (B) and the OFT (C, horizontal and D, vertical exploratory behavior) were performed. Bars represent the mean ± SEM (n = 10). $^{***}P < 0.001$ *vs* Nrf2 (+/+). $^{###}P < 0.001$ *vs* Nrf2 (−/−). Two-way ANOVA + Bonferroni *post hoc*. TST, tail-suspension test; ST, splash test; OFT, open-field test.

in the grooming time was observed in the ST (Fig. 1C). Nrf2 (−/−) mice did not show a significant variation in locomotion (Fig. 1B) compared with control mice. Therefore, the results obtained in the TST or ST were not due to an alteration in the locomotor activity and the depressive-like behavior in Nrf2 (−/−) mice was confirmed.

The first theory trying to explain the etiology of depression was postulated by Schildkraut (1965); this theory proposed that depression was related to the reduction of the neurotransmitters serotonin and noradrenaline in the brain (Schildkraut, 1965). Later on, an increase in glutamate and a decrease in dopamine levels were also implicated in depression (Nestler and Carlezon, 2006; Hashimoto et al., 2007). Interestingly, it has been extensively described that inflammation decreases 5-HT and DA synthesis (Capuron et al., 2003; Felger et al., 2007). In our study, Nrf2 (−/−) mice showed reduced levels of serotonin and dopamine and increased levels of glutamate in the prefrontal cortex. Because the metabolites of DA (homovanillic acid and dihydroxyphenylacetic acid) and 5-HT (5-hydroxyindoleacetic acid) were not altered in this area, the observed reduction of DA and 5-HT levels in Nrf2 (−/−) animals seems to be due to a reduction in their synthesis and not to an increase in their degradation. The reason could be that the degrading enzymes are working at the same speed despite that the neurotransmitter levels are decreased.

During the past 10 years, molecular and cellular studies have moved the field of mood disorder research beyond the monoamine hypothesis of depression toward brain neuroplasticity. The most studied neurotrophic factors linked to depression have been BDNF (Siuciak et al., 1997; Dwivedi et al., 2003), VEGF (Warner-Schmidt and Duman, 2007, 2008; Nowacka and Obuchowicz, 2012) and NGF (Hunsberger et al., 2007). For example, BDNF protein and mRNA levels in hippocampus and prefrontal cortex of post mortem brains from suicide victims have been described to be diminished (Dwivedi et al., 2003). In the hippocampus of Nrf2 (−/−) mice there was a slight reduction of BDNF levels although such reduction was not statistically significant. As for VEGF, Nrf2 (−/−) mice showed decreased levels in the hippocampus (Fig. 3B) but increased levels in the prefrontal cortex (Fig. 3E). Although this result was initially puzzling, differential levels of a certain neurotrophic factor, for example BDNF, have been reported to be reduced in the hippocampus but increased in the nucleus accumbens in postmortem depressed patients (Krishnan et al., 2007).

In the case of mayor depression, a reduction in the number of astrocytes has been described (Czeh et al., 2006). In Nrf2 (−/−) mice we observed astroglial process retraction in the hippocampus which could be indicative of astroglial atrophy (Fig. 4) and could correlate with the reduction of trophic factors observed in this area. Although Innamorato and coworkers have previously described an increase in astrocyte number in the striatum of Nrf2 KO mice (Innamorato et al., 2010), the discrepancy with our data could be due to differences in vulnerability to oxidative stress/inflammation among the different brain regions.

The causal relationship between inflammation and the pathogenesis of depression is unclear and it appears that this situation could be occurring in a subgroup of patients. Depression could result from the interaction between inflammation and the traditional systems related to depression such

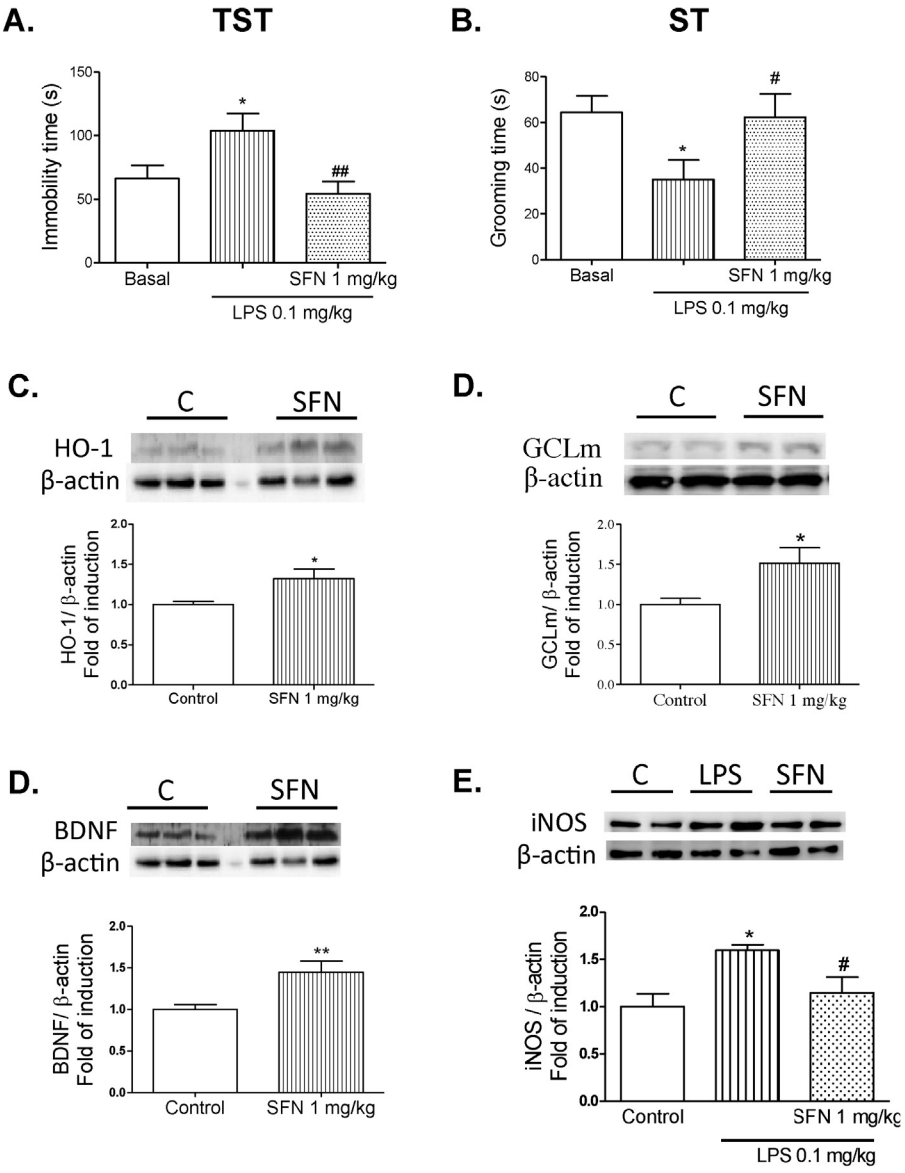

Figure 7 The Nrf2 activator sulforaphane (SFN) induces HO-1, GCLm and BDNF, and prevents LPS-induced depressive-like behavior and iNOS overexpression. Male Swiss mice were treated with a single dose per day of SFN at 1 mg/kg IP for 7 consecutive days. The day after, LPS was injected (IP) at a dose of 0.1 mg/kg and 2 h later, the tail-suspension test (TST) (A) and splash test (ST) (B) were performed. Bars represent the mean ± SEM (n = 14—16). *$P < 0.05$ *vs* Basal. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* LPS alone, One-way ANOVA + Newman-Keuls *post hoc*. (C)—(E) show HO-1, GCLm and BDNF expression in the hippocampus. Male Swiss mice were treated with a single dose per day of SFN (1 mg/kg) during 7 days, 24 h after SFN last administration hippocampi were quickly dissected and protein expression was analyzed by western blot. Bars represent the mean ± SEM (n = 5—19). *$P < 0.05$, **$P < 0.01$ *vs* Control Student's t-test was employed in C, and D and Mann—Whitney test in E. (E) After LPS administration and performance of the behavioral tests, hippocampi were quickly dissected and iNOS expression was analyzed by western blot. Bars represent the mean ± SEM (n = 4). *$P < 0.05$ *vs* Control. $^{\#}P < 0.05$ *vs* LPS alone. One-way ANOVA + Newman—Keuls *post hoc*. The images accompanying each bar graph are representative immunoblots showing levels of HO-1, GCLm, BDNF or iNOS at the top of the images and β-actin at the bottom as a loading control.

as the monoaminergic, glutamatergic and neurotrophic systems as proposed elsewhere (Maes, 2008; McNally et al., 2008). In 1991 Smith proposed the macrophage theory of depression (Smith, 1991) which postulates that excessive secretion of activated macrophage monokines is implicated in the pathophysiology of depression. Thus, when microglia become activated they proliferate and migrate to the site of injury were they express pro-inflammatory cytokines among other toxic molecules. In this respect, it has been documented that patients who suffer from refractory depression (30—40%) show higher levels of acute phase response markers (IL-6, reactive C protein, *etc.*) (Sluzewska et al., 1995; Maes et al., 1997). In our study we have corroborated the inflammatory reaction in Nrf2 (−/−) mice, shown as an increase in mRNA of the microglial and macrophage marker F4/80 in hippocampus and prefrontal cortex (Fig. 5E and F) and also by

a retraction of microglial processes and microglial cell body enlargement (Fig. 5B–D). In line with these observations, it has been described that Nrf2 (−/−) mice exhibit an increase of inflammatory markers like iNOS, IL-6 and TNFα (Thimmulappa et al., 2006; Innamorato et al., 2008; Rojo et al., 2010), giving consistency to our hypothesis that the depressive-like phenotype of Nrf2 (−/−) mice could have an inflammatory origin. The presence of microgliosis could be related to the chronic exposure to oxidative stress due to the lack of Nrf2, that leads to activation of NF-κB, AP-1 and their up-regulating kinases (Chen and Kunsch, 2004) which, in turn, increase the production of pro-inflammatory mediators such as cytokines (TNFα, IL-1β, IL-6, *etc.*), iNOS or COX-2.

The participation of inflammation in the depressive behavior of Nrf2 (−/−) mice was further reinforced when we used anti-inflammatory treatment. We selected the drug rofecoxib because it has been used as an effective anti-inflammatory treatment by others (Jain et al., 2001; Singal et al., 2004) and because it is known to cross the blood–brain barrier (Dembo et al., 2005). Rofecoxib had no effect in wild-type animals in the TST (Fig. 6A), consistent with previous data; however, in Nrf2 (−/−) mice this drug significantly decreased the immobility time (Fig. 6A). Moreover, non-significant alterations in the number of crossings and rearings in the OFT were observed (Fig. 6C and D), indicating that locomotor activity could not account for the results found in the TST. In the ST, there was a tendency for improvement in rofecoxib treated Nrf2 (−/−) animals, although it did not reach statistical significance (Fig. 6B). Taken together, the anti-inflammatory treatment improved the depressive-like behavior of Nrf2 (−/−) mice.

At this point, our results indicate that ablation of Nrf2 provokes a depressive like behavior related to an increased inflammatory state. Therefore, induction of Nrf2 could presumably improve the depressive behavior in an inflammatory model. Indeed, activation of Nrf2 with sulforaphane proved to be antidepressant in the LPS inflammatory model of depression (Fig. 7). Sulforaphane is a drug known to cross the blood brain barrier and to induce a battery of proteins encoded by Nrf2 in the CNS, although these effects are lost in Nrf2 (−/−) mice (Innamorato et al., 2008; Jazwa et al., 2011). In fact, ablation of Nrf2 leads to total inhibition of the protective effects of sulforaphane in different CNS injury models (Zhao et al., 2007a,b; Rojo et al., 2008; Jazwa et al., 2011). Our treatment protocol with sulforaphane employed lower doses than those previously described (Innamorato et al., 2008; Jazwa et al., 2011); however, Nrf2 was being induced since HO-1 and GCLm expression was increased 24 h after the last administration of sulforaphane (Fig. 7C and D). Although HO-1 can also be regulated by other transcription factors, different from Nrf2 (Alam and Cook, 2007), induction of HO-1, GCLm and other Nrf2-related proteins by sulforaphane are known to be lost in Nrf2 (−/−) mice (Innamorato et al., 2008; Jazwa et al., 2011; Rojo et al., 2010). Therefore, the antidepressant effects of sulforaphane in the LPS model can be linked to Nrf2 induction. Furthermore, in the context of depression, we also found other beneficial effects of sulforaphane treatment such as increased BDNF production and reduced iNOS induction (Fig. 7E and F).

In conclusion, we believe that the results of this study provide clear and novel data on the participation of the transcription factor Nrf2 in depression. It would therefore be interesting to study whether patients suffering from depression with augmented levels of inflammatory markers show an alteration in the expression/functionality of Nrf2 and if pharmacological induction of Nrf2 could benefit the subset of refractory patients to classical antidepressants.

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Conflict of interest

The authors declare that there are no actual or potential conflicts of interest. The authors affirm that there are no financial, personal or other relationships with other people or organizations that have inappropriately influenced or biased their research.

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