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CANNABINOID CB_2 RECEPTOR MODULATION BY THE TRANSCRIPTION FACTOR NRF2 IS SPECIFIC IN MICROGLIAL CELLS

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ABSTRACT

Nuclear factor-erythroid 2-related factor 2 (NRF2) is a pleiotropic transcription factor that has neuroprotective and anti-inflammatory effects, regulating more than 250 genes. As NRF2, cannabinoid receptor type 2 (CB_2) is also implicated in the preservation of neurons against glia-driven inflammation. To this concern, little is known about the regulation pathways implicated in CB_2 receptor expression. In this study, we analyze whether NRF2 could modulate the transcription of CB_2 in neuronal and microglial cells. Bioinformatics analysis revealed an antioxidant response element (ARE) in the promoter sequence of the CB_2 receptor gene (*CNR2*). Further analysis by chemical and genetic manipulations of this transcription factor demonstrated that NRF2 is not able to modulate the expression of CB_2 in neurons. On the other hand, at the level of microglia, the expression of CB_2 is NRF2-dependent. These results are related to the differential levels of expression of both genes regarding the brain cell type. Since modulation of CB_2 receptor signaling may represent a promising therapeutic target with minimal psychotropic effects that can be used to modulate endocannabinoid-based therapeutic approaches and to reduce neurodegeneration, our findings will contribute to disclose the potential of CB_2 as a novel target for treating different pathologies.

INTRODUCTION

In recent years, the transcription factor Nuclear Factor erythroid-derived 2-like 2 (herein referred as NRF2, encoded by *NFE2L2* gene) has emerged as an essential factor in modulating the expression of genes involved in a broad spectrum of cellular functions. Although NRF2 was originally described as the master regulator of redox homeostasis (Itoh et al. 1997; Itoh et al. 1995), its role in mechanisms involved in neuroinflammation, proteasome/autophagy, DNA repair, apoptosis, iron and heme metabolism as well as phase I, II, and III drug/xenobiotic metabolism has now been described (Hayes and Dinkova-Kostova 2014; Schmidlin et al. 2019). In basal conditions, there are low levels of NRF2 due to the action of an E3 ubiquitin ligase complex containing a substrate adaptor protein, Kelch-like ECH-associated protein 1 (KEAP1), that binds to and negatively regulates NRF2 (Itoh et al. 1999). Oxidative or electrophilic stress induces NRF2 signaling through modifications of key cysteine residues in KEAP1 that induce conformational changes in the binding of NRF2-KEAP1 that avoids the degradation of NRF2. This allows the accumulation of newly synthesized NRF2, which can then translocate to the nucleus and bind to the antioxidant response element (ARE) sequence in the promoter regions of NRF2-dependent genes, and recruit transcriptional machinery (Itoh et al. 1997).

Because NRF2 is able to regulate the expression of more than 250 genes, its capacity for action is very broad. At the level of the central nervous system (CNS), it has been described that the activation of NRF2 has beneficial effects against the main hallmarks of neurodegeneration. Pharmacological activation of NRF2 induces proteasome and autophagy enhancing the degradation of protein aggregates (Lastres-Becker et al. 2016; Pajares et al. 2016; Rojo et al. 2017), has anti-inflammatory effects (Castro-Sánchez et al. 2019; Cuadrado et al. 2018a; Rojo et al. 2018) and reduces

oxidative stress (Lastres-Becker 2017; Cuadrado et al. 2018b). On the other hand, deficiency in NRF2 worsens all these parameters exacerbating the neurodegenerative process. Therefore, modulation of NRF2 activity has the potential to alter neurodegenerative disease course (Burnside and Hardingham 2017; Cuadrado et al. 2018a; Lastres-Becker 2017; Lastres-Becker et al. 2016).

Like the NRF2 pathway, the endocannabinoid system has emerged as an important neuromodulation system for many brain functions (Zanettini et al. 2011). The endocannabinoid system consists of cannabinoid receptors type 1 and 2 (CB_1 and CB_2), their endogenous ligands, and the enzymes for synthesis and metabolism of the endocannabinoids (Di Marzo 2018). The CB_2 receptor, a seven-transmembrane and G protein-coupled receptor, is generally expressed in immune tissues and cells but is also present at low levels in neuronal and non-neuronal (quiescent microglia, for example) brain cells (Navarro et al. 2016). As NRF2, CB_2 receptor activation has exhibited great potential as anti-oxidative stress and anti-inflammation in various disease models (Di Marzo 2018) and its potential as a therapeutic target is being investigated. An important aspect in this research is the fact that, despite the modest expression of CB_2 receptors in glial elements in normal conditions of the CNS, they experience a notable up-regulation in response to different neurotoxic (e.g. proinflammatory, oxidative, traumatic, infectious) insults. This up-regulation involves the induction of CB_2 receptor gene expression, although little is known about the elements (transcription factors) involved in this response. In this respect, it has been described that the promoter region of human *CNR2* (CB_2 receptor gene) contains several boxes with a transcription binding site for stress response such as AP-1 or AP-4 (activator protein 1 or 4), HSF (heat shock factor) and STRE (stress response element), depending on the isoform (Onaivi et al. 2012). As both the activation of NRF2 and CB_2 have similar anti-oxidative and anti-inflammatory

actions, this evidence prompted us to investigate whether NRF2 could be one of the key transcription factors implicated in the expression of CB_2.

MATERIAL AND METHODS

Bioinformatics analysis. A putative antioxidant response element (ARE) in *CNR2* gene promoter was identified in The Encyclopedia of DNA Elements at UCSC (ENCODE) (http://genome.ucsc.edu) for the human genome (Feb. 2009), taking as reference the available information from chromatin immunoprecipitation (ChIP) of ARE-binding factors MAFK and BACH1. The putative MAFK was localized in a 280-base pair long DNase-sensitive and H3K27Ac-rich region, i.e., most likely regulatory promoter regions. In addition, a frequency matrix of the consensus ARE sequence based on the JASPAR database (http://jaspar.genereg.net) was converted to a position-specific scoring matrix (PSSM) by turning the frequencies into scores through the log(2) [odd-ratio (odd ratio: observed frequency/expected frequency)]. One unit was added to each frequency to avoid log(0). Then a script was generated with the Python 3.4 program to scan the promoter sequences with candidate AREs retrieved from ENCODE with the PSSM. The max score was calculated by adding the independent scores for each of the 11 base pairs of the consensus ARE sequence with the PSSM. The relative score (score relative) was calculated from this max score (score of the sequence $_{max}$) as: score relative = (score of the sequence $_{max}$ − score $_{min\ possible}$)/(score $_{max\ possible}$ − score $_{min\ possible}$). The min possible score (score $_{min\ possible}$) is calculated as the lowest possible number obtained for a sequence from the PSSM and the max possible score (score $_{max\ possible}$) is the highest possible score that can be obtained. We considered putative ARE sequences those with a score relative over 80%, which is a commonly used threshold for the computational framework for transcription factor binding site (TFBS) analyses using PSSM.

Cell culture. Primary microglia were prepared from neonatal (P0-P2) mouse cortex from *Nfe2l2*$^{+/+}$ and *Nfe2l2*$^{-/-}$ mice (obtained from colonies of *Nfe2l2*$^{-/-}$ mice and *Nfe2l2*$^{+/+}$ littermates established from founders kindly provided by Dr. Masayuki Yamamoto (Tohoku University Graduate School of Medicine, Sendai, Japan)) (Itoh et al. 1997) and grown and isolated as described in (Lastres-Becker et al. 2014). Briefly, neonatal (P0-P2) mouse cortex was mechanically dissociated and the cells were seeded onto 75 cm^2 flasks in Dulbecco's Modified Eagle Medium:F12 (DMEM:F12) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin. After 2 weeks in culture, flasks were trypsinized and separated using CD11b MicroBeads for magnetic cell sorting (MACS Miltenyi Biotec, Germany). Microglial cultures were at least 99% pure, as judged by immunocytochemical criteria. Medium was changed to DMEM:F12 serum-free without antibiotics 16 h before treatment. Immortalized microglial cell line (IMG), isolated from the brains of adult mice, was purchased from Kerafast Inc. HT22 mouse hippocampal neuronal cell line was obtained from Dr. Ana Pérez laboratory (Biomedical Research Institute, Madrid, Spain). IMG and HT22 cells were grown in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin and 2 mM L-glutamine, in 5% CO_2 at 37ºC, 50 % relative humidity. Primary neuronal cultures were prepared from the cerebral cortex of 18-day-old Wistar rat embryos (E18), both genders being indistinctly used. Dissected cerebral cortices were mechanically dissociated in culture medium (Minimum Essential Medium, Life Technologies) supplemented with 22.2 mM glucose, 0.1 mM glutamax, 5% fetal bovine serum, 5% donor horse serum, 100 U/ml penicillin and 100 µg/ml streptomycin similarly as described before (Choi et al. 1987). The cell suspension was seeded at a density of $1x10^6$ cells/ml in the same medium using plates previously treated with poly-L-lysine (100 µg/ml, Sigma-Aldrich) and laminin (4 µg/ml, Sigma-Aldrich) overnight at

37 °C. After 4 h, culture medium was changed to Neurobasal (Life Technologies) containing B27 serum-free supplement (Life Technologies), 2 mM glutamax (Life Technologies), 100 U/ml penicillin and 100 µg/ml streptomycin. Growth continued until DIV 7, when medium was changed to fresh Neurobasal medium completed as before but omitting glutamax. Experimental treatments took place after 12 DIVs by adding reactives directly to the growth medium. Medium was changed to serum-free DMEM without antibiotics 16 h before treatments for IMG and HT22 cells. Dimethyl fumarate (DMF) was obtained from Sigma-Aldrich (Cat number 242926) and used at 20 µM.

Analysis of mRNA levels by quantitative real-time PCR. Total RNA extraction, reverse transcription and quantitative polymerase chain reaction (qPCR) were done as detailed in previous articles (Lastres-Becker et al. 2014). Primer sequences are shown in Supplementary Table S1. Data analysis was based on the ΔΔCT method with normalization of the raw data to housekeeping genes (Applied Biosystems). All PCRs were performed in triplicates.

Immunoblotting. Whole cell lysates were prepared in RIPA-Buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EGTA, 1% Igepal, 1% sodium deoxycholate, 0.1 % SDS, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin). Whole cell lysates, cytosolic and nuclear fractions containing 25 µg of whole proteins from IMG-treated cells were loaded for SDS-PAGE electrophoresis. Immunoblots were performed as described in (Cuadrado et al. 2014). The primary antibodies used are described in Supplementary Table S2.

NRF2 overexpression. HT22 cells were seeded on 6-well plates (300,000 cell/well) and transfected with an *NFE2L2* expression plasmid (8 µg) that lacks the high affinity binding site for KEAP1 and contains a V5 tag ($NRF2^{\Delta ETGE}$-V5) (McMahon et al. 2003) kindly provided by Dr. John D. Hayes (Biomedical Research Institute, Ninewells

Hospital and Medical School, University of Dundee) or pcDNA3.1/V5HisB. After 24 h from transfection with Lipofectamine 2000 Reagent (Invitrogen Life Technologies, Cat number 116668–019), cells were lysed for analysis of mRNA levels by quantitative real-time PCR.

Production of lentiviral stocks and infection of HT22 cells. Recombinant lentiviral stocks were produced in HEK293T cells by cotransfecting shCtrl or shNRF2 (shCLN_NM_010902, MISSION shRNA, Sigma) (Robledinos-Anton et al. 2017), 6 µg of envelope plasmid pMD2.G (Addgene; deposited by Dr. Didier Trono) and 6 µg of packaging plasmid pSPAX2 (Addgene; deposited by Dr. Didier Trono), using Lipofectamine 2000 Reagent (Invitrogen Life Technologies). After 12 h at 37 °C, the medium was replaced with fresh DMEM containing 10% fetal bovine serum. Virus particles were harvested at 24 h and 48 h post-transfection. HT22 cells were incubated in the presence of 2 µg/ml polybrene (Sigma-Aldrich, TR-1003-G) with the lentivirus during 24 h, and then selected with Puromycin (5 µg/mL) for 48 h. mRNA was extracted 7 days after lentiviral transduction.

RNA-Seq of cell types isolated from mouse. Differential distribution of the mRNA for *Cnr2* and *Nfe2l2* in the mouse brain were obtained from the Brain-RNAseq database. For details see Ref. (Zhang et al. 2014).

Statistical analyses. Data are presented as mean ± SEM. To determine the statistical test to be used, we employed GraphPad Instat 3, which includes the analysis of the data to normal distribution via the Kolmogorov-Smirnov test. In addition, statistical assessments of differences between groups were analyzed (GraphPad Prism 5, San Diego, CA) by unpaired Student's t-tests when normal distribution and equal variances were

fulfilled, or by the non-parametric Mann–Whitney test. One and two-way ANOVA with *post hoc* Tukey test were used, as appropriate.

RESULTS

Identification of putative AREs in the cannabinoid receptor *CNR2* gene

To define comprehensively the role of *NFE2L2* in the transcriptional regulation of the CB_2 receptor, we searched the Encyclopedia of DNA Elements at UCSC (ENCODE) (An integrated encyclopedia of DNA elements in the human genome 2012) of the human genome (Feb. 2009) for *CNR2* with putative AREs (Figure 1A). The ENCODE database gathers experimental data from chromatin immunoprecipitation (ChIP) analysis of ARE-binding transcription factors MAFF, MAFK, and BACH1, although *NFE2L2* is not analyzed. As shown in Figure 1B, the binding site was located at histone acetylated and DNase-sensitive regions in the *CNR2* gene. Then, we used Python-based bioinformatics analysis to scan this binding region for the consensus ARE as established in the JASPAR database (Mathelier et al. 2014). We detected one putative ARE in the *CNR2* gene with a relative score higher than 80%, a commonly used threshold for transcription factor binding-site analysis (Figure 1C) (Andersen et al. 2008; Kwon et al. 2012). This putative ARE sequence in the *CNR2* promotor region has a high degree of similarity with the consensus ARE sequence (Figure 1D) described by (Hirotsu et al. 2012).

CB_2 expression in neurons is not NRF2 dependent

Recently, it has been described that the CB_2 receptor could have distinct roles in neuronal and microglial cells (Li and Kim 2017). Therefore, to determine whether the transcription factor NRF2 could modulate CB_2 receptor expression in neuronal cells we

followed different strategies by chemical and genetic manipulations of this transcription factor. First, we employed a pharmacological strategy by using dimethyl fumarate (DMF) (20 μM), a well-known NRF2 inducer (Cuadrado et al. 2018a; Lastres-Becker et al. 2016). Hippocampal neuronal cell line HT22 cells were maintained under serum-free conditions for 16 h and then treated with DMF and data were collected at different time points. NRF2 signaling was activated by DMF treatment (Figure 2 A-C) corroborated by the observation that mRNA and protein levels of NRF2, heme oxygenase 1 (HO-1) and NAD(P)H dehydrogenase quinone 1 (NQO1) were increased in a time-dependent fashion. However, neither mRNA or protein levels of CB_2 receptor were modified by DMF treatment. Similar results were obtained in primary neuronal cultures, where DMF treatment for 4 h increased mRNA levels of *Hmox1* but not *Cnr2* (Figure 2D). Next, we overexpressed NRF2 by using a stable mutant, $NRF2^{\Delta ETGE}$-V5, that lacks four residues (ETGE) essential for recognition by the E3 ligase complex Cul3/KEAP1. More than 80-fold of change of *Nfe2l2* mRNA expression did not induce any modification of *Cnr2* expression (Figure 2E). Finally, we silenced NRF2 with a lentiviral shRNA vector in the hippocampal HT22 cell line. Evidence of NRF2-knockdown was provided by analysis of mRNA expression levels, where we observed around 50% of decreased expression of *Nfe2l2* (Figure 2F). Knockdown of NRF2 did not lead to modifications of *Cnr2* mRNA expression levels. Taken together these results suggest that NRF2 is not capable of modulating the expression of the CB_2 receptor at the neuronal level.

NRF2 modulates CB_2 expression in microglial cells

CB_2 receptors are largely found on microglial cells when activated by different neurotoxic stimuli, then playing important protective roles against these stimuli, in particular against neuroinflammation (Cassano et al. 2017). This fueled the idea of

targeting the CB_2 receptor as a promising treatment for glia-driven inflammation and neuronal degeneration (Turcotte et al. 2016). Because NRF2 has an important anti-inflammatory effect, we wanted to analyze if at the level of microglia NRF2 could modulate the expression of the CB_2 receptor. As before, we followed different strategies based on chemical and genetic manipulations of this transcription factor to determine whether NRF2 could modulate CB_2 receptor expression in microglial cells. First, we treated immortalized microglial cells (IMG) with DMF (20 μM). IMG cells were maintained under serum-free conditions for 16 h and then treated with DMF and data were collected at different time points. We corroborated that NRF2 signaling was activated by DMF treatment (Figure 3 A-C) by analyzing the mRNA and protein levels of NRF2, HO-1, and NQO1 and we demonstrated that they were increased in a time-dependent way. Interestingly, treatment with DMF first modulates the protein levels of NRF2 by increasing the stability of the protein (4 h and 8 h) and subsequently induces its transcriptional expression (24h). In relation to these results, we observed that, at 8 h of treatment, increased protein expression of CB_2 is delayed to that of NRF2 (Figure 3B-C). At the mRNA level, we observed a biphasic effect of DMF: first, it induces a decrease in the expression of *Cnr2* at 4 h and a subsequent induction at 24 h (Figure 3A).

Then, we analyzed *Cnr2* mRNA expression levels in primary microglial cells obtained from *Nfe2l2*$^{+/+}$ and *Nfe2l2*$^{-/-}$ mice. Our results demonstrated that NRF2-deficient microglial cells barely express *Cnr2* (approx. 90% of reduced expression) in comparison to wild type microglial cells (Figure 3D). In this case, we chose not to transfect microglia, since it has been described (and we ourselves have observed) that current transfection methodologies provide low transfection efficiency and induce cell death and/or inflammatory activation of the microglia. Anyway, the results we have obtained clearly indicate that the expression of the CB_2 receptor is modulated by NRF2 in microglia. These

data become even more relevant when we compare the expression levels of *Nfe2l2* and *Cnr2* in the different brain cell types by RNA-seq obtained from (Zhang et al. 2014) (Figure 3E). Figure 3E clearly shows that both genes are co-expressed only at the level of the microglia, but not in neurons or astrocytes.

DISCUSSION

During the last decades, knowledge about the endocannabinoid system has increased exponentially, although the CB_2 receptor has been less well characterized in comparison with the CB_1 receptor. However, recently, CB_2 receptors have gained attention, primarily due to their promising therapeutic potential for treating various pathologies while avoiding the adverse psychotropic effects that can accompany CB1 receptor-based therapies (Dhopeshwarkar and Mackie 2014b), although we cannot ignore the possibility of provoking immunosuppression. Therefore, it is very important to know the mechanisms involved in CB_2 transcriptional regulation. Thus, we have analyzed the implication of the transcription factor NRF2 in the expression of CB_2 at the level of neuronal and microglial cells. We showed for the first time that in the promoter region of CB_2 there is an ARE sequence and that NRF2 is able to specifically modulate the expression of CB_2 in microglia (Figures 1 and 3).

It is interesting to note that the regulation of CB_2 by NRF2 only occurs at the microglia level, which is the cell type where both genes are most highly expressed in the brain (Figure 3E). It is also the type of neural cells in which CB_2 receptor expression is most significantly elevated after their activation. In addition, this result is even more relevant when we analyze the involvement of both NRF2 and CB_2 in neuroinflammation processes associated with microglia activation. NRF2 has been demonstrated to counteract inflammation in several neurodegenerative disorders like Alzheimer's disease

(Cuadrado et al. 2018a; Lastres-Becker et al. 2014; Rojo et al. 2018; Rojo et al. 2017; Castro-Sánchez et al. 2019) and Parkinson's disease (Lastres-Becker et al. 2016; Lastres-Becker et al. 2012; Jazwa et al. 2011; Rojo et al. 2010; Lastres-Becker 2017). NRF2 modulates redox homeostasis and phagocytosis in microglia and deficiency in this protein results in exacerbated inflammatory response (Vilhardt et al. 2017), This indicates that NRF2 is implicated in modulating microglial dynamics (Innamorato et al. 2009; Rojo et al. 2010) through, at least, its interaction with the transcription factor NF-κB (Cuadrado et al. 2014), master regulator of inflammation. Lack of NRF2 can magnify NF-κB activity primarly increasing cytokine production, whereas NF-κB can modulate NRF2 transcription and activity (*NFE2L2* promoter has a κB site), having both positive and negative effects on the gene expression (Wardyn et al. 2015). Related to CB_2, it has been observed an up-regulation in reactive microglia in the spinal cord of TDP-43 (A315T) transgenic mice, an experimental model of amyotrophic lateral sclerosis (Espejo-Porras et al. 2019). CB_2 up-regulation has been also observed in the context of amyloid-triggered neuroinflammation (Lopez et al. 2018), and glia-driven inflammation in CNS structures affected, for example, in Parkinson's disease (Gomez-Galvez et al. 2016) and Huntington's disease (Sagredo et al. 2009; Palazuelos et al. 2009). Such responses have been also found in post-mortem tissues from patients affected by these diseases (Jordan and Xi 2019). However, it is still unknown what is the molecular mechanism by which CB_2 activation has anti-inflammatory effects (Wu et al. 2017; Cakir et al. 2019). One possibility is that it acts as an inhibitor of the control of proinflammatory cytokines and, as NRF2, promotes the shift from M1 to M2 phenotypes. It has been described that peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) may mediate CB_2 receptor agonist AM1241-induced anti-inflammation in microglial cells, and the mechanism might be associated with the augmentation of mitochondria biogenesis (Ma

et al. 2018). Therefore, the fact that NRF2 is able to induce the expression of CB_2 in microglia could shed light on the signaling pathways involved in the anti-inflammatory processes. In addition, the induction of CB_2 expression at the microglial level would be a powerful therapeutic target to treat microgliosis associated with pathologies (Navarro et al. 2016; Soethoudt et al. 2017).

Although in principle it has always been described that the CB_2 receptor is preferentially expressed in cells of the immune system (macrophages and microglia), neuronal expression of CB_2 cannabinoid receptor mRNAs has been recently observed in the mouse hippocampus (Li and Kim 2015). It is interesting to note that the function of CB_2 depends on the cell type where it is expressed. For example, CB_2 expression in different types of cells in the mature hippocampus plays diverse roles in the regulation of memory and anxiety (Li and Kim 2017). It is also expressed in other neuronal subpopulations although with a more restricted distribution compared to CB_1 receptors (Dhopeshwarkar and Mackie 2014a; Hu and Mackie 2015). Our results show a very interesting pattern of modulation of CB_2 expression: at the neuronal level NRF2 is not able to alter the expression of CB_2 (Figure 2) suggesting that there must be other transcription factors involved.

Overall, the present work suggests the involvement of NRF2 activity in CB_2 modulation in microglia, and points to NRF2/CB_2 as promising pharmacological targets for therapeutic strategies to modulate neuroinflammation.

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Compliance with Ethical Standards: All experiments were performed by certified researchers according to regional, national and European regulations concerning animal welfare and animal experimentation, and were authorized by the Ethics Committee for Research of the Universidad Autónoma de Madrid and the Comunidad Autónoma de Madrid, Spain, with Ref PROEX 279/14 and Ref PROEX 221/14, following institutional, Spanish and European guidelines (Boletín Oficial del Estado (BOE) of 18 March 1988 and 86/609/EEC, 2003/65/EC European Council Directives). The housing facilities at the Institute were approved by Comunidad de Madrid (# ES 280790000188) and comply with official regulations.

Conflict of interest: None of the authors has a conflict of interest to declare. The authors alone are responsible for the content and writing of the paper.

FIGURE LEGENDS

Figure 1: *Bioinformatic analysis of putative AREs in the cannabinoid receptor CNR2 gene promoter*. **(A)** Scheme of the *CNR2* gene location at Chr.1p36.11 from the Encyclopedia of DNA Elements at UCSC (ENCODE) for the human genome. **(B)** Putative AREs in the *CNR2* gene were identified taking as reference the available information from ChIP of ARE-binding factors MAFK, MAFF, and BACH1. A MAFK binding site was found in a DNase-sensitive and H3K27Ac-rich region upstream the CNR2 gene (i.e. most likely promoter region). **(C)** Table showing the putative ARE sequence identified in the *CNR2* promoter with a relative score over 80%. **(D)** Original

ARE and Core ARE highlighting the main bases involved in NRF2 binding demonstrated by (Hirotsu et al. 2012).

Figure 2: *Modulation of NRF2 does not change CB_2 expression in neurons.* **(A)** HT22 cells were incubated in the presence of dimethyl fumarate (DMF) at 20 µM for 4, 8 and 24 h. Quantitative real-time PCR determination of messenger RNA levels of *Cnr2*, *Nfe2l2* and NRF2-regulated genes coding *Hmox1* and *Nqo1*, normalized by *Tbp* (TATA-box binding protein) messenger RNA levels. The experiments were performed twice (each experiment with n=4). **(B)** Immunoblot analysis in whole cell lysates of protein levels of CB_2, NRF2, HO-1, NQO1, and β-ACTIN as a loading control. Representative blots are presented. **(C)** Densitometric quantification of protein levels normalized for β-ACTIN. The experiments were performed twice (each experiment with n=2), mean ± SEM. Asterisks denote significant differences *p<0.05 **p<0.01 and ***p<0.001, comparing the indicated groups with the basal condition according to a one-way ANOVA followed by Tukey post-test. (**D**) Primary culture of rat cortical neurons were incubated in the presence of DMF at 20 µM for 4 h. Quantitative real-time PCR determination of messenger RNA levels of *Cnr2* and *Hmox1*, normalized by *Tbp* (TATA-box binding protein) messenger RNA levels. Bars indicate n=4, mean ± SEM**.** **(E)** HT22 cells were transfected with 8 µg of pcDNA3.1/V5HisB-mNRF2$^{\Delta ETGE}$ plasmid or the pcDNA3.1/V5HisB, as a negative control. Cells were lysed 24 h after transfection. Quantitative real-time PCR determination of messenger RNA levels of *Cnr2* and *Nfe2l2* normalized by *Tbp* messenger RNA levels. **(F)** HT22 were transduced with lentiviral vectors carrying *Nfe2l2* shRNA or a control scrambled shRNA. Cells were lysed 7 days after infection. The experiments were performed twice (each experiment with n=4, mean ± SEM). Asterisks denote significant differences *p<0.05 and ****p<0.0001, comparing the indicated groups with the control condition according to Student's t-test.

Figure 3: *NRF2 modulates CB_2 expression in microglia.* **(A)** IMG cells were incubated in the presence of DMF at 20 µM for 4, 8 and 24 h. Quantitative real-time PCR determination of messenger RNA levels of *Cnr2*, *Nfe2l2* and NRF2-regulated genes coding *Hmox1* and *Nqo1*, normalized by *Tbp* messenger RNA levels. The experiments were performed twice (each experiment with n=4). **(B)** Immunoblot analysis in whole cell lysates of protein levels of CB_2, NRF2, HO-1, NQO1, and β-ACTIN as a loading control. Representative blots are presented. **(C)** Densitometric quantification of protein levels normalized for β-ACTIN. The experiments were performed twice (each experiment with n=2), mean ± SEM. Asterisks denote significant differences *p<0.05 **p<0.01, ***p<0.001 and ****p<0.0001, comparing the indicated groups with the basal condition according to a one-way ANOVA followed by Tukey post-test. **(D)** Primary cultures of microglia from control wild-type mice ($Nfe2l2^{+/+}$) and NRF2-knockout mice ($Nfe2l2^{-/-}$) were used. Bars indicate n=4, mean ± SEM. Asterisks denote significant differences ****p<0.0001, comparing the indicated groups with the control condition according to Student's t-test. **(E)** Differential expression of *Cnr2* and *Nfe2l2* in the mouse brain according to the Brain-RNAseq database (Zhang et al. 2014). Both transcripts are higher in microglia; whereas *Nfe2l2* levels are lower and *Cnr2* levels drop sharply to undetectable levels in both neurons and astrocytes. Expression level estimation was reported as fragments per kilobase of transcript sequence per million mapped fragments (FPKM) value together with confidence intervals for each sample.

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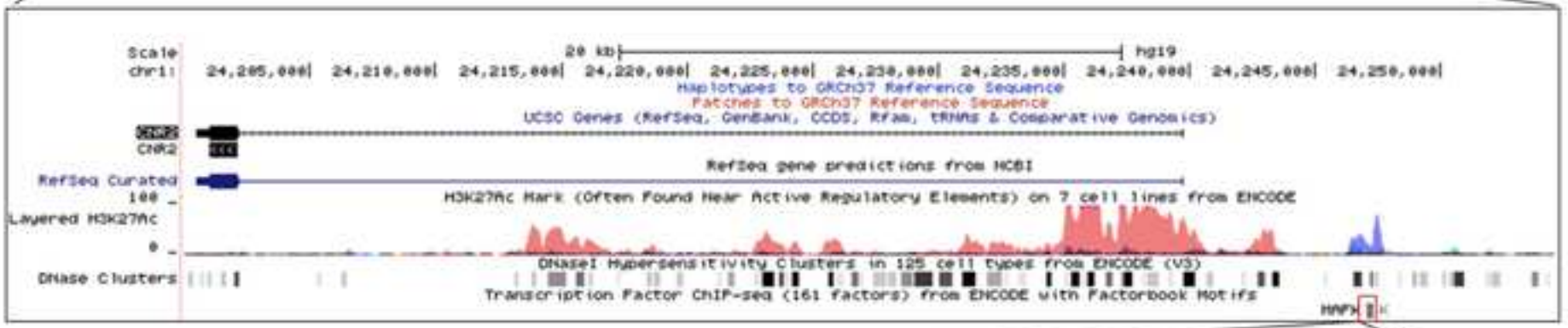
Figure 1

A)

B)



C)

Gene (Human)	Localization in the human genome	Max. score	Relative score	ARE putative binding sequence
CNR2	chr1:24247275-24247264	12.46	0.84	ATGACTTAGCA

D)

Original ARE	RGTGACNNNGC
Core ARE	TGACNNNGC

Figure 2

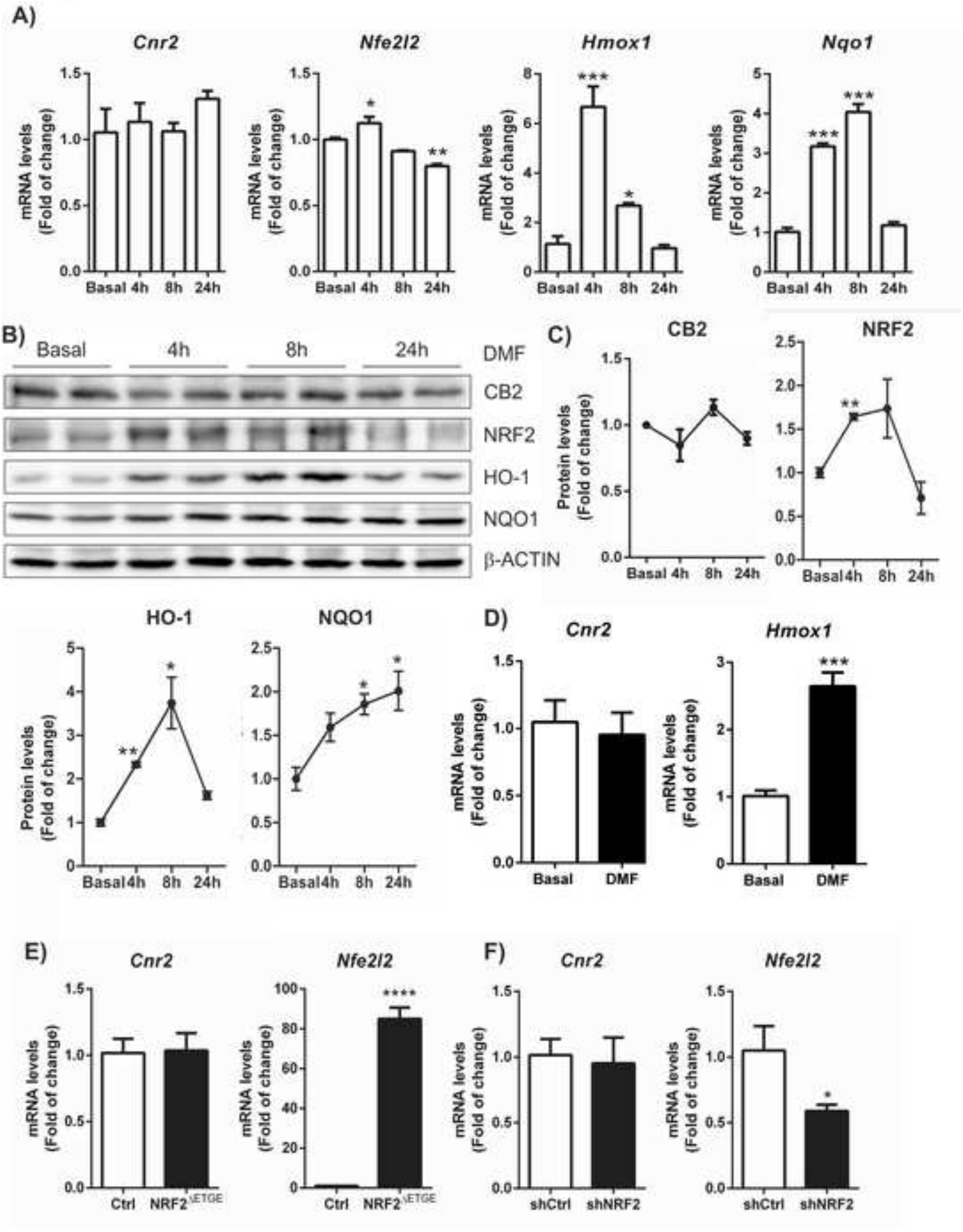


Figure 3