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RESEARCH ARTICLE

NR1D1 downregulation in astrocytes induces a phenotype that is detrimental to cocultured motor neurons

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Abstract

Nuclear receptor subfamily 1 group D member 1 (NR1D1, also known as Rev-erbα) is a nuclear transcription factor that is part of the molecular clock encoding circadian rhythms and may link daily rhythms with metabolism and inflammation. NR1D1, unlike most nuclear receptors, lacks a ligand-dependent activation function domain 2 and is a constitutive transcriptional repressor. Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease, caused by the progressive degeneration of motor neurons in the spinal cord, brain stem, and motor cortex. Approximately 10%–20% of familial ALS is caused by a toxic gain-of-function induced by mutations of the Cu/Zn superoxide dismutase (SOD1). Dysregulated clock and clock-controlled gene expression occur in multiple tissues from mutant hSOD1-linked ALS mouse models. Here we explore NR1D1 dysregulation in the spinal cord of ALS mouse models and its consequences on astrocyte–motor neuron interaction. NR1D1 protein and mRNA expression are significantly downregulated in the spinal cord of symptomatic mice expressing mutant hSOD1, while no changes were observed in age-matched animals overexpressing wild-type hSOD1. In addition, NR1D1 downregulation in primary astrocyte cultures induces a pro-inflammatory phenotype and decreases the survival of cocultured motor neurons. NR1D1 orchestrates the cross talk between physiological pathways identified to be disrupted in ALS (e.g., metabolism, inflammation, redox homeostasis, and circadian rhythms) and we observed that downregulation of NR1D1 alters astrocyte–motor neuron interaction. Our results suggest that NR1D1 could be a potential therapeutic target to prevent astrocyte-mediated motor neuron toxicity in ALS.

KEYWORDS
amyotrophic lateral sclerosis, astrocytes, inflammation, motor neurons, NR1D1, Rev-erbα

Abbreviations: ALS, amyotrophic lateral sclerosis; NR1D1, nuclear receptor subfamily 1 Group D member 1; ROR, RAR-related orphan receptor; SOD1, superoxide dismutase 1.

1 | INTRODUCTION

NR1D1 (also known as Rev-erbα) is a transcription factor that belongs to the nuclear hormone receptor family. NR1D1 is a constitutive transcriptional repressor and was initially classified as an orphan receptor until the discovery of its ligand, heme, in 2007.[1,2] NR1D1, unlike most nuclear receptors, lacks a ligand-dependent activation function domain 2 (AF-2).[3] NR1D1 binds as a monomer or homodimer to the ROR response element (RORE) or Rev-DR2 motif, respectively. During promoter binding, nuclear receptor corepressor 1 (NCoR1) and histone deacetylase 3 (HDAC3) are recruited by NR1D1 to repress transcription.[4,5]

NR1D1 represses the expression of genes involved in numerous physiological functions, including circadian rhythm. Circadian rhythms are genetically encoded by a molecular clock composed of interlocked positive and negative transcriptional/translational feedback loops (TTFL), which induce and repress the transcription of target genes, respectively. The positive arm of the core clock consists of brain and muscle ARNT-like 1 (Bmal1) and circadian locomotor output cycles protein kaput (Clock), while the negative arm of the core clock consists of Period 1/Period 2 (Per1/2) and Cryptochrome 1/Cryptochrome 2 (Cry1/2).[6] NR1D1 participates in a second interlocking TTFL, where it suppresses the positive arm of the core clock. NR1D1 and RAR-related orphan receptor α (Rorα) compete for binding to the RORE motif located in the Bmal1 promoter to directly repress or stimulate transcription, respectively.[7-9]

In addition to its role in the molecular clock, NR1D1 is also a known repressor of metabolic and inflammatory genes whose expressions also fluctuate in a time-of-day-dependent manner.[10-12] Therefore, NR1D1 can potentially be the link coordinating circadian rhythm, metabolism, and inflammation. In vivo, NR1D1 is not required for circadian rhythm generation[7]; however, Nr1d1 knockout mice display spontaneous microglial and astrocyte activation and dysregulated NF-κB signaling.[13]

Amyotrophic lateral sclerosis (ALS) is characterized by the progressive loss of motor neurons in the motor cortex, brain stem, and spinal cord. No cure or effective treatment is currently available, although two drugs (riluzole and edaravone) appear to slightly slow disease progression.[14,15] Approximately 10% of the ALS cases present with a familial history of the disease (familial ALS (FALS)) and are most frequently linked to dominant mutations. The remaining ALS cases do not have a familial history (sporadic ALS (SALS)) and may result from yet unidentified interaction between environmental and genetic risk factors.[16] The first gene linked to ALS was superoxide dismutase 1 (SOD1)[17] and mice overexpressing ALS-linked mutant hSOD1 develop an ALS-like phenotype.[18] The pathophysiological analysis of these mouse models has contributed to a significant portion of our mechanistic understanding of ALS pathology.

In ALS patients and animal models, astrocytes display increased inflammatory markers.[19-22] Upregulation of inflammatory genes and downregulation of metabolic genes appear to be among the earlier transcriptional changes observed in astrocytes from ALS mice.[23] The molecular mechanism underlying the death of motor neurons in ALS remains uncertain. However, the phenotype acquired by ALS astrocytes likely contributes to motor neuron demise—an observation that has been clearly established in FALS and SALS models.[24-30]

We recently analyzed the expression of clock and clock-controlled genes in multiple tissues from mutant hSOD1-linked ALS mouse models.[31] Interestingly, NR1D1 was the only core clock gene that displayed significant expression changes in all tissue types analyzed from early symptomatic mice overexpressing the ALS-linked hSOD1^{G93A} mutation. Here we further explore NR1D1 dysregulation in the spinal cord of ALS mouse models and its consequences on astrocyte–motor neuron interaction.

2 | METHODS AND MATERIALS

2.1 | Reagents

All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise specified. Culture media and supplements were from ThermoFisher Scientific unless otherwise specified. Primers sequences were previously described[31] and were obtained from Integrated DNA Technologies.

2.2 | Animals

B6.Cg-Tg(SOD1*G93A)1Gur/J and B6. Cg-Tg(SOD1) 2Gur/J mice[18] were obtained from The Jackson Laboratory and maintained as hemizygous animals in a C57BL/6J background. hSOD1$^{H46R/H48Q}$mice were provided by Dr. David Borchelt[32] and have been backcrossed into C57BL/6J pure background for more than 10 generations. Tissue harvesting was performed at the same time of the day for all animals in this study. All animal procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH. The Animal Care and Use Committee of UW-Madison approved the animal protocol pertinent to the experiments reported in this publication.

2.3 | Primary astrocyte cultures and astrocyte–motor neuron cocultures

Primary astrocyte cultures were prepared from 1-day-old pups according to the procedure of Saneto and De Vellis.[33] Briefly, spinal cords were dissociated and incubated in 0.25% trypsin in PBS for 25 min at 37°C. Dissociated cells were passed through a 70-μm sieve, centrifuged and plated at a density of 1.5×10^6 cells per 25 cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, HEPES (3.6 g/L), penicillin (100 IU/ml), and streptomycin (100 μg/ml). When confluent, cultures were shaken for 48 h at 250 rpm at 37°C, followed by a 48-h treatment with 10 μM cytosine arabinoside. After 48 h, cytosine removal cells were replated at a density of 2×10^6 cells/cm^2. Experiments were performed when cultures reach confluency. Control induced pluripotent stem cells (iPSCs) lines were obtained from the NINDS Human Cell and Data Repository or commercial vendors. Details of iPSC lines are as follows: line 1 (iPSC ID# FA0000011, control), line 2 (iPSC ID# ND38555, control), line 3 (XCL-1, Stemcell #70901, control). iPSCs were differentiated into induced NPCs using an embryoid body formation protocol in the presence of SMAD signaling inhibitors (STEMdiff SMADi Media, Stemcell). Induction was confirmed by an increase in *MAP2*, *PAX6*, and *NESTIN* gene expression and concurrent decrease in *SOX2*, *OCT3*, and *NANOG* expression. Induced NPCs were cultured for 3 weeks in astrocyte differentiation media (STEMdif Astrocyte Differentiation Media, Stemcell), following by 3 weeks in astrocyte maturation media (STEMdif Astrocyte Maturation Media, Stemcell). Astrocyte differentiation was confirmed by assessing *GFAP*, *S100B*, and *ALDH1L1* gene expression. Following differentiation, iAs were cultured in DMEM-F12 supplemented with 10% FBS and 0.3% N2 supplement. Motor neuron cultures were prepared from 12.5-embryonic-day mouse spinal cords. Motor neurons were purified by a combination of differential centrifugation on BSA cushions and an OptiPrep gradient as previously described.[34] For coculture experiments, motor neurons were plated on primary astrocyte monolayers at a density of 300 cells/cm^2 and maintained for 16 or 72 h in L15 medium supplemented with 0.63 mg/ml bicarbonate, 5 μg/ml insulin, 0.1 mg/ml conalbumin, 0.1 mM putrescine, 30 nM sodium selenite, 20 nM progesterone, 20 mM glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2% horse serum. For coculture experiments with iPSC-derived astrocytes, motor neurons were plated on astrocyte monolayers at a density of 1500 cells/cm^2 and maintained for 72 h in Neurobasal medium supplemented with 1X B27 supplement and GDNF (1 ng/ml). Motor neurons in cocultures with mouse primary astrocytes were identified by immunostaining against βIII-tubulin (Millipore, 05-559, lot:2757108), and survival was determined by counting all cells displaying intact neurites longer than 4 cell bodies in diameter. Counts were performed over an area of 0.90 cm^2 in 24-well plates. The cocultures with iPSC-derived astrocytes were performed with motor neurons isolated from $ROSA^{mT/mG}$ transgenic mice (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J; Stock No. 007676, The Jackson Laboratory), which express a cell membrane-localized tdTomato (mT) fluorescent protein. $ROSA^{mT/mG}$ motor neurons in the coculture were identified by immunostaining with a CF594-conjugated anti-Red Fluorescent Protein antibody (Biotium, 20422, lot: 20C1003). Motor neuron counting was performed as described above.

2.4 | Immunofluorescence

Antigen retrieval and staining in paraffin-embedded tissues was performed as previously described.[35] Mice lumbar spinal cord sections were stained with anti-GFAP (Novus, NB300-141, lot 179-071616) or anti-NeuN (Cell Signaling, 24307, lot 2) and anti-NR1D1 (Abnova, H00009572-MO2, lot J9051-4F6) antibodies. Nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride). Images were acquired on an LSM 880 microscope (Zeiss) with Zen Black (v2.3) image software. To determine the percent of NR1D1-positive nuclei, images were analyzed using ImageJ (version 1.52p). After setting a threshold, individual image channels were converted to binary, overlaid, and then the number of nuclei containing overlapping areas were obtained using the Analyze Particles function. In each group, there were four or five mice and two to four images were analyzed per animal.

2.5 | Real-time PCR and western blot

RNA extraction, RNA retrotranscription, real-time PCR, and western blot analysis were performed as previously described.[36] Membranes were incubated overnight with anti-NR1D1 (Novus, H00009572-M02) and anti-Actin (Sigma, A5441, lot: 061M4808). Image acquisition was performed in a chemiluminescent western blot scanner (Li-Cor) or exposed on Kodak BioMax Light film. The western blot image presented in Figure 4A was quantified using ImageJ Software (NIH), all other western blot quantifications were performed using the Image Studio Software (Li-Cor).

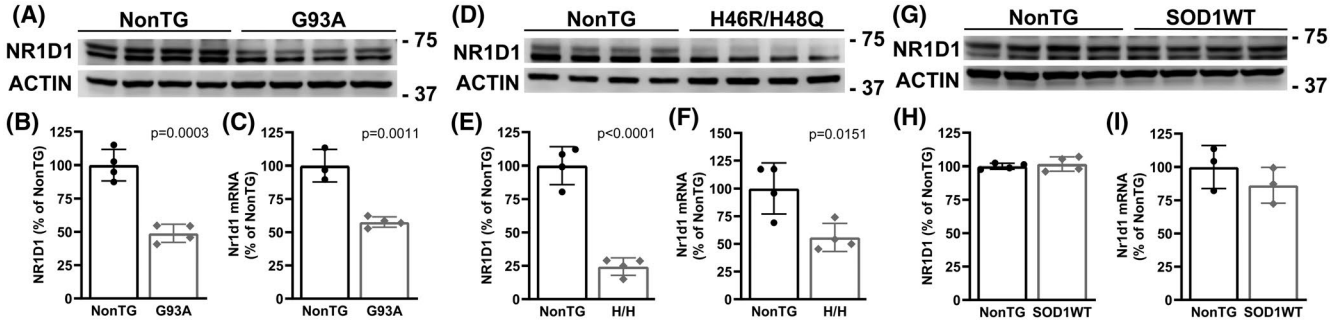

FIGURE 1 Decreased NR1D1 expression in the spinal cord of mutant hSOD1-linked mouse models. (A, D, and G) Western blot analysis of NR1D1 expression in the lumbar spinal cord of age-matched nontransgenic (NonTG) and symptomatic $hSOD1^{G93A}$ (G93A, about 140 days old) mice (A) or symptomatic $hSOD1^{H46R/H48Q}$ (H46R/H48Q, about 210 days old) mice (D) or age-matched control $hSOD1^{WT}$ mice (about 210 days old) (G). (B, E, and H) Quantification of NR1D1 protein levels shown in A, D, and G, respectively. NR1D1 expression was quantified, normalized by ACTIN levels and expressed as a percentage of age-matched NonTG mice (each lane corresponds to a different animal, mean ± SD). Both bands shown in the NR1D1 western blot were used for intensity quantification. (C, F, and I) *Nr1d1* mRNA levels in the lumbar spinal cord of age-matched NonTG and symptomatic G93A (C), or symptomatic H46R/H48Q (F), or age-matched $hSOD1^{WT}$ (I) mice. *Nr1d1* mRNA levels were determined by real-time PCR and corrected by *Actin* mRNA levels ($n = 3$ or 4 mice, mean ± SD)

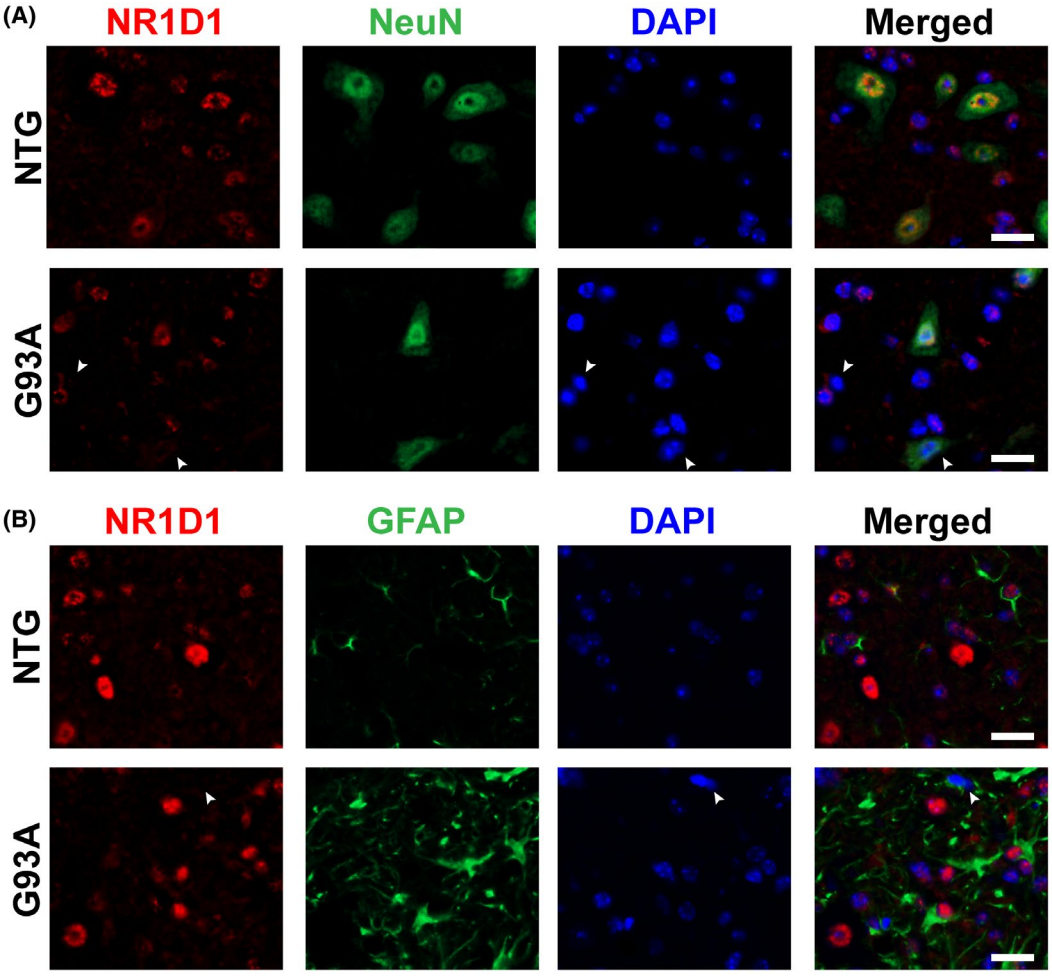

FIGURE 2 Decreased NR1D1 expression in the spinal cord of symptomatic $hSOD1^{G93A}$ mice. (A) Representative images showing NR1D1 (red) and NeuN (green) immunofluorescence in the ventral horn of the lumbar spinal cord from symptomatic $hSOD1^{G93A}$ (G93A) and age-matched nontransgenic (NTG) mice. Nuclei were counterstained with DAPI (blue). The arrowheads point to NR1D1-negative nuclei from NeuN-negative and NeuN-positive cells. Scale bar: 20 μm. (B) Representative images showing NR1D1 (red) and GFAP (green) immunofluorescence in the ventral horn of the lumbar spinal cord from symptomatic G93A and age-matched NonTG mice. Nuclei were counterstained with DAPI (blue). The arrowhead points to a NR1D1-negative, GFAP-positive nucleus. Scale bar: 20 μm

2.6 | Cell treatment and transfections

siRNA transfections with a mouse-specific Nr1d1-siRNA (duplex sequence: 5′-AGAAUAUCCAGUACAAACGGUG UCT-3′ and 5′-AGACACCGUUUGUACUGGAUAUUCUG U-3′) or human-specific Nr1d1-siRNA (Horizon, ID: L-003411-00-0005) were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. Astrocytes were transfected with 25 nM of a negative control siRNA (NC-siRNA) or siRNA for Nr1d1 (Nr1d1-siRNA). After 48 h, astrocytes were harvested for analysis or used in coculture experiments.

2.7 | NF-κB reporter assay

Adenovirus expressing a firefly luciferase gene under the control of a synthetic promoter that contains direct

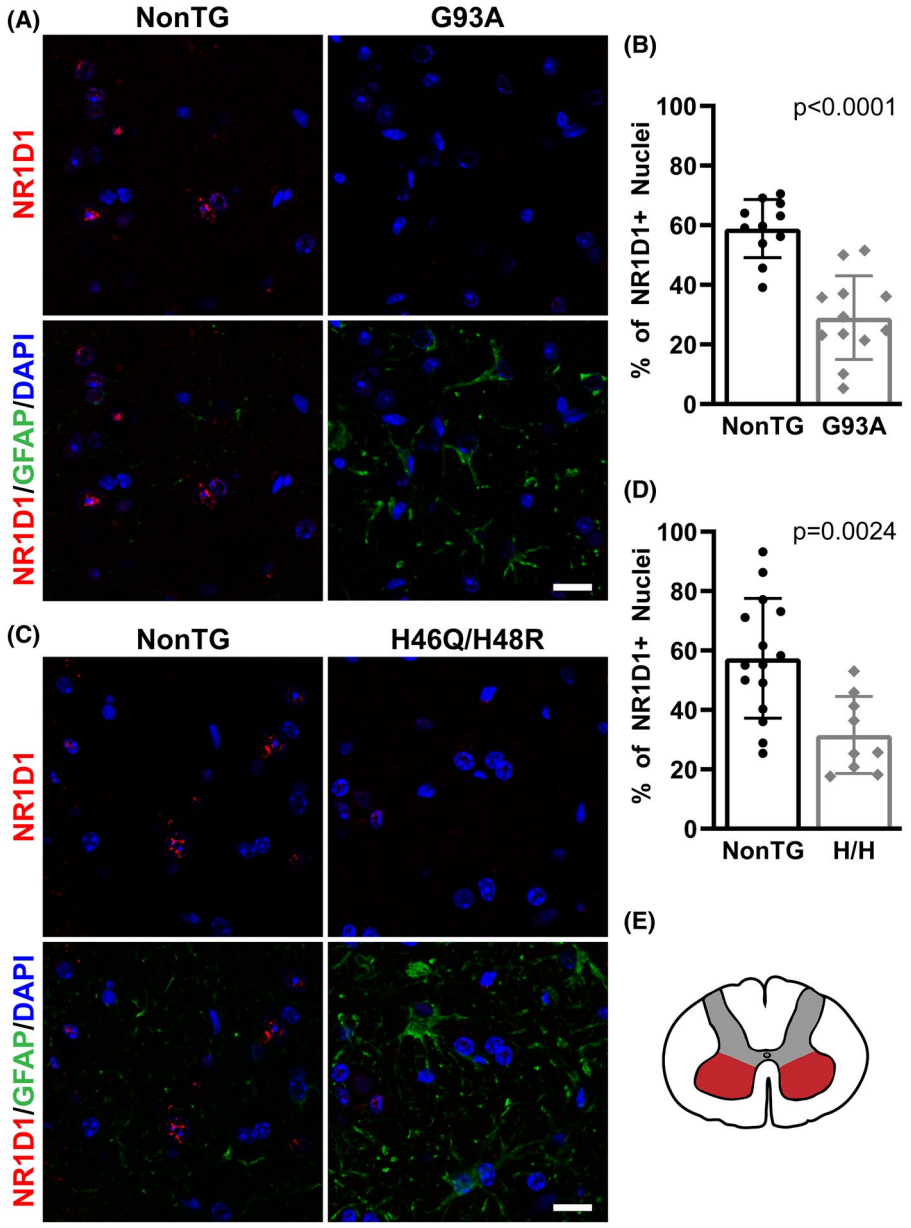

FIGURE 3 Decreased NR1D1 immunostaining in the spinal cord of mutant hSOD1-linked mouse models. (A and C) Representative images showing GFAP (green) and NR1D1 (red) immunofluorescence in the ventral horn of the lumbar spinal cord from symptomatic $hSOD1^{G93A}$ (G93A), $hSOD1^{H46R/H48Q}$ (H46R/H48Q), and age-matched nontransgenic (NonTG) mice. Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm. (B, D) Quantification of NR1D1-positive nuclei in the ventral horn of the lumbar spinal cord from mice in A and C. Data are expressed as the percentage of NR1D1-positive nuclei from the total number of nuclei analyzed for each genotype ($n = 4$ or 5 mice per group and two to four images per animal; mean ± SD). (E) Schematic representation of the spinal cord indicating the area (red) in which the analysis was performed

repeats of the NF-κB binding site (Ad-NFkb-Luc) or a *Renilla* luciferase under a constitutive promoter (Ad-pRL-Luc) were obtained from Vector BioLabs. Adenovirus-mediated transductions were performed at a multiplicity of infection of 10 and 3, respectively. Seventy-two hours after viral transduction, cultures were transfected with NC-siRNA or Nr1d1-siRNA as described above, and firefly and *Renilla* luciferase activity were consecutively assayed 48 h later with the Dual-Glo luciferase system (Promega).

2.8 | Statistical analysis

Groups of three or four animals were used for biochemical analysis. Unless otherwise noted, cell culture experiments were repeated in at least three independent primary culture preparations, and values from each independent experiment were combined for data reporting. All data are reported as mean ± SD. Comparisons between two groups were performed with an unpaired *t*-test. Multiple group comparisons were performed by one-way ANOVA with Tukey's posttest. When comparing the effect of combinations of treatments, two-way ANOVA was used followed by Tukey's posttest. Differences were declared statistically significant if $p < .05$. All statistical computations were performed using Prism 9.0 (GraphPad Software).

3 | RESULTS

We have previously shown evidence of dysregulated clock and clock-controlled gene expression at mRNA level in early symptomatic animal models of ALS.[31] Here, we examined NR1D1 expression in the spinal cord of overtly symptomatic hSOD1^{G93A} mice (about 140-day-old). Compared to nontransgenic controls, hSOD1^{G93A} mice showed significant downregulation in both NR1D1 protein (Figure 1A,B) and mRNA levels (Figure 1C). Likewise, NR1D1 protein and mRNA expression were also significantly downregulated in the spinal cord of symptomatic hSOD1$^{H46R/H48Q}$ mice (about 210-day-old, Figure 1D–F). Spinal cords from 7-month-old mice overexpressing wild-type hSOD1, which do not develop overt motor neuron degeneration, showed no difference in NR1D1 expression levels (Figure 1G–I); suggesting that NR1D1 downregulation is linked to the expression of the mutant hSOD1 protein in these mouse models. Consistent with the downregulation in NR1D1 protein expression described in Figure 1, immunostaining in the ventral horn of the spinal cord of hSOD1^{G93A} mice shows that NR1D1 downregulation appears to occur in both neuronal and nonneuronal cells (Figure 2). Moreover, when quantified, we observed a significant decrease in the number of NR1D1-positive nuclei in the ventral horn of the spinal cord from both hSOD1^{G93A} and hSOD1$^{H46R/H48Q}$ mice (Figure 3).

NR1D1 downregulation in the spinal cord of these two ALS models does not seem to be restricted to a specific cell type. However, since NR1D1 displays altered rhythmicity in iPSC-derived astrocytes from ALS patients[31] and decreased NR1D1 expression can lead to dysregulated inflammation,[13,37] we sought to determine the effects of decreasing NR1D1 expression on the inflammatory profile of spinal cord astrocytes in culture. We transfected primary nontransgenic astrocyte cultures with a negative control siRNA (NC-siRNA) or an Nr1d1-specific siRNA (Nr1d1-siRNA). After 48 h, we observed a significant NR1D1 downregulation in cultures treated with Nr1d1-siRNA (Figure 4). In addition, we observed that the downregulation of NR1D1 lead to increased luciferase expression from an NF-κB-driven promoter (Figure 5A). Accordingly, mRNA of NF-κB target genes, including *Nos2*, *Il6*, *Ptgs2*, *Ccl5*, and *Cxcl10* were significantly upregulated in astrocytes with decreased NR1D1 expression (Figure 5B),

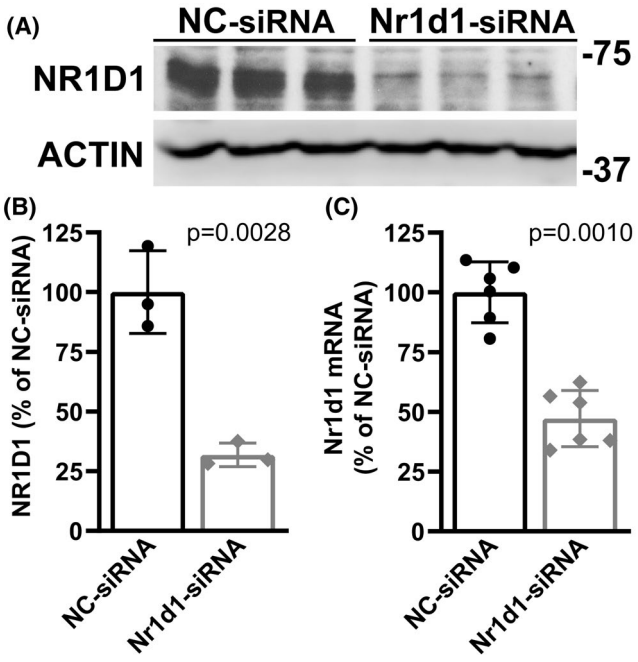

FIGURE 4 NR1D1 silencing in primary nontransgenic spinal cord astrocytes. Confluent primary astrocyte cultures from nontransgenic mice were treated with a negative control siRNA (NC-siRNA) or an Nr1d1-siRNA for 48 h. (A) Western bolt analysis of NR1D1 protein levels in astrocytes following siRNA treatment. (B) Quantification of NR1D1 protein levels shown in A. NR1D1 expression was quantified, normalized by ACTIN levels, and expressed as a percentage of NC-siRNA-treated cultures (mean ± SD). (C) *Nr1d1* mRNA levels in Nr1d1-siRNA-treated astrocytes. *Nr1d1* mRNA levels were determined by real-time PCR and corrected by *Actin* mRNA levels (mean ± SD)

indicating that NR1D1 downregulation induces a pro-inflammatory phenotype in astrocytes.

It has been previously reported that NR1D1 has a key role in regulating the expression of fatty acid-binding protein 7 (FABP7) in astrocytes.[38,39] Accordingly, we observed that Nr1d1-siRNA-treated spinal cord astrocytes displayed upregulated FABP7 expression (Figure 5B–D). We have previously shown that FABP7 upregulation induces a phenotype that is detrimental to the survival of cocultured motor neurons.[40] To examine the effect of NR1D1 silencing on the way astrocytes interact with neighboring neurons, we cocultured motor neurons with astrocytes pretreated with Nr1d1-siRNA or NC-siRNA. Downregulation of NR1D1 in astrocytes does not alter the number of motor neurons able to attach to the astrocyte monolayer, since a similar number of motor neurons was observed after 16 h in coculture with Nr1d1-siRNA or NC-siRNA (Figure 6A). However, after 3 days in culture, a significant decrease in motor neuron survival was observed in cocultures with astrocytes pretreated with Nr1d1-siRNA, compared to cocultures with NC-siRNA-treated astrocytes (Figure 6B). Moreover, the decrease in motor neuron survival does not appear to be a consequence of decreased trophic support, since the addition of exogenous trophic factors (GDNF 10 ng/ml and BDNF 0.1 ng/ml), capable of supporting motor neuron survival in pure cultures, did not prevent motor neuron loss in cocultures with Nr1d1-silenced astrocytes (Figure 6B). In transdifferentiated astrocytes obtained from three different human iPSC lines from healthy subjects, silencing of Nr1d1 expression also leads to increased luciferase expression from an NF-κB-driven promoter (Figure 7A–C). In addition, a significant

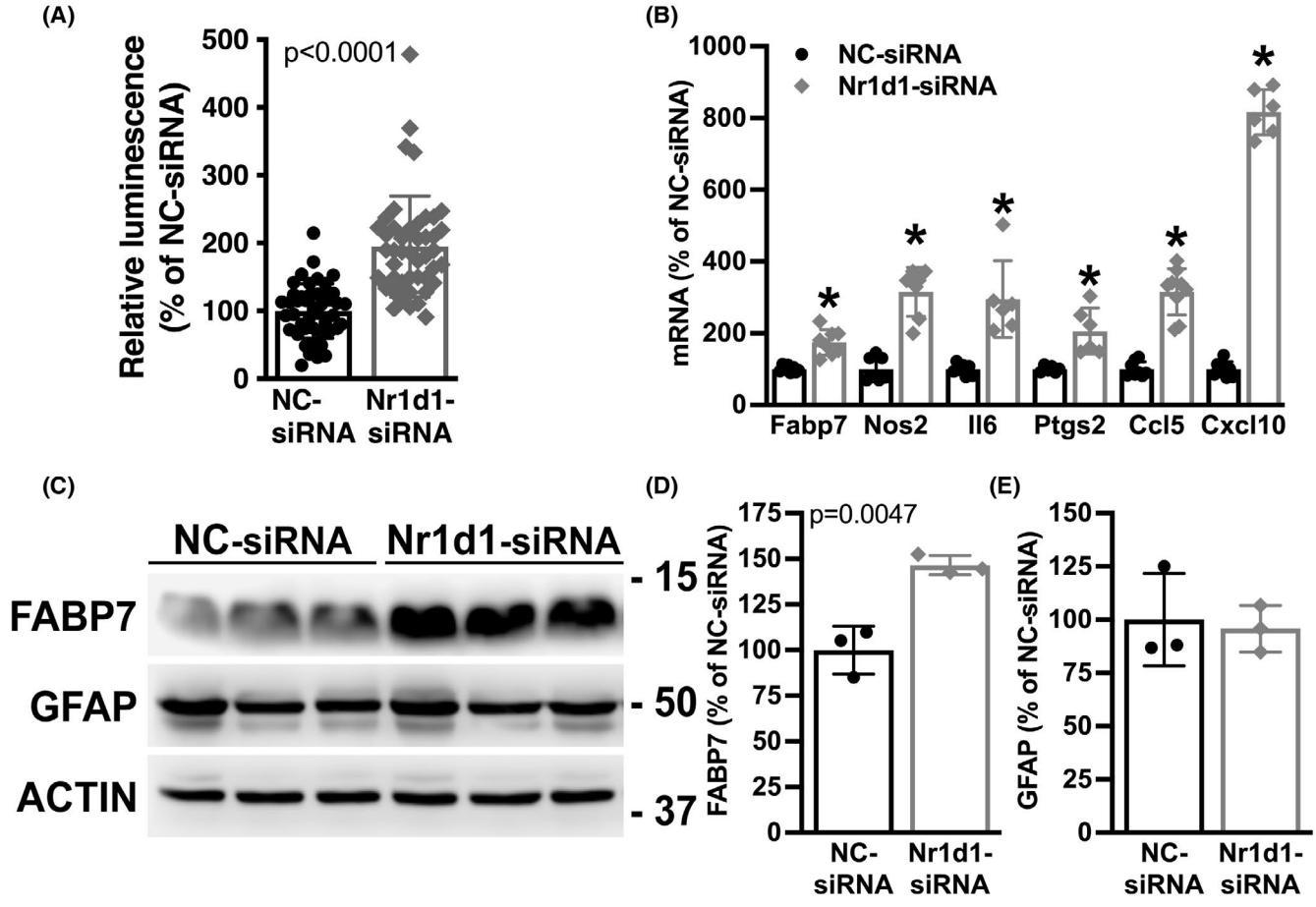

FIGURE 5 NR1D1 downregulation induces a pro-inflammatory phenotype in nontransgenic astrocytes. (A) Relative luminescence produced by a firefly luciferase expressed under an NF-κB-driven promoter 48 h after NC-siRNA or Nr1d1-siRNA treatment of nontransgenic astrocytes. Relative firefly luciferase luminescence was corrected by the amount of *Renilla* luciferase activity controlled by a constitutive promoter and expressed as a percentage of NC-siRNA-treated control cells. (B) *Fabp7*, *Nos2*, *Il6*, *Ptgs2*, *Ccl5*, and *Cxcl10* mRNA levels in astrocytes 48 h after NC-siRNA or Nr1d1-siRNA treatment. mRNA levels were determined by real-time PCR and corrected by *Actin* mRNA levels. Data are expressed as a percentage of NC-siRNA-treated control cells. (C) Western blot analysis of FABP7 and GFAP expression in astrocytes treated as in A. (D, E) Quantification of the images shown in (C). FABP7 and GFAP protein levels were quantified, corrected by ACTIN levels and expressed as a percentage of NC-siRNA-treated control cells. For all graph panels, data are expressed as mean ± SD (*$p < .05$, significantly different from NC-siRNA-treated cells)

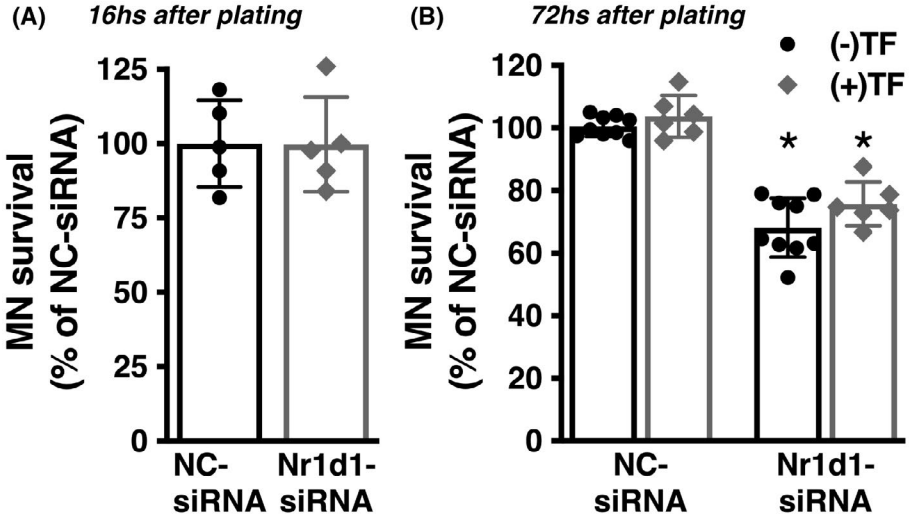

FIGURE 6 The phenotype induced by NR1D1 downregulation in primary nontransgenic astrocytes is detrimental to cocultured motor neurons. (A) Spinal cord nontransgenic astrocytes were treated with NC-siRNA or Nr1d1-siRNA. Forty-eight hours later embryonic motor neurons were plated on top. Motor neuron (MN) survival was determined 16 h later (data obtained from two independent coculture experiments, two or three replicas per coculture). (B) The same coculture setup as in A but motor neuron survival was determined 72 h later. The addition of trophic factors (TF: GDNF 10 ng/ml and BDNF 0.1 ng/ml) to the coculture does not prevent the motor neuron loss induced by Nr1d1-siRNA treated astrocytes. Cocultures were treated at the time of motor neuron plating with (+TF) or without (−TF) trophic factors, and motor neuron survival was determined 72 h later. Data were obtained from at least three independent coculture experiments, two or three replicas per coculture. For all panels, data are expressed as percentage of NC-siRNA control mean ± SD (*$p < .05$, significantly different from NC-siRNA-treated cells)

decrease in motor neuron survival was also observed in cocultures with transdifferentiated astrocytes pretreated with Nr1d1-siRNA, compared to cocultures with NC-siRNA-treated astrocytes.

4 | DISCUSSION

Astrocyte-mediated motor neuron death is a feature observed in many ALS models[24,25,27,29,30,41,42,43,44,46] and strategies aimed at curbing this neurotoxic phenotype have been shown to increase motor neuron survival when translated into ALS mouse models.[35,45,47,48,49] Here we show that NR1D1 (also known as Rev-erbα) expression is significantly downregulated in the spinal cord of symptomatic mice expressing mutant hSOD1 and that this decrease in NR1D1 expression could potentially impact the way astrocytes interact with motor neurons.

In FALS and SALS patients, reactive astrocytes surround both upper and lower degenerating motor neurons, as well as the region where the descending fibers of corticospinal tracts enter the gray matter.[50-53] The astrocyte reactivity observed in ALS patients is recapitulated in mutant hSOD1 transgenic rodents.[32,54,55,56,57,58,59] Reactive astrocytes in patients and ALS animal models express inflammatory makers such as cyclooxygenase-2 (Ptgs2) and inducible nitric oxide synthase (Nos2 or iNos).[19-22] Many of these markers are under the direct control of the transcription factor NF-κB and activation of the canonical NF-κB pathway is a prominent feature of activated glia in ALS patients and animal models.[23,27,60] Moreover, translational profiling of astrocytes from hSOD1^{G37R}-overexpressing mice identified upregulation of inflammatory genes and downregulation of metabolic genes as the earlier changes observed.[23]

Since silencing NR1D1 expression in astrocytes causes an inflammatory profile similar to the one seen in ALS animal models, our data suggest that the decrease in NR1D1 expression observed in symptomatic ALS mice may be contributing to the altered inflammatory profile of ALS astrocytes. Moreover, the consequence of this decrease in NR1D1 expression is likely detrimental, since the phenotype induced by silencing NR1D1 in nontransgenic spinal cord murine astrocytes causes a decrease in the survival of cocultured motor neurons in the absence of an exogenous noxious stimulus. Since motor neurons in coculture with these astrocytes cannot be rescued with the addition of exogenous trophic factors, this observation likely reflects the acquisition of a neurotoxic phenotype in Nr1d1-silenced astrocytes, rather than a decrease in trophic support. Moreover, this negative effect of decreased NR1D1 expression in astrocyte–neuron interaction is also observed in human transdifferentiated astrocytes, suggesting that it could potentially contribute to motor neuron toxicity in the context of ALS pathology.

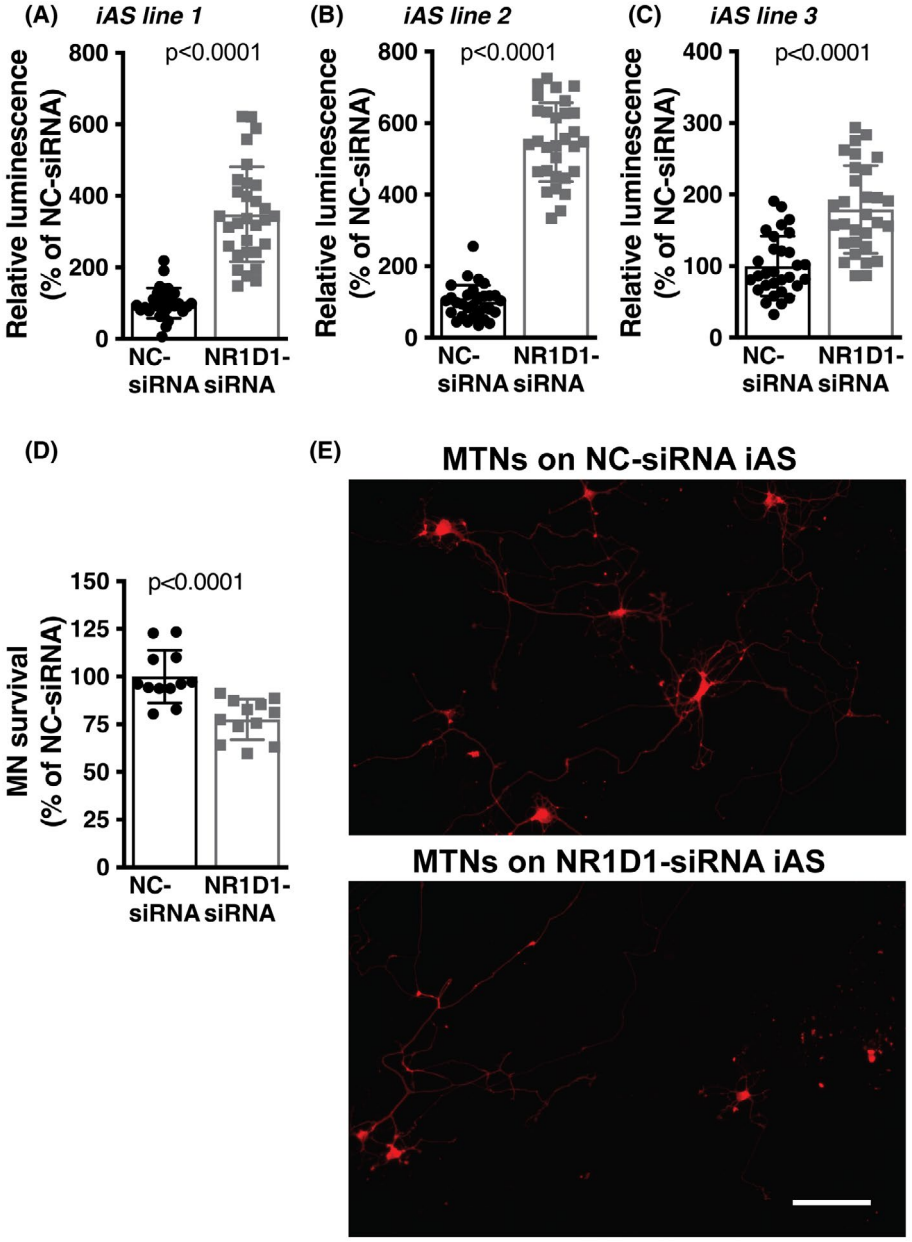

FIGURE 7 NR1D1 downregulation in transdifferentiated human astrocytes is detrimental to cocultured motor neurons. (A, B, C) Relative luminescence produced by a firefly luciferase expressed under an NF-κB-driven promoter 48 h after NC-siRNA or Nr1d1-siRNA treatment of three different transdifferentiated human astrocytes (iAs) lines derived from control (nondisease) healthy subjects. Relative firefly luciferase luminescence was corrected by the amount of *Renilla* luciferase activity controlled by a constitutive promoter and expressed as a percentage of NC-siRNA-treated cells. (D) Control iAs (line 1) were treated with NC-siRNA or Nr1d1-siRNA. Forty-eight hours later embryonic motor neurons were plated on top. Motor neuron (MN) survival was determined 72 h later. Data were obtained from four independent coculture experiments, three replicas per coculture. For all panels, data are expressed as percentage of NC-siRNA control (mean ± SD). (E) Representative images showing motor neurons cultured on the top of NC-siRNA or Nr1d1-siRNA-treated iAs. Scale bar: 20 μm

NR1D1-KO mice display spontaneous glial activation in the hippocampus and dysregulated NF-κB signaling.[13] In addition, primary neurons exposed to conditioned media from mixed glia cultures treated with an Nr1d1-siRNA are more susceptible to hydrogen peroxide toxicity than neurons cultured in conditioned media from control glia.[13] These results suggest that glia NR1D1 deficiency may impact neuronal health in response to oxidative stress in vitro. On the other hand, pharmacological inhibition or genetic knockdown of NR1D1 stimulates microglial amyloid-β clearance and reduces amyloid plaque deposition in the 5XFAD mouse model of Alzheimer's disease.[61] While these observations reflect that NR1D1 may likely have a complex role in glia biology, they also point

at the potential therapeutic role of NR1D1 manipulation during neurodegeneration.

Circadian rhythm coordinates different physiological functions to recurring daily environmental changes like light/dark cycles and food availability.[62] The ability to anticipate these changes increases the fitness of the organism, while impairment of normal circadian rhythmicity leads to a range of metabolic defects.[63-66] Beyond progressive motor impairment, patients with ALS suffer from major, yet incompletely characterized, defects in energy metabolism.[67,68] Since NR1D1 appears to coordinate metabolism and circadian rhythms, it is plausible that NR1D1 dysregulation contributes to the metabolic and redox impairment observed in ALS patients and mouse models.[69-82] Moreover, based on the current understanding of the role of astrocytes in circadian rhythm,[83-86] it would be important to determine the potential effect that circadian rhythms have in astrocyte–neuron metabolic and redox coupling.

Overall, our data suggest that NR1D1 downregulation promotes a pro-inflammatory response in astrocytes that is ultimately detrimental for motor neuron survival. Thus, modulating NR1D1 expression could be a potential therapeutic approach to prevent astrocyte-mediated motor neuron toxicity in the context of ALS pathology.

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DISCLOSURES

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Kelby M. Killoy, Benjamin A. Harlan, Mariana Pehar and Marcelo R. Vargas performed the experiments. Kelby M. Killoy, Mariana Pehar, and Marcelo R. Vargas analyzed the data and wrote the paper. All authors reviewed and approved the content of the manuscript.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article. The data are available from the corresponding author upon reasonable request.

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