Nitric Oxide-Induced Neuronal to Glial Lineage Fate-Change Depends on NRSF/REST Function in Neural Progenitor Cells

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INTRODUCTION

Neural stem/progenitor cells (NPCs) are characterized by their ability to divide and differentiate into the three major cell types that constitute the mammalian brain: astrocytes, oligodendrocytes, and neurons [1]. In the normal adult mammalian brain, NPCs originating from the subventricular zone (SVZ) give rise to neuroblasts, which then migrate to the olfactory bulb and striatum [2, 3]. However, upon cell loss in central nervous system (CNS) tissue, NPCs migrate toward the site of injury. These areas have been shown to possess a substantial number of newly formed oligodendrocytes and astrocytes, whereas neurons are rarely generated at these sites [3, 4]. This suggests that the local environment is influencing specific NPC lineage fate decisions during neuroinflammatory conditions. NPCs of the SVZ are located in close proximity to the ventricle system and are generally believed to be exposed to the cerebral spinal fluid (CSF) and its contents. Moreover, a substantial portion of CSF cycles through the brain interstitial space during normal conditions, indicating constant CSF-contact to all brain regions including other NPC populations [5]. During neuroinflammatory conditions, such as multiple sclerosis (MS) and neurotrauma, close access to CSF that contains inflammatory components will potentially influence NPC-lineage fate decisions.

The free radical nitric oxide (NO) is produced in various tissues and functions normally both as a mediator of neural transmission as well as a regulator of other physiological events, such as vascular toning and adult neurogenesis [6–8]. However, during innate immune responses, NO exerts other functions as it is released in excessive amounts through induction of the inducible form of nitric oxide synthase (iNOS) [9]. In contrast to the other two isoforms of NOS (endothelial NOS; eNOS and neuronal NOS; nNOS), the production of NO via iNOS-synthase in various brain regions including other NPC populations [5]. During neuroinflammatory conditions, such as multiple sclerosis (MS) and neurotrauma, close access to CSF that contains inflammatory components will potentially influence NPC-lineage fate decisions.

Degeneration of central nervous system tissue commonly occurs during neuroinflammatory conditions, such as multiple sclerosis and neurotrauma. During such conditions, neural stem/progenitor cell (NPC) populations have been suggested to provide new cells to degenerated areas. In the normal brain, NPCs from the subventricular zone generate neurons that settle in the olfactory bulb or striatum. However, during neuroinflammatory conditions NPCs migrate toward the site of injury to form oligodendrocytes and astrocytes, whereas newly formed neurons are less abundant. Thus, the specific NPC lineage fate decisions appear to respond to signals from the local environment. The instructive signals from inflammation have been suggested to rely on excess levels of the free radical nitric oxide (NO), which is an essential component of the innate immune response, as NO promotes neuronal to glial cell fate conversion of differentiating NPCs in vitro. Here, we demonstrate that the NO-induced neuronal to glial fate conversion is dependent on the transcription factor neuron-restrictive silencing factor-1 (NRSF/REST) chromatin modification status of a number of neuronal and glial lineage restricted genes was altered upon NO-exposure. These changes coincided with gene expression alterations, demonstrating a global shift toward glial potential. Interestingly, by blocking the function of NRSF/REST, alterations in chromatin modifications were lost and the NO-induced neuronal to glial switch was suppressed. This implicates NRSF/REST as a key factor in the NPC-specific response to innate immunity and suggests a novel mechanism by which signaling from inflamed tissue promotes the formation of glial cells.
cells is induced by the JAK/STAT pathway and regulated at a transcriptional level [10, 11]. This accounts for a relatively slow production, but in excessive amounts that can be 1,000-fold higher than the NO released under normal noninflammatory conditions [10]. While excessive amounts of NO exert antibacterial, antiparasitic, and tumoricidal effects, induction of iNOS in microglia and astrocytes within the CNS induces damage to neurons and oligodendrocytes [12–15]. Importantly, NO production is excessively increased in the CNS under various neuroinflammatory conditions, such as MS, meningitis, and neurotrauma [16–20]. For example, in MS lesions iNOS expression is increased in astrocytes and, interestingly, the level of NO production in the human CSF correlates with disease severity [19, 21, 22]. Accordingly, this raises the question of how pathologically relevant levels of NO affect the NPC population of the adult brain. Previously, we showed that adult rat NPCs that had been exposed to clinically relevant but pathological levels of NO in vitro were less prone to form neurons [23]. Relatively short exposure induced fate changes from proneuronal to proglial fate, as demonstrated by the downregulation of proneural gene Neurogenin 2 (Ngn2) and the concentration-dependent reduction of neuron formation in the differentiated cultures [23].

The transcription factor NRSF/REST (neuron-restrictive silencing factor-1/repressor element-1 silencing transcription) has been shown to play an essential role in the regulation of neurogenesis [24, 25]. NRSF/REST is expressed in NPCs and in non-neuronal tissues during embryonic development, whereby it acts as a repressor of neuronal genes [26]. As the NPCs differentiate toward the neuronal lineage, NRSF/REST is downregulated. Interestingly, recent studies in developing rodent brain demonstrate the requirement of NRSF/REST for oligodendrocyte differentiation and formation of the proper ratio of neurons and glial cells [27, 28]. NRSF/REST regulates target genes through the binding to specific RE-1 (repressor element-1) sites and the recruitment of cofactors including histone deacetylases, demethylases, and methyltransferases, which induce changes in chromatin status and nucleosome repositioning [29]. A number of different histone modifications have been coupled to NRSF/REST function, both of which are associated with active and repressed chromatin such as acetylation and trimethylation of Lysine 27 on Histone 3 (H3K27Ac and H3K27me3) [29, 30].

Here, we show that NRSF/REST is upregulated in rat and human primary NPC cultures that have been exposed to pathological levels of NO. This coincides with alterations in chromatin status and gene expression of a number of neuronal and glial lineage-specific genes, demonstrating a shift toward glial potential of the NPC cultures. The changes in histone modification status were reversed by the introduction of a dominant negative version of NRSF/REST (dnREST). We provided functional evidence that NRSF/REST is required for the NO-induced neuronal to glial fate change in differentiating rat NPCs, suggesting a role for NRSF/REST in the innate immunity-driven NPC response.

**Materials and Methods**

**Rat Neural Progenitor Cell Cultures, NO-Exposure, and Transfection**

Neural progenitor cells were isolated from adult Dark Agouti (DA) rats (Scanbur B&K, Sollentuna, Sweden, http://www.scanbur.eu/) and cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium (GIBCO cat no 31331-028, Life Technologies, Carlsbad, CA, www.lifetechnologies.com) with B27 supplement without vitamin A (Life Technologies, cat no 12587-010), Penicillin-Streptomycin (100 U/ml), 20 ng/ml of epidermal growth factor (EGF), and 10 ng/ml of Basic fibroblast growth factor (FGF2) (FGF2 and EGF; added every second day). For all experiments, cells had undergone two passages before they were plated onto poly(o-lysine) (PDL) glass coverslips. For NO-exposure, we used the NO-donor DETA-NONOate (Alexis Biochemicals, Goteborg, Sweden, http://www.axoxa.com/) in a total concentration of 0.1 mM (corresponding to approximately 500 nM of NO) for 4–20 hours (see reference for measured NO concentration) [23]. As control medium, we used 0.1 mM of depleted DETA-NONOate (incubated for 10 days in +4 °C before usage). After NO-exposure, cells were either processed for analysis (immunohistochemistry, Western blot, ChIP analysis, or qPCR) or cultured for 5 days under differentiation conditions (DDC) (containing 1% Fetal calf serum (FCS) and EGF, FGF withdrawal) before being processed for immunohistochemistry. For transfection experiments, we used Lipofectamine 2000 (Invitrogen, Life Technologies) and 300 ng/30,000 cells of either pCAGG-dnRESTmyc, pCAGG-REST, or pCAGG-GFP expression constructs [31] for 12–15 hours directly after NO-exposure but before initiation of differentiation conditions. For siRNA experiments, we used 20 pmol siRNA/10,000 cells of either two different NRSF/REST siRNA (Silencer Select Predefined siRNA, siRNA id s136125, and siRNA id s136124, 10 pmol of each used/10,000 cells, cat no 4390771, Life Technologies) or control siRNA (Silencer Select Negative Control No. 1, cat no 4390843, Life Technologies). Transfected cells were analyzed by immunohistochemistry either directly after transfection or after 5 DDC. All animal experiments were carried out according to the Karolinska Institute rules and guidelines for animal care. The protocol was approved by the Stockholm Animal Ethics committee (permit number 379/10).

**Quantitative Polymerase Chain Reaction**

QPCR procedures have been previously described [23]. Primers used for NRSF/REST detection were: Rat; Forward: 5'‐AGCAGATA CCACTGGCCGAA, Reverse: 5'‐CGCATGTTGCTAGTAGAT, Human; Forward: 5’‐TCGATGGTGGCCAAAATTAC, Reverse: 5’‐CAGCAGCTGTCCTTATCT; Rat Ngn2; Forward: 5’‐CCA ACT CCA GTT CCC CAT AC, Reverse: 5’‐GAG GTG CAT AAC GGT GCT TCT C, and Ascl1: Forward: 5’‐ACCCCTACTAGTCCAGAAG A, Reverse: 5’‐CTGCTGGTCT GTTTGGT; rat Sox9: Forward: 5’‐TCTCTTAAGCCCATCTTCAAGG; Reverse: 5’‐AGCTTGAC CTGTC TTTTG; rat Olig2: Forward: 5’‐TTAACAGAGACCCGAC CAAC, Reverse: 5’‐AGAC GATCA GCGTTCTG GAAG; rat NeuroD1: Forward: 5’‐TCTGAGATATGGAATGTCAC, Reverse: 5’‐TCTGATCATTGCTTCAAGC; rat Hes1: Forward: 5’‐CAGA AAGTC ATCAA GCTATCATG, Reverse: 5’‐TCGATGGTTTCAGTGGC TCAAC. The relative expression of the target genes was expressed as the quantity ratio of the respective target gene and housekeeping gene for each individual sample. The housekeeping genes used throughout included β-actin or 18S.

**Western Blot**

Rat NPCs (500,000 cells/experiment) were lysed with 100 μl RIPA buffer (cat no R0278, Sigma-Aldrich, Buchs, Switzerland, www.sigmaaldrich.com) including protease inhibitors (Complete, Cat no 13006200, Roche Diagnostics, Rotkreuz, Switzerland, 2540 NO-Induced NPC Fate-Change Is Mediated by REST RIPA buffer (cat no R0278, Sigma-Aldrich, Buchs, Switzerland, www.sigmaaldrich.com) including protease inhibitors (Complete, Cat no 13006200, Roche Diagnostics, Rotkreuz, Switzerland, 2540 NO-Induced NPC Fate-Change Is Mediated by REST
www.roche-diagnostics.com). Samples were run on NuPAGE 3%–8% gels (Invitrogen, cat no. EA03752) with running buffer Novex Tris-Acetate (Invitrogen, cat no. LA0041) according to the manufacturer’s recommendation. Proteins were transferred to a membrane using the iBlot system (Invitrogen, cat no. IB3010-01), developed and visualized using ECL Select Detection Reagent (cat no. RPN 2235, GE life science, Uppsala, Sweden, www.gelifescience.com) and Luminescent Image Analyzer (Image Quant LAS 4000, GE Life Science). Antibodies used for Western blot were anti-REST (Millipore, Billerica, MA, www.millipore.com, 2 μg/ml) and anti-Actin (Sigma, 1:10,000).

Cell Death Assay
Cells were fixed with 2% paraformaldehyde for 20 minutes at RT and measured for apoptosis by Click-IT TUNEL Alexa Flour 488 Imaging Assay (Invitrogen, cat no C10245) according to the manufacturer’s recommendation. Apoptotic cells were imaged using florescence microscopy and cell-counting was performed from five individual experiments.

Immunohistochemistry and Microscopy
Cells were fixed in 2% paraformaldehyde for 20 minutes at RT followed by three washes in Phosphate buffered saline (PBS) and incubated with blocking solution 0.3% Triton X-100 (Life Technologies) and 10% FCS in PBS for 20 minutes at RT. Antibodies were diluted in blocking solution including 1% FCS and incubated at 4°C for 8–10 hours. After three washes in PBS, incubation with secondary antibodies (Alexa Flour, Life Technologies) was performed at RT for 1 hour in PBS. Following antibodies were used: mouse anti-Tuj1 (Covance, MRB-435, Covance, Princetown, New Jersey, www.covance.com), rabbit anti-Sox2 (Millipore, cat no AB5603), rabbit anti-Sox3 (gift from J. Muhr, Karolinska Institutet, Sweden), mouse anti-O4 (MAB345, Millipore, Billerica, MA, www.millipore.com), rabbit anti-glia fibrillary acidic protein (GFAP) (Dako, cat no Z0334, Dako, Glorstrup, Denmark, www.dako.com), mouse anti-Ki67 (Abcam, Ab16667, Abcam, Cambridge, UK, www.abcam.com), goat anti-Olig2 (R&D Systems, cat no AF2418, R&D Systems, Minneapolis, MN, www.rndsystems.com), and mouse anti-NeuN (Chemicon, cat no MAB377, Chemicon, Millipore). All images were received using confocal microscope LSM 5 EXCITER (Carl Zeiss, Oberkochen, Germany, www.zeiss.com). Graphs indicate the mean value of the total cell number in percentage and SD from 5 to 12 individual experiments if not stated otherwise. Statistical analysis was performed using two-tailed Student’s t test, ***, p < .001; **, p < .01; *, p < .05.

Human Cell Cultures
Tissue samples from two adult patients that had undergone temporal lobe resection for treatment of epilepsy were received and samples for experiments were restricted to the wall of the lateral ventricle (SVZ area). Both patients underwent MR scans and pathology screening to exclude tumors and were screened for infectious diseases. The used tissue was considered biological waste and would otherwise be discarded. The research protocol was approved by the local ethical committee (permit number 01–294). Biopsies were transported in Hibernate-A medium (GIBCO, cat no A12475) from operation theater. The tissue was dissociated mechanically with a scalpel followed by ready-to-use Accutase (Sigma-Aldrich, cat no A6964) in 37°C for 10 minutes. Propagation culture conditions used were DMEM/F-12 medium (GIBCO cat no 31331-028) with B27 supplement without vitamin A (Life Technologies, cat no 12587-010), HEPES buffer (1 M, 0.9%, Gibco), Penicillin-Streptomycin (100 U/ml), 20 ng/ml of EGF, and 10 ng/ml of FGF2 (added two to three times weekly) and have been described previously [32]. After 4–6 weeks in propagation conditions, human neuro spheres were dissociated into single cells by Accutase, plated on PDL-coated coverslips in 50 μl droplets, and left for 24 hours to attach following NO-exposure via DETA-NONO-ate 0.1 mM according to description for rat cells. Cultures were either analyzed directly after 24 hours of NO-exposure or left for 11 days under differentiation conditions (propagation medium containing 1% FCS and EGF, FGF2 withdrawal) before fixation with 2% paraformaldehyde for 20 minutes at RT and immunohistochemistry analysis.

Chromatin Immunoprecipitation
ChiPs were performed using Millipore ChiP assay kit (cat no 17–295, Millipore) and the procedures have been described elsewhere [33]. Antibodies used were anti-H3K27me3 (cat no C15410069, Diagenen, Liege, Belgium, www.diagenen.com), anti-H3K27Ac (cat no C15410174, Diagenen), and anti-H3 (cat no C15310135, Diagenen). Detection of ChiP signals was done by qPCR (Rotor Gene RG-3000A, Corbett Life Science, Mortlake, NSW, www.corbettlifescience.com) using SYBR Green (Kapa Biosystems, Wilmington, MA, www.kabapisosystems.com). Primers used for detection were: rat Sox9; forward 5’-TGCTGAAAGGAGACCGAGAA, reverse 5’-GGGGTGCTTCTTGGTC; Hess; forward 5’-CAGCTCCAGATCGTGGTA, reverse 5’-AAGTTCCTCACGAGCCGT; NeuroD1; forward 5’-TACCCCTCTCAGTTACCC, reverse 5’-ATAGCAGGTCAGTGTC; NGN2; forward 5’-CTTCGGCCACCTCCTAGTG, reverse 5’-AACCCAGCAGCATCAGTACC; Ascl1; forward 5’-GCTCTTGGCAAATCTCCAT, reverse 5’-TGGGATTCCGCGGAGGT; Olig2; forward 5’-CGAAGGCCTAGATCGTCTCCG, reverse 5’-CACGTTCCCTCTGTAAGGC; TNF; forward 5’-GGGTGAATGAGAGCTTTTCC, reverse 5’-ATAGCAGGTCAGTGTC. Signals are presented as 2^{Ct Sample} \div 2^{Ct IgG} = 2^{Ct Sample - Ct IgG} from one representative experiment out of four.

Results

NRSF/REST is Upregulated in NO-Exposed NPCs
To better understand how NO can act as an instructive cue in causing inflammation-induced NPC fate-change, we aimed to characterize the mechanism for the reduced neurogenesis [23]. Since the transcription factor NRSF/REST is known to prevent premature expression of neuronal genes in NPCs and it has been shown to be required for oligodendrocyte development in neonatal rat CNS [25, 27, 34], we performed a series of experiments to investigate the involvement of NRSF/REST in the changed neuronal/glial ratio observed in the NO exposed NPC cultures. NPCs from SVZ of adult DA rats were exposed to the NO-donor DETA-NONO-ate 0.1 mM or control medium (containing 0.1 mM of depleted DETA-NONO-ate) for 20 hours followed by fixation and characterization. For the used cell culture conditions, 0.1 mM DETA-NONO-ate corresponds to approximately 500 nM NO that sub-sequentially decreases to undetectable levels over a period of 24 hours [23]. Interestingly, we detected a more than twofold increase in the expression of NRSF/REST already 4 hours after initiation of NO-
exposure and after 20 hours of NO-exposure, \textit{NRSF/REST} expression was increased more than threefold compared to control cultures (Fig. 1A). Furthermore, Western blot analysis confirmed an increase in \textit{NRSF/REST} protein in rat NPCs cultures after 20 hours of NO-exposure versus control medium. (C, D): Brightfield photos of NPC cultures under control conditions (D) or after NO-exposure via DETA-NONO:ate 0.1 mM (C). (E–J): Expression of Sox2 (E, F, G) and cell-cycle marker Ki67 (H, I, J) was not changed in cells exposed to NO compared to control cells. Results are presented as mean ± SD of 6–10 experiments. Bars = C, D, 60 μm; E, F, H, I, 15 μm. Abbreviations: NRSF, neuron-restrictive silencing factor-1; REST, repressor element-1 silencing transcription.

Increased Number of Oligodendrocytes After NO Exposure

We have previously reported that NO-exposure to NPCs leads to decreased neuronal differentiation paralleled by an increase in astroglial differentiation, demonstrated by an increase in GFAP protein levels [23]. Since the focus in the previous study was on measuring protein levels, we now investigated the number of astrocytes and oligodendrocytes within the cultures. Following 5 days of differentiation of the NO-exposed NPC cultures, the percentage of cells expressing the oligodendrocyte marker O4 was increased in NO-exposed cultures compared to control (18% ± 4% and 8% ± 3% O4+ cells, respectively) (Fig. 2B, 2E, 2N). Furthermore, also the proportion of cells expressing the oligodendrocyte precursor marker Olig2 was increased after NO-exposure (Supporting Information Fig. S2A–S2F). This suggests that NO-exposed NPCs are more prone to
differentiate toward the oligodendrocyte lineage compared to nonexposed cells. The percentage of cells expressing the astrocyte marker GFAP was 82% ± 7% and 78% ± 9% after NO-exposure and control conditions, respectively (Fig. 2J, 2M, 2P). The reduction in the percentage of neurons after NO-exposure was confirmed by neuronal markers Tuj1 and NeuN (Fig 2F, 2I, 2O and Supporting Information Fig. S2G–S2L). Importantly, the decrease in generated neurons was not due an increased cell-death or an inability of NO-exposed NPCs to initiate the differentiation process, as the majority of NO-exposed and control cells had downregulated the progenitor marker Sox2 after 5 days of differentiation (Supporting Information Fig. S3A–S3F).

Blocking of NRSF/REST Function Rescues the NO-Induced Effect on NPC Differentiation

To determine the involvement of NRSF/REST in the NO-induced neuronal-to-glial fate change, we next examined if the suppression of NRSF/REST activity would restore the neuronal/glial ratio. To examine this we used a dominant negative variant of NRSF/REST (dnREST), which includes the Zn-finger domain of NRSF/REST (aa 203–440) fused to a Myc-tag for detection. The N- and C-terminal repression domains of NRSF/REST had been deleted to avoid repression of target genes, while it retains its DNA binding capacity to the RE-1 target.
Thus, dnREST competes with endogenous NRSF/REST for the binding to the RE-1 sites and thereby impedes NRSF/REST from carrying out its function. Following 20 hours of NO-exposure, rat NPCs were transiently transfected with dnREST expression construct or a control green fluorescent protein (GFP)-vector. A transfection efficiency of 80% was detected 24 hours post-transfection (Supporting Information Fig. S4A–S4G). After transfection, EGF and FGF were withdrawn and the NPCs were cultured in serum-containing medium and differentiated for 5 days (Fig. 2A). Interestingly, under conditions when the function of NRSF/REST was blocked, the capacity of NO-exposed rat NPCs to differentiate toward the neuronal lineage was restored. After 5 days of differentiation, the proportion of Tuj1 expressing cells in dnREST-transfected populations was comparable to cells cultured in the absence of NO (Fig. 2B–2I, 2O). This event was not observed in the GFP-transfected control cultures (Fig. 2H, 2O) where the effect from NO-exposure was comparable to nontransfected NO-exposed cultures (Fig. 2F, 2O). Furthermore, during conditions when NRSF/REST siRNA was cotransfected with a NRSF/REST expression vector (entitled “REST”), the proportion of Tuj1-expressing cells was further decreased (4% ± 3%) and was lower than in those cultures transfected with NRSF/REST siRNA only (Fig. 3D, 3E). In addition, we observed a trend of increased percentage of O4-expressing oligodendrocytes in the siRNA-transfected cultures compared to control siRNA (9% ± 5% and 17% ± 7%; p-value = .0514) (Fig. 3F–3I), whereas the proportion of O4-positive cells in the REST cotransfected rescue experiment was 15% ± 4% (Fig. 3H, 3I). The number of GFAP expressing astrocytes remained unchanged (Fig. 3J–3M). Altogether, these results suggest that the effects achieved by decreasing the levels of NRSF/REST are similar to those when NRSF/REST activity was blocked and thus implicate NRSF/REST as a mediator of NO-induced NPC differentiation.

![Figure 3. Increased number of neurons after NRSF/REST siRNA transfection.](image)

(A): Western blot analysis shows downregulation of NRSF/REST protein in rat neural stem/progenitor cell cultures after 20 hours of nitric oxide (NO)-exposure and NRSF/REST siRNA transfection compared to control siRNA or nontransfected cultures. (B–E): Tuj1 expression after NO-exposure and transfection of NRSF/REST siRNA (B), control siRNA (C), or cotransfection of NRSF/REST siRNA and NRSF/REST expression vectors (D). (F–I): O4 expression after NO-exposure and transfection of NRSF/REST siRNA (F), control siRNA (G), or cotransfection of NRSF/REST siRNA and NRSF/REST expression vectors (H). (J–M): GFAP expression after NO-exposure and transfection of NRSF/REST siRNA (J), control siRNA (K), or cotransfection of NRSF/REST siRNA and NRSF/REST expression vectors (L). Results are presented as mean ± SD of four experiments, **, p < .01 (Student’s t test). Bars = B–D, F–H, J–L, 25 μm. Abbreviations: GFAP, glial fibrillary acidic protein; REST, repressor element-1 silencing transcription factor.
Altered Chromatin Modifications Are Reversed After Blocking of NRSF/REST Function

In order to understand the molecular mechanisms underlying the neuronal to glial fate-change after NO-exposure, we investigated chromatin modifications and expression levels of genes that are specifying the neuronal and glial lineages. The examined genes included the proneural factors Neurog2 (Ngn2), Ascl1, and NeuroD1 as well as the glial lineage genes Olig2 and Sox9 [35, 37]. In the analysis, we also included Hes1, which blocks proneural gene expression, and thus plays an important role in the acquisition of the glial lineage [36]. Most of these genes (Ascl1, NeuroD1, Olig2, and Hes1) have previously been reported to be bound by NRSF/REST in mouse embryonic stem (ES) cells, where RE-1 sites can be found within the reported NRSF/REST binding areas for NeuroD1 and Hes1 [30, 38]. Moreover, several of the genes have also been reported to be bound by NRSF/REST in NPCs (Ascl1, NeuroD1, and Hes1) [38, 39]. In addition, expression levels of Hes1, Olig2, and Sox9 were shown to be upregulated following reduction in NRSF/REST levels in a NPC like cell line (NT2) [30]. Thus, previous studies indicate direct as well as indirect NRSF/REST involvement of regulation of these genes. We performed ChIP-qPCR experiments targeting the repressive histone modification mark H3K27me3, as well as the activating modification H3K27Ac, both of which previously have been connected to NRSF/REST activity [29, 30]. Acetylation and methylation of H3K27 are mutually exclusive events at promoter sites of active and silent genes, respectively [40, 41], and we therefore focused on the promoter regions of the investigated genes. Interestingly, within the promoter regions of the tested neuronal lineage restricted genes, Ngn2, NeuroD1, and Ascl1, and for the proneural blocking gene Hes1 we found H3K27me3 levels to be increased, whereas the levels of the active mark H3K27Ac were decreased after NO-exposure (Fig. 4A, 4B). Furthermore, in NO-exposed cultures where NRSF/REST function was blocked by dnREST transfection, the changes in H3K27me3 and H3K27Ac levels were less dramatic, resembling those levels observed in the control cultures (Fig. 4A, 4B). However, within the promoter regions of the tested glial restricted genes, Sox9 and Olig2, the situation was opposite and H3K27me3 levels were decreased, whereas H3K27Ac levels were found to be increased. Also for the glial-restricted genes these histone modification changes were reversed by transfection with dnREST. We did not identify any NO-induced histone modification changes within the negative control region; the promoter region of the cytokine gene TNF (tumor necrosis factor) (Fig. 4A, 4B). Furthermore, no significant changes in expression levels of these genes were observed. However, there was a trend that corresponded to increased/decreased levels of H3K27me3 and H3K27Ac; Ngn2, Ascl1, and Hes1 had decreased expression levels whereas the glial-restricted genes Sox9 and Olig2 had increased expression levels after NO-exposure (Fig. 4C). NeuroD1 levels could not be detected in undifferentiated cultures (Fig. 4C). In conclusion, the increased glial potential in NO-exposed NPCs involves NRSF/REST function at certain glial and neuronal gene promoter regions and suggests NRSF/REST as an important target for effects of innate immunity.

NRSF/REST Is Upregulated in NPCs from Adult Human After NO-Exposure

As the level of NO production in the human CNS correlates with disease activity in various neuroinflammatory conditions [16]-[20, 42] it is reasonable to question whether adult human NPCs are affected by pathological levels of NO. To address this question we analyzed if NRSF/REST was upregulated after NO-exposure also in adult human NPCs and if the differentiation capacity of these cells was altered after NO-exposure. Human tissue biopsies, restricted to the wall of the lateral ventricle (SVZ area), were dissociated and cells cultured as neurospheres during 4–6 weeks following dissociation to single cells and plating onto PDL-coated coverslips. After 24 hours, cells were fixed and immune-reactively labeled for NPC markers Sox2 and Pax6 (Fig. 5A–5D), or exposed to NO via DETA-NONO:ate 0.1 mM (or control medium) in a similar fashion as previously described experiments in rat NPCs. After 24 hours of NO-exposure, a 2.4-fold increase in NRSF/REST expression was detected compared to control cells (Fig. 5E), suggesting a possible function of NRSF/REST after NO-exposure in human NPCs. Moreover, after 11 days of differentiation fewer TuJ1-positive neurons could be detected in the NO-exposed cultures compared to control cultures (2% ± 4% and 26% ± 10%, respectively) (Fig. 5F, 5I, 5H). Cells expressing the astrocyte marker GFAP occurred in both NO-exposed and control cultures (Fig. 5G, 5J, 5K). However, oligodendrocytes expressing O4 could not be detected in the control or NO-exposed cultures after 11 days under differentiation conditions (data not shown). Taken together, these results demonstrate that pathological levels of NO increase the expression of NRSF/REST and affect lineage specification of the neural progenitor population from the adult human brain.

DISCUSSION

After CNS injury and in other neuroinflammatory conditions, the environment within the CNS changes rapidly. Innate immune responses are activated, releasing free radicals and reactive oxygen species in the surrounding tissue [16, 43]. We have previously hypothesized that inflammatory cues may act to direct NPC-fate specification and part of the innate inflammatory response is the excess release of NO via iNOS synthase [9], which has been shown to affect NPCs in vitro by reducing their neurogenic capacity and promoting gliogenesis [23]. Here, we demonstrate that the NO-induced neuronal to glial lineage change in NPCs is regulated at the transcriptional level and depends on the function of the transcription factor NRSF/REST.

Degeneration of CNS tissue commonly occurs during various neuroinflammatory conditions. In MS, for example, oligodendrocyte and myelin destruction lead to demyelination of neuronal axons and subsequent neuronal damage [44]. Moreover, the formation of glial scars after neurotrauma is important to limit the damage and inhibit axonal loss [45, 46]. Glial scar tissue consists of several different cell types where astrocytes derived from resident NPCs constitute a considerable portion [46, 47]. In MS, conversely, replacement of degenerated oligodendrocytes is evident within the MS lesions [48, 49]. This suggests that the affected tissue requires a rapid contribution of glial cells and several studies demonstrate an increased formation of glial cells that migrate from proliferative areas toward the site
of injury [3, 47, 50, 51]. In addition, in mice induced with experimental autoimmune encephalomyelitis, progenitor cells from the SVZ showed less neurogenic capacity, but an increased capacity of generating Olig2-positive oligodendrocyte precursors [52], which is in line with the NPC fate-changes observed in this study after NO-exposure in vitro.

The transcription factor NRSF/REST is required for oligodendrocyte differentiation and the formation of proper ratio of neurons and glia in the developing rodent CNS [27, 28]. Here, we show that NRSF/REST is upregulated in NPC cultures that have been exposed to pathological levels of NO. Furthermore, we present functional evidence that NRSF/REST is required for the NO-induced neuronal to glial fate-change in differentiating rat NPCs. Blocking of NRSF/REST function, as well as reducing the NRSF/REST levels with siRNA in NO-exposed NPCs, reversed the effect from NO, which suggests that NRSF/REST is a key factor in converting the NPC lineage specification from neuronal to glial.

NRSF/REST has an essential role during NPC differentiation in the adult CNS, where one of its functions is to repress proneural gene expression [39]. Here, we show that the chromatin at the promoter regions of Ngn2, Ascl1, Hes1, NeuroD1, Sox9, and Olig2, is set in a repressive state following NO-exposure, demonstrated by an increase in H3K27me3 and decrease in H3K27Ac modifications. In contrast, at the promoter regions of the glial lineage genes, Olig2 and Sox9, NO-exposure instead leads to the formation of an active chromatin state. We noticed a trend in gene expression changes that coincided with the changed levels of H3K27 methylation and acetylation. However, the changes were not significant and were possibly reflecting the parts of the NO-exposed

**Figure 4.** Altered histone modifications at specific gene promoters after nitric oxide (NO) exposure. (A, B): ChIP analysis on rat neural stem/progenitor cells (NPCs) exposed to NO (empty bars), nonexposed (checked bars), or exposed to NO and transfected with dnREST (black bars) was performed using antibodies against H3K27me3 (A) or H3K27Ac (B) for Ngn2, Ascl1, Hes1, NeuroD1, Sox9, and Olig2 promoter regions. The promoter region for the TNF gene has been included as a negative control. Results are presented as fold enrichment over IgG for the specific antibody, relative to the fold enrichment over IgG for H3 from each experiment. Error bars represent the SD of triplicate qPCR measurements from one representative experiment out of four, ***, p < .001; **, p < .01; *, p < .05 (Student’s t-test). (C): Gene expression analysis (Ngn2, Ascl1, Hes1, NeuroD1, Sox9, and Olig2) on rat NPCs exposed to NO, nonexposed, or exposed to NO and transfected with dnREST. NeuroD1 levels could not yet be detected in the NPC cultures. Results are presented as log scale mean ± SEM relative to the control cultures. Abbreviations: REST, repressor element-1 silencing transcription; TNF, tumor necrosis factor.
populations in where cell-fate changes were observed (Fig. 2). We cannot exclude the possibility that alterations in gene expression and histone modifications at specific gene promoters may depend on the changes in expression of NPC genes rather than a direct effect of NRSF/REST. For instance, previous reports suggest that Sox9 is indirectly regulated by NRSF/REST via miR-124, whereby NRSF/REST repression of miR-124 allows Sox9 expression, which in turn promotes glial differentiation [53, 54]. Thus, it is reasonable that the increased Sox9 levels after NO-exposure are mediated through miR-124 repression by NRSF/REST. Furthermore, the regulation of Ngn2 could also be an indirect event since the binding of NRSF/REST to this gene has not been identified in any cell-type as far as we know. However, a low affinity NRSF/REST binding motif has been identified within this gene, suggesting that NRSF/REST activity could include additional binding motifs and target genes [55]. Nevertheless, together our data provide evidence for the involvement of NRSF/REST in establishing glial commitment in NO-exposed NPCs.

CONCLUSION

Our results demonstrate that pathological levels of NO promote the expression of NRSF/REST, which affect lineage specification of the neural progenitor population from the adult brain. In conclusion, our findings implicate NRSF/REST as a key factor in the crosstalk between the NPC population and innate immunity and suggest a molecular mechanism by which signaling from inflamed tissue regulates the lineage fate of NPCs of the adult CNS.

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AUTHOR CONTRIBUTION

M.B.: conception and design, financial support, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; R.C.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, and final approval of manuscript; C.P.E.: data analysis and interpretation and final approval of manuscript; M.S.: financial support, provision of study material, manuscript
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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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