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A Nurr1/CoREST transrepression pathway attenuates neurotoxic inflammation in activated microglia and astrocytes

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Abstract

Nurr1, an orphan nuclear receptor, plays an essential role in the generation and maintenance of dopaminergic neurons in the brain. Rare mutations in *Nurr1* are associated with familial Parkinson's disease, but the underlying basis for this relationship has not been established. Here, we demonstrate that Nurr1 unexpectedly functions to inhibit expression of pro-inflammatory neurotoxic mediators in both microglia and astrocytes. Reduced Nurr1 expression results in exaggerated inflammatory responses in microglia that are further amplified by astrocytes, leading to the production of factors that cause death of tyrosine hydroxylase-expressing neurons. Nurr1 exerts anti-inflammatory effects by docking to NF- κ B-p65 on target inflammatory gene promoters in a signal-dependent manner. Subsequently, Nurr1 recruits the CoREST corepressor complex, resulting in clearance of NF- κ B-p65 and transcriptional repression. These studies suggest that Nurr1 protects against loss of dopaminergic neurons in Parkinson's disease in part by limiting the production of neurotoxic mediators by microglia and astrocytes.

INTRODUCTION

Parkinson's disease (PD) is the most prevalent movement disorder among people over 65 years old. Denervation of dopaminergic neurons in the substantia nigra (SN) results in severely debilitating motor symptoms such as bradykinesia, resting tremor and rigidity (Farrer, 2006; Fearnley and Lees, 1991). Although the etiologies of most common forms of PD remain poorly understood, the disease is generally associated with an inflammatory component that is

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manifested in part by the presence of activated microglia (central nervous system-resident macrophages) and elevated serum or cerebrospinal fluid levels of pro-inflammatory factors (Block et al., 2007; McGeer and McGeer, 2008; Nagatsu and Sawada, 2005). Several lines of evidence suggest that inflammatory mediators such as tumor necrosis factor (TNF) α , nitric oxide (NO) and Interleukin (IL)-1 β derived from non-neuronal cells, including microglia, modulate the progression of PD (Brown, 2007; Hartmann et al., 2003; Whitton, 2007). Whether inflammation is an initiating factor of PD in humans is unclear, but intracranial infusion of bacterial lipopolysaccharide (LPS), a ligand for Toll-like receptor (TLR)4 and a potent activator of microglia, is sufficient to induce the loss of TH+ neurons in rodents (Meredith et al., 2008). LPS-induced inflammation can also synergize with α -Synuclein and Parkin mutations associated with familial PD to potentiate the loss of tyrosine hydroxylase (TH)+ neurons in animal models (Frank-Cannon et al., 2008; Gao et al., 2008). These observations are consistent with the possibility that environmental factors, such as infection, may interact with common but less penetrant susceptibility genes to influence the onset of most commonly observed sporadic PD cases (Tansey et al., 2007).

TLRs induce inflammatory gene expression by regulating the activities and expression of signal-dependent transcription factors that include members of the NF- κ B, AP-1 and IRF families (Kawai and Akira, 2007). The TLRs play essential roles in innate immune responses to microbial pathogens based on their ability to recognize pathogen-associated molecular patterns (Akira et al., 2006; Medzhitov, 2007). More recently, genetic loss-of-function experiments in mice have shown that TLRs contribute to the pathogenesis of a number of diseases in which inflammation is known to play a pathogenic role, including atherosclerosis, inflammatory bowel disease, and liver fibrosis (Atkinson, 2008). These results are consistent with TLRs being able to signal in response to the generation of endogenously derived ligands, such as components of necrotic cells.

Nurr1 (NR4A2) belongs to the nuclear receptor (NR)4 family of orphan nuclear receptors and is known to function as a constitutively active transcription factor by binding to target genes as a monomer, homodimer or heterodimer with retinoid X receptors (RXRs) (Aarnisalo et al., 2002; Maira et al., 1999; Wang et al., 2003). Deletion of the *Nurr1* gene in mice results in a severe reduction in dopaminergic neurons and perinatal lethality (Zetterstrom et al., 1997), consistent with an essential role for Nurr1 in the development and/or maintenance of dopaminergic neurons. Human mutations resulting in reduced expression of Nurr1 are associated with late-onset familial PD (Le et al., 2003), indicating that Nurr1 may play a protective role.

Nurr1 is also expressed in non-neuronal cell types and *Nurr1* mRNA is induced by inflammatory stimuli, including LPS, in macrophages (Barish et al., 2005; Pei et al., 2005). Intriguingly, recent observations suggest that members of the NR4 family can function as both activators and repressors of cell type-specific inflammatory responses. For instance, Nurr1 suppresses expression of inflammatory response genes in human macrophages that are implicated in the development of atherosclerosis (Bonta et al., 2006). In contrast, Nurr1 promotes the development of mouse and human Th17 T cells that contribute to the pathogenesis of multiple sclerosis (Doi et al., 2008). However, the molecular mechanisms by which Nurr1 controls transcriptional activation or repression of inflammatory responses have not been established, and the potential impact of Nurr1 function on the inflammatory component of PD has not been evaluated.

Here, we present evidence that Nurr1 plays an essential role in both microglia and astrocytes as a signal-dependent transcriptional repressor of genes that encode pro-inflammatory neurotoxic factors. Nurr1 functions as a key component of a negative feedback loop in both microglia and astrocytes by recruiting CoREST co-repressor complexes to NF- κ B target genes.

CoREST complexes, in turn, mediate the turnover of NF- κ B and restore activated gene expression to a basal state. Loss of Nurr1 function in extraneuronal cells of the SN results in exaggerated and prolonged inflammatory responses that accelerate the loss of dopaminergic neurons in response to LPS or overexpression of a mutant form of α -Synuclein (A30P) associated with familial PD (Kruger et. al., 1998).

RESULTS

Nurr1 protects TH+ neurons from LPS-induced inflammation *in vivo*

Studies demonstrating that Nurr1 mRNA is upregulated by LPS in macrophages (Barish et al., 2005; Pei et al., 2005) raised the question of whether it might also be expressed in non-neuronal cells and influence the development of PD. Analysis of Nurr1 protein and mRNA levels in primary human and mouse microglia, primary human astrocytes, and the BV2 microglia cell line demonstrated significant protein expression under basal conditions and induction of Nurr1 mRNA in microglia in response to LPS (Fig. S1A–E and data not shown). Similarly, Nurr1 protein colocalized with the microglia marker F4/80 (Fig. S2E) and Nurr1 mRNA was induced approximately 2-fold in the SN 6h following stereotaxic injection of LPS (Fig. S1F). To investigate the potential role of Nurr1 in PD pathology, we evaluated the impact of reducing Nurr1 expression. Since Nurr1-deficient mice die shortly after birth, we performed stereotaxic injections of lentiviruses encoding two independent shRNAs against Nurr1 (shNurr1-1 and shNurr1-2) or control shRNA (shCtrl) into the SN of adult wild-type mice (Fig S2A). shNurr1-1 and -2 efficiently and specifically reduced Nurr1 mRNA and protein expression in the SN, as determined by qPCR and immunostaining (Fig. S2B–E). A comparison of lentivirus-directed GFP expression with cell-specific markers indicated preferential transduction of non-neuronal cells, including microglia and astrocytes (Fig. S2G). The lentiviral injection was followed two days later by injection of LPS into the same coordinates. We then analyzed the magnitude of the inflammatory response by qPCR 6h after LPS injection and quantified TH+ neurons by immunohistochemistry (IHC) 7 days after LPS injection.

Loss of TH+ neurons following LPS injection normally takes 2–3 weeks (Meredith et al., 2008). However, stereological analysis revealed a significant decrease in TH+ neurons in the SN of shNurr1 lentivirus-injected mice compared to shCtrl-injected animals after only 7 days of LPS treatment (Fig. 1A and B). Interestingly, a pathological morphology of TH+ neurons with reduced or absent processes and alterations in the size and shape of the cells was observed more often in the shNurr1 groups (Supp Fig. 2E and Fig. 1B). In addition, pathological TH+ cells were observed close to activated microglia (Fig. S2F). The accelerated loss of TH+ neurons following Nurr1 knockdown required LPS injection, as it was not observed in buffer (PBS)-injected animals (Fig. 1C and D). In addition, LPS injection was associated with detection of caspase-3 cleavage, suggesting that loss of TH+ cells was due to cell death rather than to loss of TH expression (Fig. S2G) (Sakurada et al., 1999). Reduction of Nurr1 expression in the SN also resulted in exaggerated expression of inflammatory mediators in response to LPS injection, including *iNOS*, *TNF α* and *IL1 β* (Fig. 1E–G).

We also examined whether Nurr1 exerted neuro-protective effects in the context of overexpression of an α -Synuclein mutant (A30P) associated with familial PD by combining stereotaxic injection of shNurr1- or shCtrl-lentivirus with a lentivirus encoding mutant α -Synuclein (A30P). A30P expression alone caused weak inflammation in the SN, whereas reduction of Nurr1 expression in the context of A30P expression resulted in a dramatic increase in expression of numerous inflammatory response genes, including *TNF α* and *IL1 β* , and significant loss of TH+ neurons (Fig. S3A–C and data not shown). In concert, these experiments indicate that Nurr1 limits inflammatory responses in the CNS and protects TH+ neurons from LPS- and α -Synuclein (A30P)-induced toxicity.

Glia-mediated inflammation contributes to the death of TH+ neurons

To define the cell types responsible for LPS-mediated inflammation in the SN, we evaluated the responses of human and mouse microglia, astrocytes and neurons to LPS. These experiments demonstrated that microglia are orders of magnitude more responsive than astrocytes or neurons, exemplified by the pattern of TNF α induction in primary mouse and human microglia and astrocytes and the neuronal Neuro 2A (mouse neuroblastoma) cell line (Fig. 2A, Fig. S4F). These results are consistent with the expression patterns of TLR4, co-receptors and down-stream signaling molecules in neurons and glial cells (Fig. S4A–E). Although, TLR4 expression was virtually absent from the neuronal cell lines examined, we tested whether LPS could directly induce the death of these cells. Three different neuronal cell lines, Neuro2A, SK-N-SH and PC12, were incubated with LPS for 24h. No significant cell death was observed by TUNEL assay or caspase-3 cleavage, in contrast to the effects of TNF α plus cyclohexamide (CHX) treatment (Fig. 2B and Fig. S4G). In addition, knockdown of Nurr1 in Neuro2A cells did not increase the sensitivity to LPS or death signaling (TNF α plus CHX) as determined by TUNEL assay (Fig. 2C).

Based on these results, we evaluated the consequences of reducing Nurr1 expression in microglia on LPS responses. Knockdown of Nurr1 expression in BV2 microglia using specific lentivirus-encoded shRNAs led to significant increases in LPS-dependent expression of inflammatory mediators, including TNF α , iNOS and IL-1 β (Fig. 2D–F). Similar results were observed in the primary mouse (Fig. S5 and S6A–C) and human (data not shown) microglia. To explore whether loss of Nurr1 in microglia resulted in secretion of mediators exhibiting preferential toxicity for TH+ neurons, we knocked down Nurr1 expression using lentivirus-encoded shRNAs in BV2 cells and tested the activity of conditioned media (CM) after LPS stimulation on *in vitro* differentiated neurons and glial cells derived from mouse neuronal stem cells (NSC). CM from shNurr1-BV2 cells resulted in the death of nearly all TH+ neurons, with a significantly smaller effect on gamma-aminobutyric acid (GABA)-positive neurons and no significant effect on glial fibrillary acidic protein (GFAP)-positive astroglial cells (Fig. 2G–I).

Experiments using neuron and glia co-culture *in vitro* suggest that activation of innate immunity in the CNS can trigger neuronal death (Lehnardt et al., 2003). Since NSC-derived neurons always co-exist with astrocytes, it is possible that astrocytes contributed to the neurotoxic effect of the microglia CM. To explore this possibility, we performed sequential CM experiments employing isolated primary microglia and astrocytes and using Neuro2A cells as a read-out for neurotoxicity. Primary murine astrocytes and microglia were infected with shCtrl- or shNurr1-lentivirus used for the injection into the SN. Cells were then stimulated with LPS and CM was harvested as described in Figure 2G. CM of microglia infected with shNurr1 induced significant cell death in Neuro2A cultures, whereas CM of astrocytes infected with shNurr1 had much less effect on the death of Neuro2A cells. Intriguingly, sequential conditioning of media from microglia to astrocytes indicated that astrocytes significantly amplified the production of neurotoxic factors when exposed to microglia-conditioned media (Fig. 2J lane 2 to lane 6 and 7). This effect was further increased when expression of Nurr1 was reduced in astrocytes (Fig. 2J lane 3 to lane 8 and 9). We conclude that microglia are the initial responders to LPS-mediated inflammation and that astrocytes amplify the production of neurotoxic factors after the microglial activation. The knockdown of Nurr1 in either microglia or astrocytes increases the toxicity of CM, suggesting that Nurr1 inhibits the production of neurotoxic factors in both cell types.

Nurr1-mediated transrepression requires GSK3 β -dependent recruitment of Nurr1 monomers to p65

Chromatin immunoprecipitation (ChIP) experiments indicated that Nurr1 was recruited to LPS-responsive promoters following LPS treatment, exemplified by the TNF α promoter (Fig.

3A), suggesting that it was acting locally to repress transcription. Two different general mechanisms of NR-mediated repression have been described: active repression, involving sequence-specific DNA binding, and transrepression, involving tethering of NRs to negatively regulated target genes via protein-protein interactions (Glass and Ogawa, 2006). A mutant of Nurr1 (Nurr1^{C280A/E281A}, CEAA), defective for sequence-specific DNA binding and unable to activate NGFI-B responsive element (NBRE)-luciferase, a reporter for Nurr1 monomer-binding, was fully able to repress *iNOS* induction by LPS (Fig. 3B and Fig. S6D). In addition, mutations directed at the heterodimerization (I-box) domain (Aarnisalo et al., 2002) of Nurr1 (Nurr1^{K555A/L556A/L557A}, KLL) that prevented its ability to activate a Nurr1/RXR-dependent (DR5)-promoter did not interfere with Nurr1-mediated repression of *iNOS* (Fig. 3B). On the other hand, this I-box mutation increased transcriptional activation of Nurr1 monomers through the NBRE element, as previously reported (Aarnisalo et al., 2002) (Fig. S6D).

SUMOylation of NRs has recently been established to play important roles in transrepression (Pascual et al., 2005). Since it is known that Nurr1 interacts with the protein inhibitor of activated STAT (PIAS) 4 (Galleguillos et al., 2004), which is a SUMO E3 ligase, we examined whether SUMOylation is also involved in Nurr1-mediated repression. As shown in Figure 3C, knockdown of Ubc9, an essential E2 enzyme for SUMOylation (Hay, 2005), reversed Nurr1-mediated repression of *iNOS*, suggesting that SUMOylation is required. Next, we confirmed that Nurr1 could be SUMOylated with SUMO2 and SUMO3 using PIAS4 as an E3 ligase (Fig. S6E and F) and found that IL1 β stimulation could induce SUMOylation of Nurr1 in the absence of overexpression of PIAS4 (Fig. S6F). Mutational studies demonstrated that lysine 558 and, to a lesser extent lysine 576, are essential SUMO sites of Nurr1 (Fig. 3D). Since both K558R and K576R mutants are located in the ligand binding domain and close to the I-box and RXR is not required for repression activity (Fig. 3B and Fig. S6D), we hypothesize that SUMOylation is required for monomerization of Nurr1. Consistent with this, K558R and K576R mutants were less able to activate the NBRE reporter and preferentially activated the DR5 reporter (Fig. S6G). In addition, reconstitution of BV2 microglia cells expressing the Nurr1 shRNA-2 with a non-targeted (NT) form of WT Nurr1 reversed hyperactivation of the endogenous *iNOS* and *TNF α* genes, while the non-targeted forms of the SUMOylation mutants (Nurr1 K558R and K576R) did not (Fig. 3E, Fig S6H–I).

Since transrepression requires the tethering of NRs to other transcription factors, we tested whether Nurr1 could bind to transcription factors involved in inflammation, such as NF- κ B. Co-immunoprecipitation (Co-IP) assays of Nurr1 in BV2 cells showed interaction with NF- κ B-p65 that was significantly enhanced by LPS treatment and independent of changes in Nurr1 protein levels (Fig. 3F and Fig. S6A). Phosphorylation of Serine-468 (S468) in p65 is associated with negative regulation of NF- κ B signaling (Buss et al., 2004) and can be mediated by GSK3 β , which is activated following TLR4 stimulation in human monocytes (Martin et al., 2005). Furthermore, inactivation of GSK3 β results in increased NF- κ B-dependent transcription of *TNF α* without changing the kinase activity of the IKK complex or the nuclear translocation of p65 (Buss et al., 2004). Therefore, we hypothesized that S468 phosphorylation of p65 by GSK3 β might provide the docking site for tethering of Nurr1. Consistent with this possibility, the GSK3 β -specific inhibitor SB216763 (SB21) inhibited the interaction of Nurr1 and p65 in BV2 cells in a dose-dependent manner (Fig. 3G and Fig. S7D) and prevented the recruitment of Nurr1 to the *TNF α* -promoter, as determined by ChIP assay (Fig. 3A). To further confirm GSK3 β involvement, we performed *TNF α* -luciferase reporter assays in RAW264.7 cells cotransfected with a kinase-dead mutant of GSK3 β (GSK3 β -K85R mutant, GSK3 β -KD). GSK3 β -KD expression abolished the Nurr1-mediated transrepression of the *TNF α* -promoter in a dose-dependent manner (Fig. S7B). Furthermore, knockdown of GSK3 β completely prevented Nurr1-mediated *iNOS* repression (Fig. 3H). We further validated the contribution of phospho-S468 in p65 by exchanging S468 for alanine (S468A). The p65 S468A mutant, but not wild-type p65, reversed Nurr1-mediated *iNOS* repression in RAW264.7 cells in a dose-

dependent manner (Fig. 3I and Fig. S7E). Finally, GSK3 β stimulated the *in vitro* interaction of Nurr1 with wild-type p65 but not with p65-S486A (Fig. S7C).

The CoREST-repressor complex is required for Nurr1-mediated transcriptional repression

Transcriptional repression requires the recruitment of multiprotein complexes assembled on central scaffolding proteins referred to as co-repressors. We used siRNAs against various candidate corepressors in the *iNOS*-luciferase reporter assay and identified CoREST as being essential for Nurr1-mediated repression (Fig. 4A and Fig. S8A). CoREST has been considered to be dedicated to repression of neuronal genes in non-neuronal cells or early precursors by binding to neuron-restrictive silencer factor (NRSF)/RE1-silencing transcription factor (REST) (Ballas et al., 2005). CoREST assembles many chromatin-modifying enzymes, including histone methyltransferase G9a, histone demethylase, lysine-specific demethylase (LSD1) and histone deacetylase (HDAC) 1 and 2 (Shi et al., 2003). Using Nurr1-mediated repression of *iNOS*-luciferase as an assay, we observed that G9a, LSD1 and HDAC1 were also required for Nurr1-CoREST-mediated repression (Fig. S8B). Using co-IP, we also found that LPS stimulated interaction of Nurr1 and CoREST in BV2 cells (Fig. 4B and Fig. S8C). Although the CoREST complex consists of many proteins, the interaction between Nurr1 and CoREST seemed to be direct, as indicated by *in vitro* GST-pull down assay (Fig. S8D). This interaction was mediated by the DNA-binding domain of Nurr1 (Nurr1-DBD) (Fig. S8E). When Nurr1-DBD was overexpressed in HeLa cells, the interaction between Nurr1 and CoREST was inhibited in a dose-dependent manner (Fig. S8F). Furthermore, overexpression of the Nurr1-DBD in RAW264.7 cells altered Nurr1-mediated repression of *iNOS*-promoter activity (Fig. S8G).

Since Nurr1 can be phosphorylated by serine/threonine kinases (Nordzell et al., 2004), we speculated that signal-dependent phosphorylation might contribute to the Nurr1-CoREST interaction. Nemo-like kinase (NLK) received our attention because NLK is known to be involved in the repression of various transcription factors (Yasuda et al., 2004). NLK cooperates with TGF β -activating kinase 1 (TAK1) and homeodomain-interacting kinase 2 (HIPK2) in Wnt signaling (Kanei-Ishii et al., 2004). Knockdown of NLK abolished Nurr1 repression of *iNOS*-promoter activity, whereas HIPK2 knockdown was much less effective and TAK1 had no effect (Fig. 4C and Fig. S9A). Furthermore, overexpression of kinase-dead NLK (NLK^{K155M}, NLK-KD) in RAW264.7 cells inhibited Nurr1-mediated repression of *iNOS* in a dose-dependent manner (Fig. S9B). Kinase assays showed that Nurr1, but not CoREST, could be phosphorylated by active NLK *in vitro* (Fig. 4D). Finally, Nurr1-CoREST interaction was significantly reduced by NLK-knockdown in BV2 cells (Fig. 4E).

To confirm whether CoREST was indeed localized to NF- κ B target gene promoters in association with p65 and Nurr1, we performed ChIP assays of the *iNOS*- and *TNF α* -promoters in BV2 cells. The occupancy of NF- κ B-p65, Nurr1 and CoREST on both the *TNF α* - and *iNOS*-promoters by all three proteins was strongly increased upon LPS stimulation (Fig. 4F, Fig. S9C). On the *iNOS*-promoter, which exhibits relatively slower activation kinetics, p65 binding preceded the binding of Nurr1, which in turn preceded recruitment of CoREST (Fig. 4F). To verify whether this system is indeed functional *in vivo*, we performed ChIP assays from microdissected SN after the stereotaxic injection of LPS into the SN. Consistent with *in vitro* data, Nurr1 is recruited to the *iNOS*- and *TNF α* -promoters after LPS stimulation (Fig. 4G, Fig. S9D). Finally, we asked whether Nurr1 was indeed essential for the recruitment of the CoREST complex to target gene promoters. ChIP experiments were performed using shNurr1- or shCtrl-BV2 cells. In the absence of Nurr1, CoREST was not recruited to the *TNF α* -promoter (Fig. 4H, left panel). Interestingly, under these conditions, p65 was present at the *TNF α* -promoter for extended times (Fig. 4H, right panel). Regulation of p65 acetylation by HDACs, including HDAC1, is known to determine the duration of transcription (Ashburner et al., 2001). HDAC1

is recruited to *TNF α* or *iNOS*-promoter in an LPS-dependent manner; however, this recruitment is severely impaired in the absence of Nurr1 (Fig. S9E). Finally, to verify an *in vivo* role for the genes identified to be involved in Nurr1/CoREST transrepression pathway, BV2 cells were transfected with siRNAs targeting their corresponding mRNAs and were tested for the ability to increase the production of neurotoxic factors. As shown in Figure S10A, knockdown of each of the molecules engaged in this Nurr1/CoREST-mediated transrepression pathway induced significantly higher death of Neuro2A cells compared to control siRNA, as detected by TUNEL ELISA assay

Nurr1 represses the production of neurotoxic factors in astrocytes

The observation that astrocytes could amplify the neurotoxic effects initiated by microglia (Fig. 2J) suggested that pro-inflammatory cytokines secreted by activated microglia such as *TNF α* and *IL1 β* could activate the astrocytes and induce the transcription of inflammatory neurotoxic mediators (Fig. 2D–F and Fig. S6A–B). Consistent with this possibility, the receptors for *TNF α* and *IL1 β* are highly expressed in primary mouse and human astrocytes, but not microglia (Fig. 5A–B, Fig. S11A–B). Nurr1 protein was expressed in resting human and mouse astrocytes (Fig. S1E and F) and Nurr1 mRNA was induced by *IL1 β* or *TNF α* (Fig. 5C, D and Fig. S11C,D). To test the possibility that Nurr1 also participates in a signal-dependent negative feedback mechanism in astrocytes, primary mouse and human astrocytes were infected with shCtrl- and shNurr1-lentiviruses. Activated astrocytes can up-regulate many pro-inflammatory genes, including the *iNOS* and *Ncf1* genes upon *IL1 β* and *TNF α* stimulation, which are essential enzymes for NO and reactive oxygen species (ROS) production, respectively. Knockdown of Nurr1 in astrocytes drastically increased mRNA expression of both *iNOS* and *Ncf1* in response to *IL1 β* and *TNF α* and up-regulated NO production (Fig. 5E–G and S11E–F). Furthermore, activated astrocytes can produce macrophage colony stimulating factor (CSF1), which supports the proliferation of microglia (Thery et al., 1992), and knockdown of Nurr1 significantly up-regulated the transcription of *CSF1* gene upon both *TNF α* and *IL1 β* stimulation (Fig. 5H and S11G). In contrast, transcription of brain-derived neurotrophic factor (BDNF), a known neurotrophin for dopaminergic neurons, was not affected by knockdown of Nurr1 (Fig. 5I and S11H). These data indicate that Nurr1 also acts as a transcriptional repressor for inflammatory neurotoxic mediators in astrocytes.

The Nurr1/CoREST transrepression pathway functions in astrocytes

Finally, we asked whether the mechanism of transcriptional repression by Nurr1 in astrocytes is similar to that in microglia. Treatment of primary mouse astrocytes with *IL1 β* induced the interaction of Nurr1 with p65 (Fig. 6A) and induced recruitment of both Nurr1 and p65 to the *iNOS*-promoter (Fig. 6B). Recruitment of Nurr1 to the *iNOS* promoter was blocked by inhibition of GSK3 β by SB21 (Fig. 6C), consistent with TLR4 and *IL1 β* receptors sharing the MyD88 signaling pathway (Verstrepen et al., 2008). Nurr1 also interacted with CoREST in astrocytes in a manner that was stimulated by *IL1 β* (Fig. 6D), and both molecules were recruited to the *iNOS*-promoter, as was observed in microglia (Fig. 6E). Knockdown of the components of CoREST repressor complex such as LSD1, G9a and HDAC1 also up-regulated *iNOS*, *CSF1* and *Ncf1* genes, suggesting that the CoREST-complex is required for Nurr1-mediated transcriptional repression in astrocytes (Fig. 6F–H). Finally, knockdown of Nurr1 in astrocytes resulted in prolonged occupancy of p65 on the *iNOS* promoter (Fig. 6I), similar to results obtained in microglia (Fig. 4H).

DISCUSSION

Nurr1 exerts anti-inflammatory and neuroprotective effects in glia

Although a number of genes have been identified as causes of familial PD, the majority of cases are sporadic and of unknown etiology (Farrer, 2006; Moore et al., 2005). An improved

understanding of the causes of the more common forms of the disease will therefore be essential in developing broadly applicable treatment strategies. Here, we demonstrate that in addition to its essential roles in the development and maintenance of dopaminergic neurons, Nurr1 plays a previously unexpected role in protecting these neurons from inflammation-induced neurotoxicity. Several lines of evidence suggest that this role is due to its function as an inhibitor of inflammatory gene expression in microglia and astrocytes (Fig. 7A). First, these studies utilized a model system in which neurotoxicity was induced by LPS, which is not effectively sensed by neurons and does not directly cause neuronal death. Second, reduction of Nurr1 expression in the SN (primarily in microglia and astrocytes) did not in itself lead to reduction of TH⁺ neurons but did result in enhanced expression of inflammatory mediators and accelerated loss of TH⁺ neurons in response to LPS. Finally, reduction of Nurr1 expression in isolated microglia and astrocytes resulted in their exaggerated production of neurotoxic factors in response to inflammatory stimuli.

Experiments employing sequential transfer of cell culture media from microglia to astrocytes or vice versa indicate that astrocytes can act as amplifiers of microglia-derived mediators in the production of neurotoxic factors. Collectively, our data are consistent with a model in which LPS-induced expression of factors such as IL1 β and TNF α by microglia results in paracrine activation of astrocytes. This activation in turn is predicted to enhance production of toxic mediators by astrocytes that include NO and ROS. These factors are suggested to act additively or synergistically with neurotoxic factors produced by microglia (Fig. 7A). Experiments using mixed neuronal cultures are of particular interest in this regard because they suggest that activated microglia and astrocytes produce factors that exhibit relative specificity for TH⁺ neurons (Fig. 2H and I). Conversely, distinct neuronal cell types might exhibit different sensitivities to neurotoxic factors based on protective systems, such as those conferred by genes under the control of the PGC1 α coactivator (St-Pierre et al., 2006). Defining the specific identities of the pathologically important factors produced by microglia and astrocytes and their relative ability to exert toxic effects on different types of neurons will be important future goals.

There is currently no evidence that LPS or bacterial infection contribute directly to the pathogenesis of PD in humans. However, the emerging recognition of the roles of TLRs in a number of inflammatory diseases raises the possibility that endogenous TLR ligands are generated during aging that contribute to disease initiation or progression. For example, components of dying cells, such as Hsp60, have been shown to trigger TLR4 activity (Lehnardt et al., 2008). Other factors, such as ATP, can also induce inflammatory responses through other signaling pathways (Di Virgilio, 2007). A transient event leading to neuronal injury could thus lead to activation of microglia, with subsequent amplification of inflammation by astrocytes. The present findings suggest that Nurr1 protects the CNS from amplification of inflammatory signaling by microglia-astrocyte communication. Strategies to suppress expression of neurotoxins either directly or by interfering with microglia/astrocyte communication may thus have therapeutic utility. Furthermore, it may be necessary to suppress the production of neurotoxic mediators by microglia and astrocytes in order to obtain reconstitution of functional neuronal circuits using cell-based therapies (Brundin et al., 2008).

A Nurr1/CoREST transrepression pathway mediates feedback regulation of inflammatory responses

Members of the NR4A family have been reported to both positively and negatively regulate pro-inflammatory genes (Bonta et al., 2006; Doi et al., 2008; Pei et al., 2006). The present studies demonstrate a potent anti-inflammatory activity of Nurr1 in microglia and astrocytes. We propose that this anti-inflammatory activity is mediated by a Nurr1/CoREST transrepression pathway that operates in a feedback manner to restore transcription of NF- κ B

target genes to a basal state (Fig. 7B). In this pathway, Nurr1 is recruited to NF- κ B on inflammatory gene promoters dependent on GSK3 β -mediated phosphorylation of S468 of p65. Nurr1 subsequently recruits the CoREST co-repressor complex in an NLK-dependent manner. Since HDAC1-mediated deacetylation is known to regulate the duration of p65 transcriptional activity, and HDAC1 is a component of the CoREST complex, the Nurr1-CoREST axis might have essential roles in terminating inflammatory responses by p65 clearance from the target promoters. These studies thus establish an unexpected biological role for the CoREST complex, previously considered to mainly be involved in the repression of neuronal genes in NSCs or non-neuronal cells (Ballas et al., 2005). We also find that overexpression of Nurr77 and Nor1 in a macrophage cell line can suppress iNOS activation in response to LPS (K.S., unpublished), suggesting that the CoREST transrepression pathway may be widely used by members of the NR4A family. Of interest, reduction of most of the well-established components of the CoREST complex severely compromises the anti-inflammatory activity of Nurr1. Quantitative defects in the expression or activities of these proteins could thus predispose certain organ systems to inflammation-sensitive pathologies, such as PD.

Experimental Procedures

Mice and isolation of primary cells

C57BL/6 mice were purchased from Charles River or Harlan and housed according to UCSD or Salk Institute protocols, respectively. Mouse primary microglia cells and astrocytes from the cerebrum of P0 pups. After 10–14 days of culture, microglia cells were isolated from astrocytes by the magnetic sorting using anti-mouse CD11b beads (Miltenyi). Purity of each population was over 98%, as determined by FACS. Murine microglial BV2 cells (kindly provided by Katerina Akassoglou) and macrophage RAW264.7 cells were maintained with DMEM supplemented with 10% FBS (low endotoxin, Hyclone) and penicillin/streptomycin. For other cells, details appear in Supplemental Experimental Procedures.

Reagents

All smart-pool siRNAs and GIPZ lentivirus shRNAmir were purchased from Dharmacon and Open Biosystems, respectively. LPS *E. coli* 0111:B4 used at 0.1 μ g/ml final, SB216763 as indicated, and Cycloheximide used at 10 μ g/ml final were obtained from Sigma. Human and mouse IL1 β used at 10 ng/ml final and TNF α used at 50 ng/ml final were from R&D system.

Stereotaxic Injection of lentivirus and LPS

LPS and/or lentiviruses were delivered to the right SN by stereotaxic injection at AP–3.3 mm, ML–1.2 mm, DV –4.6 mm from bregma (Franklin and Paxinos, 2008). See Supplemental Experimental procedures for full methods.

Immunohistochemistry (IHC)

Experimental animals were anesthetized and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brain samples were postfixed with 4% paraformaldehyde overnight and equilibrated in 30% sucrose. Coronal sections of 40 μ m were prepared with a sliding microtome. IHC was performed using mouse anti-tyrosine hydroxylase (Chemicon). Unbiased quantification of TH-immunoreactive neurons in the SN was performed according to the optical fractionator principle (Gundersen et al., 1988). See Supplemental Experimental procedures for full methods.

Transfection assays

The RAW264.7 mouse macrophage cell line was transiently transfected with *iNOS*- or *TNF α* -promoters directing luciferase expression (Pascual et al., 2005) using SuperFect

(Qiagen). Double transfections with plasmid DNA and siRNAs used Transmessenger reagent (Qiagen). Flag-tagged full-length (FL) mouse Nurr1 was cloned into p3XFLAG-CMV-7.1 vector (Sigma). Mutant constructs of Nurr1 were generated with the Quick-change site-direct mutagenesis kit (Stratagene). Retrovirus production (Openbiosystems) and infection into BV2 cells were performed according to the manufacturer's protocol. Lentivirus p156RRLsinPPTCMV-GFP-PREU3Nhe and pHAGE vector packaging was done using Virapower (Invitrogen). GIPZ lentivirus shRNAmir (Openbiosystems) packaging was performed according to the manufacturer's protocols. See Supplemental Experimental procedures for full methods.

Biochemical assays

Cell death detection kit (Roche) was used for TUNEL assay. SUMOylation assays were performed as described before (Pascual et al., 2005). For endogenous co-IP experiments, anti-Nurr1 (E-20, Santa Cruz), anti-CoREST (E-15, Santa Cruz and Millipore) and anti-p65 (C-20, Santa Cruz) were used for IP and Western blotting. For immunoprecipitation of tagged proteins, M2 anti-Flag-agarose (Sigma) beads were used. NLK (Millipore) in vitro kinase reaction (Kanei-Ishii et al., 2004) was performed using GST-fusion substrates.

Chromatin immunoprecipitation (ChIP) assays

For each experimental condition, 2×10^7 BV2 cells or 6×10^6 mouse primary astrocytes were used. Cells were stimulated with LPS for BV2 cells and IL1 β for astrocytes for the indicated time before crosslinking for 10 minutes with 1% formaldehyde. Anti-Nurr1 (E-20, Santa Cruz Biotechnology) anti-p65 (C-20, Santa Cruz Biotechnology), anti-CoREST (Millipore) or control rabbit IgG (Santa Cruz Biotechnology) were used for IP. See details in Supplemental Experimental Procedures.

RNA isolation and quantitative PCR

Total RNA was isolated by RNAeasy kit (Qiagen) from cells or SN samples microdissected from the brain. One microgram of total RNA was used for cDNA synthesis using Superscript III (Invitrogen), and quantitative PCR was performed with SYBR-GreenER (Invitrogen) detected by 7300 Real Time PCR System (ABI). The sequences of qPCR primers used for mRNA quantification in this study were obtained from PrimerBank (Wang and Seed, 2003).

Statistical analyses

Standard deviation, Chi-square and two-tail *Student's t*-test were performed with the Prism 4 program. $p < 0.01$ was considered significant. For IHC and IF analyses, Bonferroni was used for *post hoc* analysis when a significant difference was found with ANOVA. Unpaired two-tailed *t* test was used for other comparisons, including comparisons between control and injected sides within one group. All data are presented as mean \pm SD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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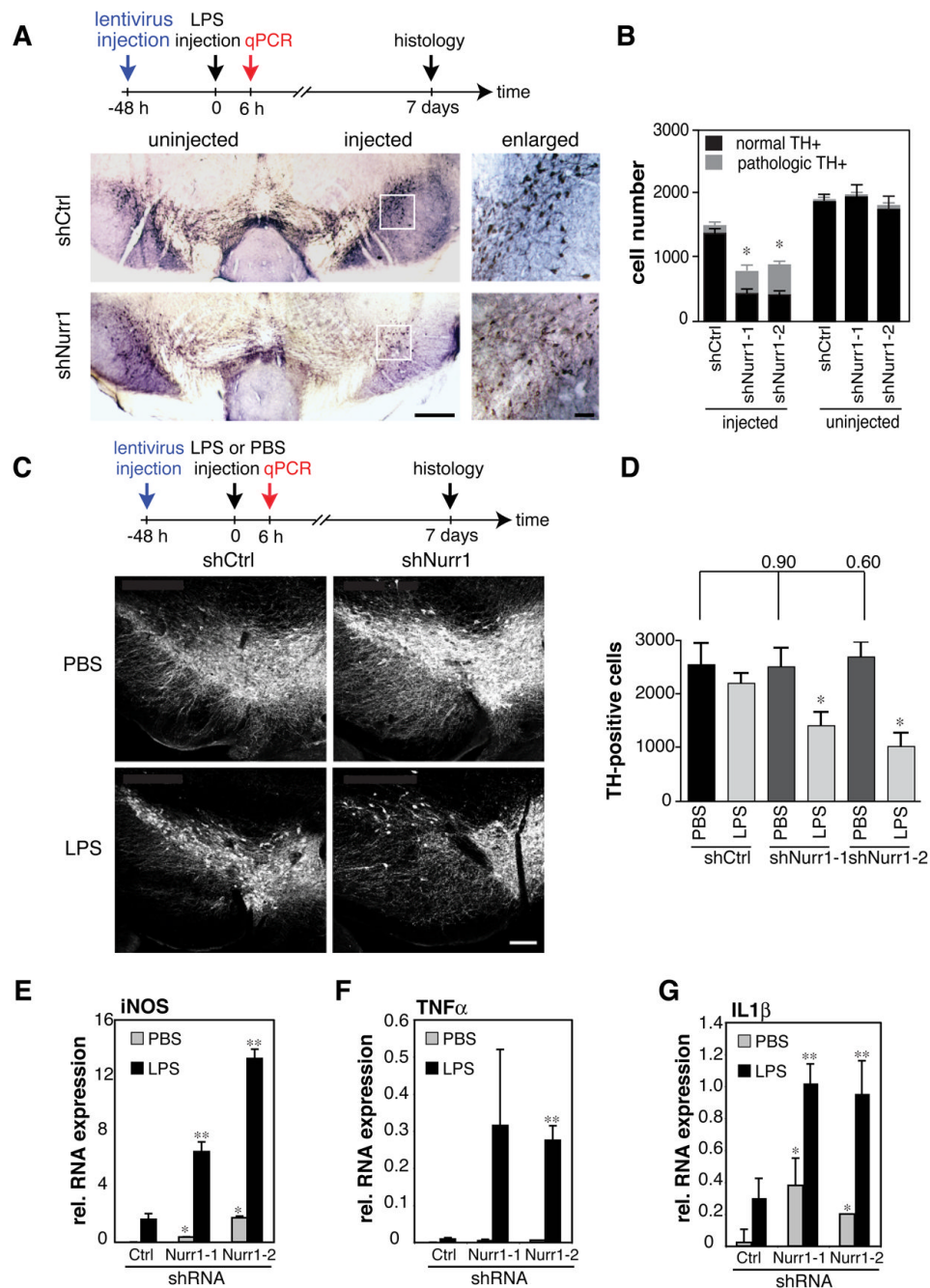


Fig. 1. Nurr1 suppresses LPS-induced inflammation and loss of TH+ neurons

A. TH-DAB staining of a representative brain section of mice injected with shCtrl- or shNurr1-lentivirus and LPS is shown at AP -3.3 mm. Regions indicated by a rectangle in the injected side of the brain are enlarged in the right panels. Scale bars: 200 μ m, right panels and 50 μ m, left panels. **B.** Quantification of TH+ cell numbers in the shNurr1 groups compared to the shCtrl-injected and the uninjected side. Bars indicate normal (black) and pathological (gray) TH+ neurons. See Supplemental Experimental Procedures for the definition of normal/pathological TH+ neurons. Asterisk, $p < 0.01$ compared to the numbers from shCtrl-lentivirus-injected. ($n = 5$) **C.** Fluorescence-TH staining of a representative brain section of mice injected with shCtrl- or shNurr1-lentivirus followed by PBS or LPS. Experimental diagram is indicated

at the top. Scale bar 200 μm . **D.** Quantification of TH+ cell numbers in the setting of Nurr1 knockdown followed by LPS or PBS injection. Asterisk, $p < 0.002$ compared to PBS injection ($n=4$). **E–G.** Expression of *iNOS* (E), *TNF α* (F) and *IL1 β* (G) mRNA in Nurr1-knockdown SN 6 hours after LPS injection as determined by qPCR and normalized to HPRT expression ($n=4$). Error bars represent SD. Asterisk, $p < 0.01$ compared to shCtrl/PBS-injected; **, $p < 0.01$ compared to shCtrl/LPS-injected samples.

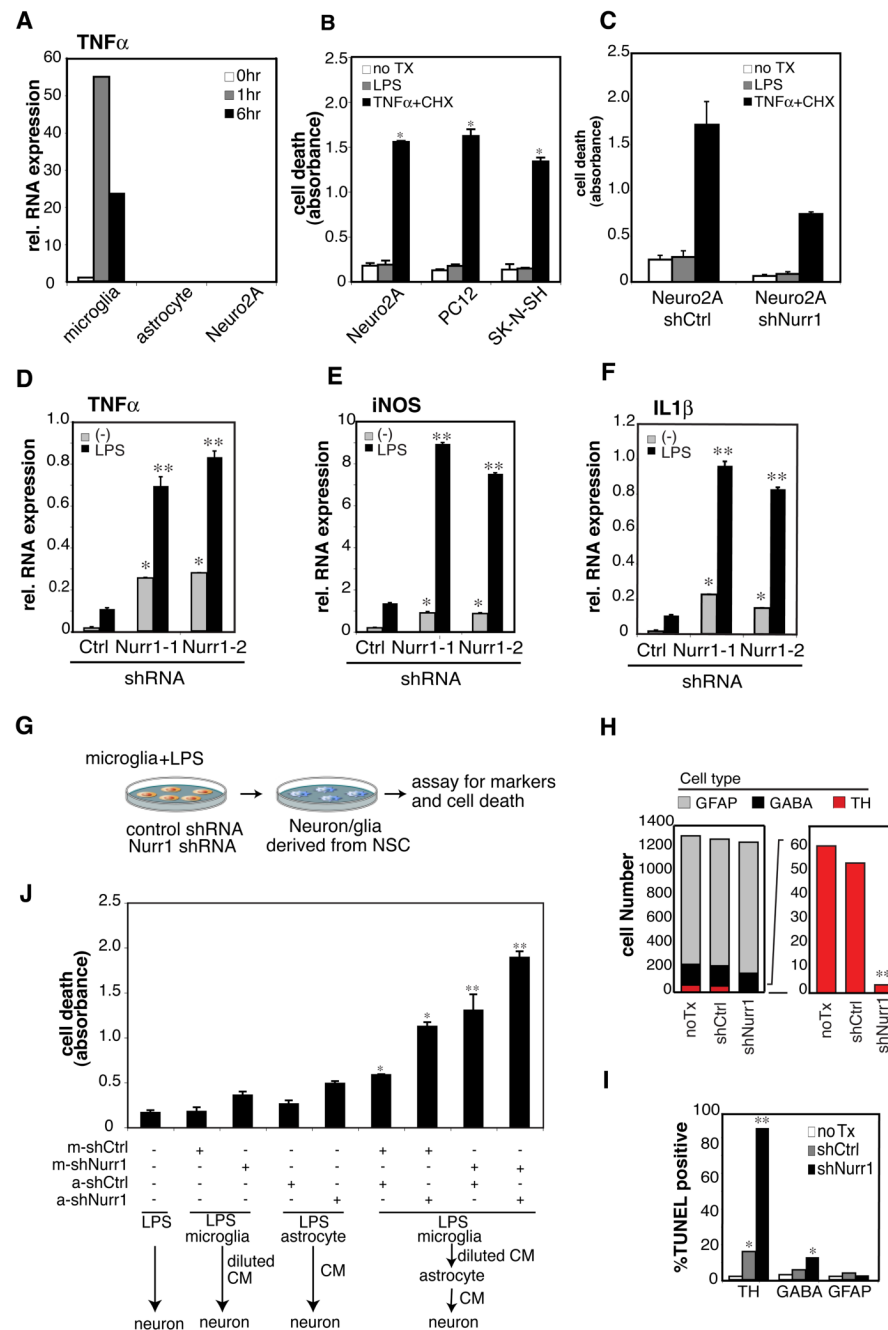


Fig. 2. Microglia initiate LPS-mediated inflammation and astrocytes propagate the production of neurotoxic factors

A. Expression of *TNF α* mRNA upon LPS stimulation in primary microglia, astrocytes and Neuro2A cells. **B.** Effect of LPS or *TNF α* + cycloheximide (CHX) on viability of the indicated neuronal cell lines determined using a TUNEL ELISA assay. *, $p < 0.01$ compared to untreated sample (no Tx) (white). **C.** Effect of Nurr1 knockdown in Neuro2A cells on sensitivity to *TNF α* + CHX assessed by TUNEL ELISA assay. **D–E.** Effect of Nurr1 knockdown in BV2 cells on LPS-induced expression of *TNF α* (D), *iNOS* (E) and *IL1 β* (F) mRNA. *, $p < 0.01$ compared to no stimulation (Ctrl); **, $p < 0.01$ compared to LPS stimulation of shCtrl-BV2 cells. **G.** Scheme of conditioned media (CM) and cell death assay. CMs were harvested from

shCtrl- and shNurr1-BV2 cells that were stimulated with LPS for 24h. Neurons or glial cells were assayed for specific markers by immunostaining and for cell death by TUNEL assay. **H.** Effect of CM from LPS-treated shCtrl-BV2 cells and a mixture of the CMs from shNurr1-1- and shNurr1-2-infected BV2 cells (shNurr1) on neurons and glial cells derived from *in vitro* differentiated neural stem cells (NSC). TUNEL assay was performed on TH-, GABA- or GFAP-positive cells derived from mouse NSC. Numbers of TUNEL-negative live cells are shown. TH-positive cells are indicated in red. **I.** The percentages of TUNEL-positive population are shown. *, $p<0.01$ and **, $p<0.001$ compared to no treatment (no TX). **J.** Effect of Nurr1 knockdown in microglia and astrocytes on the production of neurotoxic factors. Primary mouse microglia and astrocytes were infected with shCtrl- or shNurr1-lentivirus. Cells were treated with LPS for 2h and washed extensively with PBS. Cells were cultured for another 24h to generate CM. For sequential CM assay, CMs harvested from microglia were cultured with lentivirus-infected astrocytes for 24 hours. Then, CMs were harvested and tested for effect on viability of Neuro2A cells.

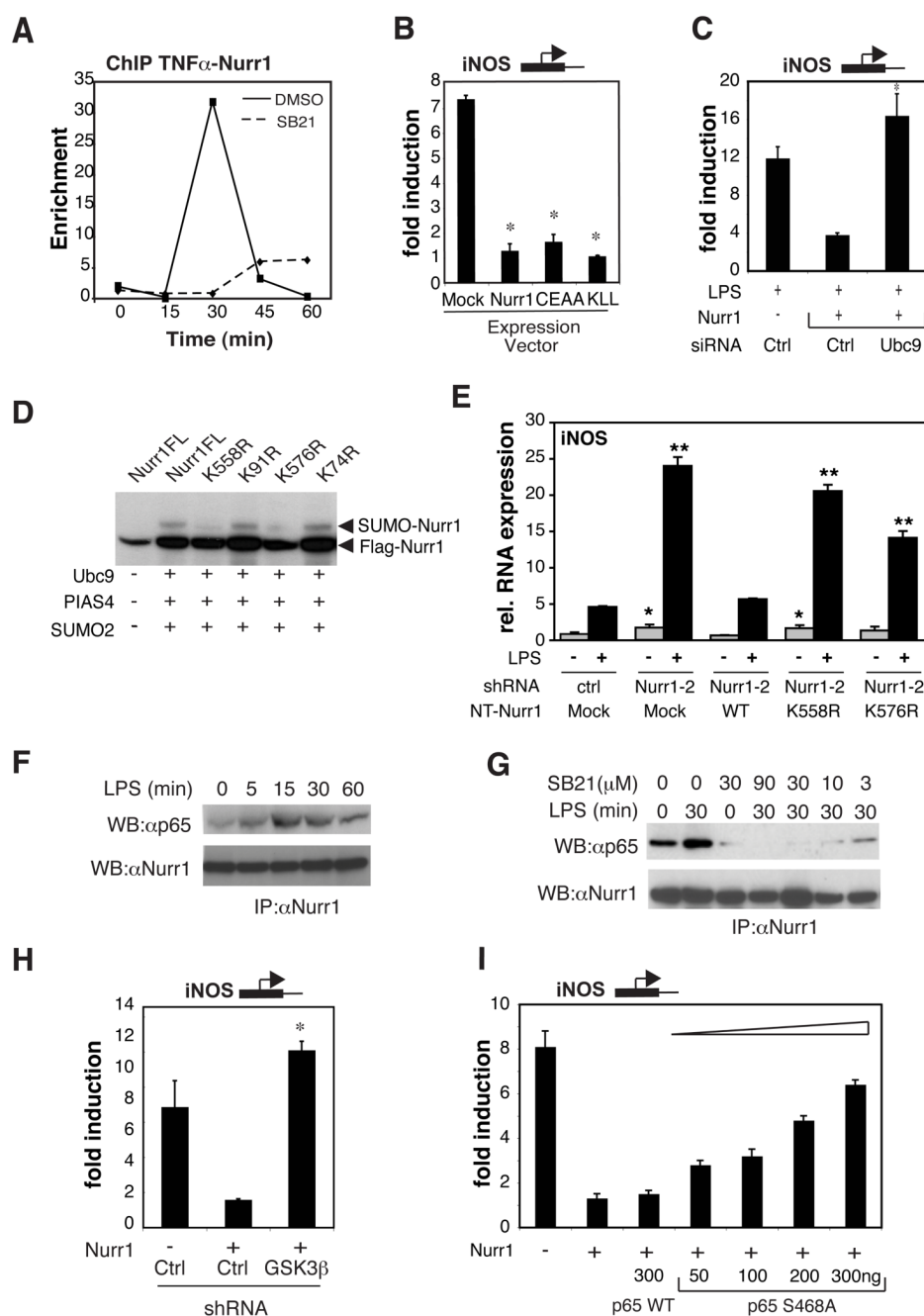


Fig. 3. Nurr1 acts as an RXR-independent, GSK3 β -dependent transrepressor for NF- κ B. **A.** ChIP assay of Nurr1 on the $TNF\alpha$ -promoter in response to LPS and effect of the GSK3 β -specific inhibitor SB216763 (SB21). BV2 cells were pre-incubated with DMSO or 30 μ M SB21 for 1h followed by LPS stimulation for the indicated times before ChIP assay. Data are displayed as fold enrichment over control IgG. **B.** Repression activities of Nurr1 mutants. RAW264.7 cells were transfected with wild type Nurr1, P-box mutant (CEAA) and I-box mutant (KLL). *iNOS*-promoter activity in RAW264.7 cells in response to LPS was measured by luciferase-reporter assay. *, $p < 0.01$ compared to control (Mock). **C.** Effect of knockdown of Ubc9 on Nurr1 repression of *iNOS*-promoter activity. *, $p < 0.01$ compared to Nurr1 with control siRNA. **D.** Identification of the predominant

SUMOylation sites of Nurr1. Flag-tag mutants of Nurr1 were transfected into Hela cells. SUMOylation assay was performed as described in Supplemental Experimental Procedure. **E.** Effect of reconstitution of Nurr1 shRNA-2 BV2 cells with non-targeted (NT) WT and mutant forms of Nurr1 that are not recognized by shNurr1-2. Endogenous iNOS mRNA levels are shown relative to levels in untreated BV2 cells transduced with control shRNA and mock Nurr1 lentivirus. *, $p < 0.01$ compared to mock control cells. **F.** Effect of LPS stimulation on interaction of Nurr1 and p65 in BV2 cells. Lysates of BV2 cells stimulated with LPS for the indicated times were immunoprecipitated with anti-Nurr1 antibody and Western blots were developed with anti-p65 antibody. **G.** Effect of SB21 on Nurr1/p65 interaction. BV2 cells were incubated with SB21 at the indicated concentrations for 1h prior to stimulation with LPS. IP and Western blotting were performed as described in F. **H.** Effect of siRNA against GSK3 β on Nurr1-mediated repression of *iNOS*-promoter activity. Nurr1 expression vector was transfected into RAW264.7 cells together with control siRNA or siRNA against GSK3 β *, $p < 0.01$ compared to Nurr1 with control siRNA. **I.** Effect of S468A mutation of p65 on Nurr1-mediated repression of *iNOS*-promoter activity.

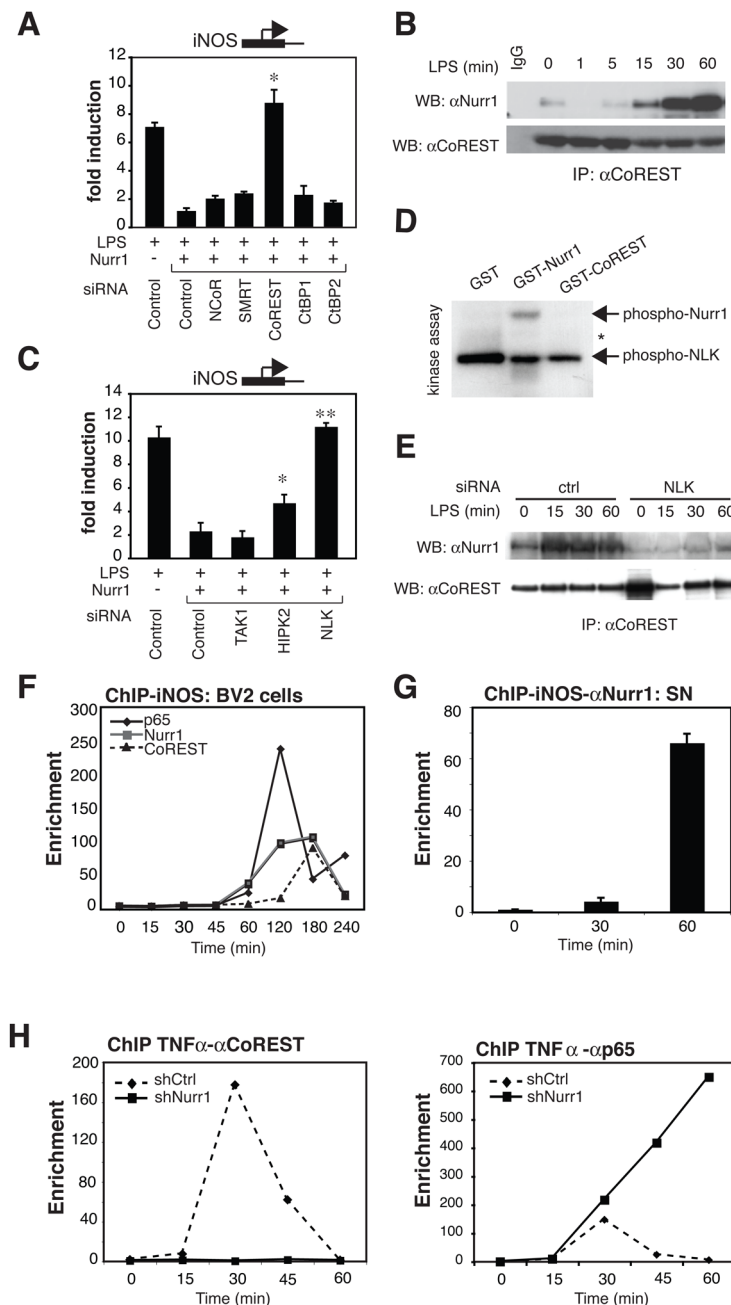


Fig. 4. CoREST repressor complex is required for Nurr1-mediated repression

A. Corepressor requirements in Nurr1-mediated repression. *iNOS*-luciferase and Nurr1-expression or control vector as well as siRNAs against the indicated corepressors were transfected into RAW264.7 cells and *iNOS*-promoter activity was assayed. *, $p < 0.01$ compared to Nurr1 with control siRNA. **B.** Interaction of Nurr1 and CoREST in BV2 cells. Co-IP was performed with anti-CoREST antibody and Western blots were developed with anti-Nurr1 antibody. **C.** Effect of siRNAs against the indicated targets on Nurr1-mediated repression of *iNOS*-promoter activity. *, $p < 0.01$. **, $p < 0.001$ compared to Nurr1 with control siRNA. **D.** NLK *in vitro* kinase assay using GST-Nurr1 and GST-CoREST as substrates. Arrows indicate phosphorylated GST-Nurr1 and autophosphorylation of NLK. The migration position of GST-

CoREST is indicated by an asterisk. GST substrates and methods are provided in Supplemental Experimental Procedures and Figure S7D. **E.** Effect of NLK knockdown on interaction of Nurr1 and CoREST. BV2 cells were transfected with siRNA against NLK or control siRNA. Co-IP of Nurr1 and CoREST was performed as described in B. **F.** Recruitment of Nurr1, CoREST and p65 to the *iNOS* promoter in BV2 cells shown by ChIP assay. Data represent fold enrichment of *iNOS*-promoter precipitated by the indicated antibodies compared to control IgG as determined by qPCR. **G.** ChIP analysis of Nurr1 on the *iNOS* promoter in the SN before and after LPS stimulation. Data are shown as averages of fold enrichment against control IgG and SD. **H.** Effect of Nurr1 knockdown on recruitment of CoREST (left) and p65 (right) to the TNF α promoter in BV2 cells. ChIP data are shown as fold enrichment over control IgG.

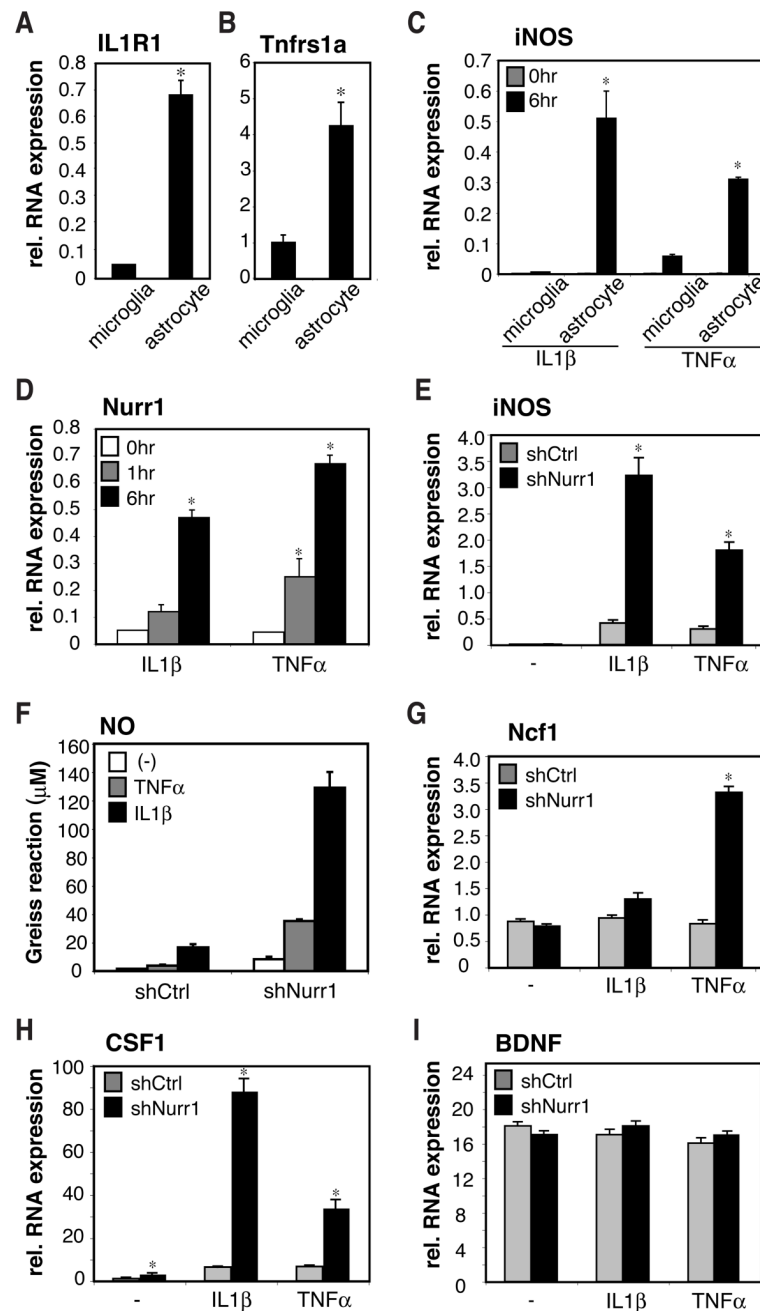


Fig. 5. Nurr1 suppresses inflammatory mediators in murine astrocytes

A and B. Expression of IL1R1 (A) and p55TNFR (B) mRNA in primary astrocytes and microglia as determined by qPCR assay. *, $p < 0.01$. **C.** Responses of primary mouse microglia or astrocytes to TNF α or IL1 β for 6h. *iNOS* mRNA level was determined by qPCR. **D.** Regulation of Nurr1 mRNA in mouse primary astrocytes stimulated with TNF α or IL1 β for the indicated times. **E–I.** Effect of knockdown of Nurr1 in astrocytes on induction of NO (F – measured by Greiss reaction) and mRNAs encoding neurotoxic mediators (E, G–I). Mouse primary astrocytes were infected with shCtrl- or shNurr1-lentivirus and cells were stimulated with TNF α or IL1 β for 6h.

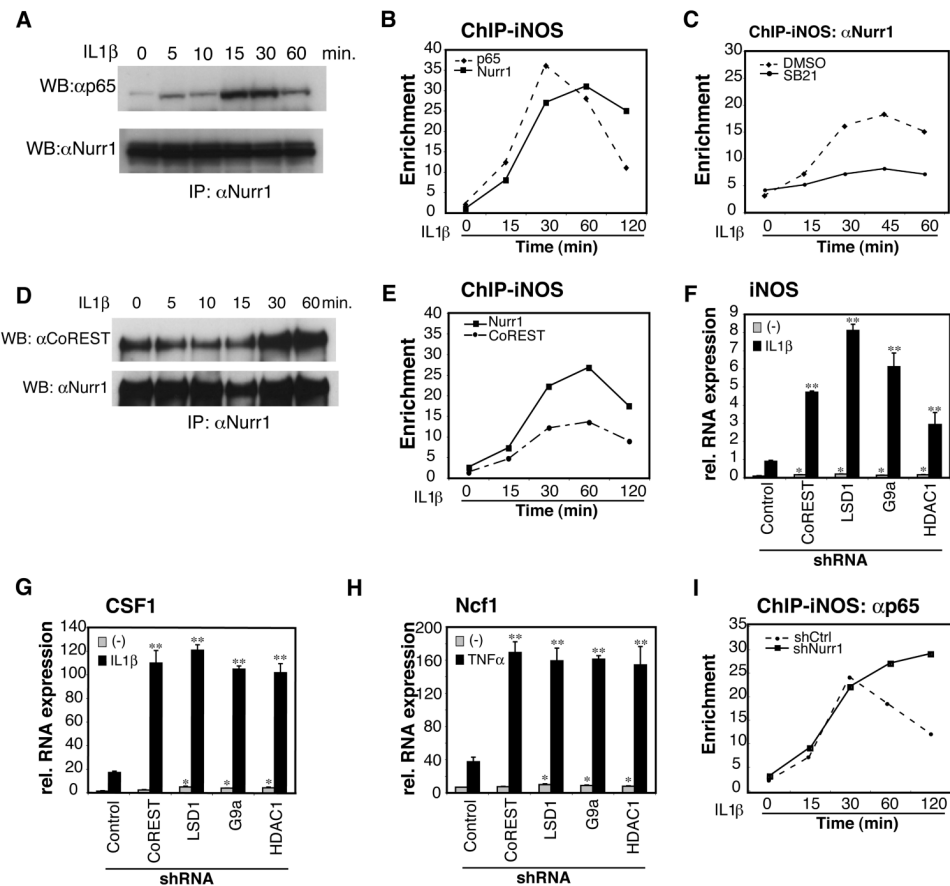


Fig. 6. The CoREST complex is required for Nurr1-mediated repression in astrocytes

A. Effect of IL1 β stimulation on association of Nurr1 and p65 in mouse primary astrocytes. Lysates of astrocytes stimulated with IL1 β for the indicated times were immunoprecipitated with anti-Nurr1 antibody and Western blots were developed with anti-p65 antibody. **B.** Recruitment of Nurr1 and p65 to *iNOS*-promoter in mouse primary astrocyte shown by ChIP assay. Data represent fold enrichment of *iNOS*-promoter precipitated with the indicated antibodies compared to control IgG as determined by qPCR. **C.** Effect of GSK3 β -specific inhibitor SB21 on recruitment of Nurr1 to *iNOS*-promoter. Data represent fold enrichment of *iNOS*-promoter precipitated with antibody against Nurr1 compared to control IgG as determined by qPCR. **D.** Interaction of Nurr1 and CoREST in mouse primary astrocytes. Co-IP was performed with anti-Nurr1 antibody and Western blots developed with anti-CoREST or anti-Nurr1 antibodies. **E.** Recruitment of Nurr1 and CoREST to *iNOS*-promoter in mouse primary astrocytes shown by ChIP assay. Data represent fold enrichment of *iNOS*-promoter precipitated with the indicated antibodies compared to control IgG as determined by qPCR. **F–H.** Effect of knockdown of the components of CoREST-repressor complex on mRNAs encoding inflammatory mediators. Mouse primary astrocytes were infected with lentivirus carrying shRNA against CoREST, LSD1, G9a, HDAC1 or control. Cells were stimulated with IL1 β for 6h and mRNA expression of *iNOS* (F), *CSF1* (G) and *Ncf1* (H) was determined by qPCR. **I.** Nurr1-dependent clearance of p65 from *iNOS* promoter. ChIP assay was performed in shNurr1- or shCtrl-astrocyte and data shown as fold enrichment over control IgG of *iNOS* promoter precipitated with antibody against p65.

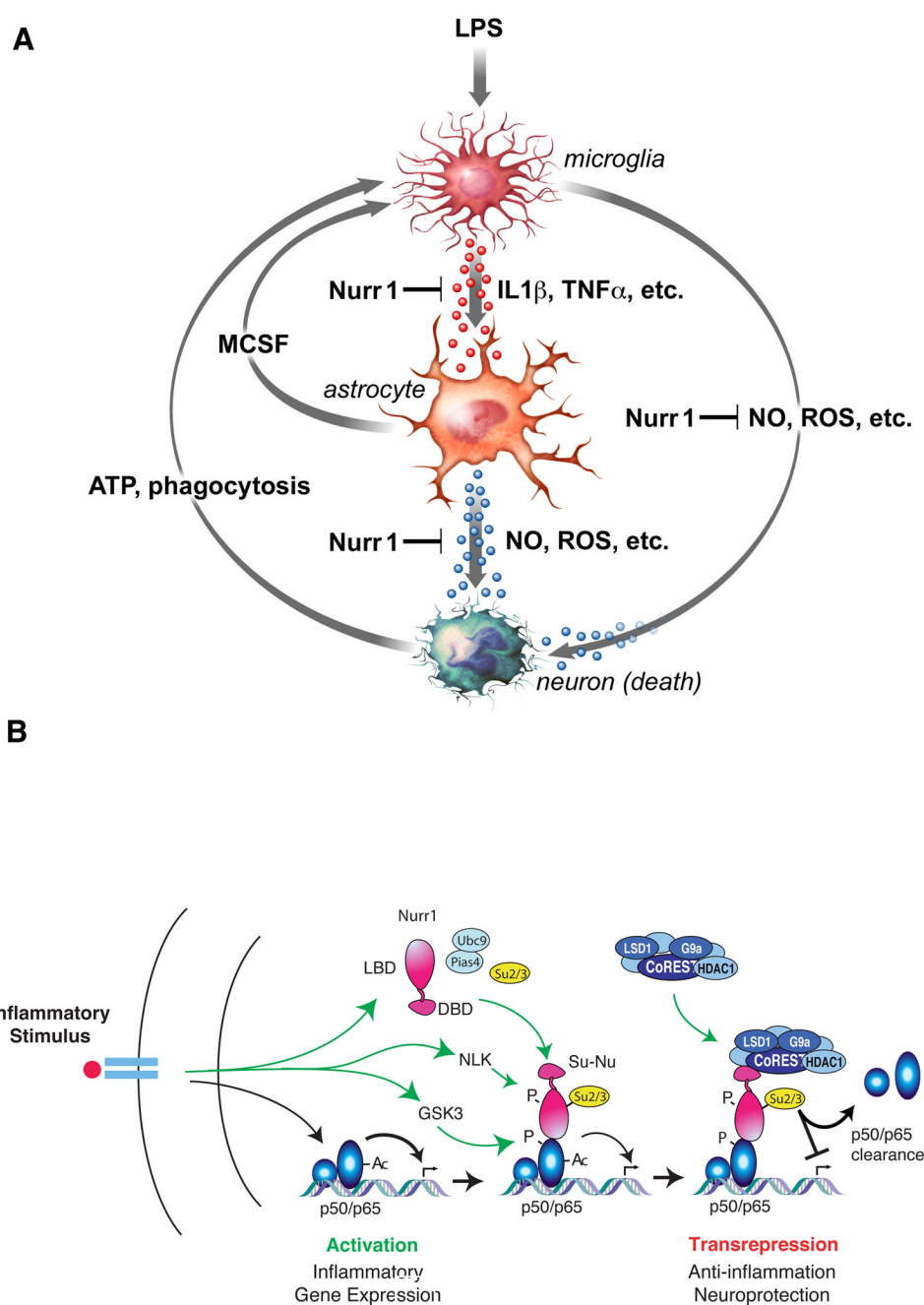


Fig. 7. Nurr1 functions to inhibit neurotoxic gene expression in microglia and astrocytes via a CoREST-dependent transrepression pathway

A. Model for communication among microglia, astrocyte and neurons. **B.** Model for Nurr1/CoREST-mediated repression. See main text for details.