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***Nurr1*^{Cd11bcre} conditional knock-out mice display inflammatory injury to nigrostriatal dopaminergic neurons**

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Abstract

Nuclear receptor related 1 protein (NURR1) is essential for the development and maintenance of midbrain dopaminergic (DAergic) neurons. NURR1 also protects DAergic neurons against neuroinflammation. However, it remains to be determined to what extent does NURR1 exerts its protective function through acting autonomously in the microglia. Using Cre/lox gene targeting system, we deleted *Nurr1* in the microglia of *Nurr1*^{Cd11bcre} conditional knockout (cKO) mice. The *Nurr1*^{Cd11bcre} cKO mice displayed age-dependent motor abnormalities and increased microglial activation, but with no obvious DAergic neurodegeneration. To boost the inflammatory injury, we systemically administered endotoxin lipopolysaccharide (LPS) to *Nurr1*^{Cd11bcre} mice. As expected, LPS treatment exacerbated the motor phenotypes and inflammatory reactions in *Nurr1*^{Cd11bcre} cKO mice. More importantly, LPS administration caused DAergic neuron loss and α -synuclein aggregation, two pathological hallmarks of Parkinson's disease (PD). Therefore, our findings provide *in vivo* evidence supporting a critical protective role of NURR1 in the microglia against inflammation-induced degeneration of DAergic neurons in PD.

Keywords

Nurr1; neuroinflammation; Parkinson's disease; microglia; neurodegeneration

Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder affecting millions of elderly people worldwide (Dauer & Przedborski, 2003). Progressive

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Data Available Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNc) and abnormal accumulation of α -synuclein-containing Lewy bodies/neurites are recognized as the two major pathological hallmarks of PD (Braak et al., 2003). Although the etiopathogenesis of PD remains elusive, both environmental factors and genetic predispositions contribute to the etiology and pathogenesis of PD (Le, Chen, & Jankovic, 2009).

Neuroinflammation is associated with the degeneration of DAergic neurons (Block & Hong, 2007; Hirsch, Vyas, & Hunot, 2012; Nagatsu & Sawada, 2005; Sampson et al., 2016). Studies of cytokines in peripheral serum/plasma and in cerebrospinal fluid demonstrate that proinflammatory cytokines, such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interferon- γ , interleukin-2 and interleukin-10, are significantly increased in PD patients (Rocha, de Miranda, & Teixeira, 2015). As the only residential immune cells in the central nervous system (CNS), microglia undergo ramified morphological change and release a variety of proinflammatory and neurotoxic molecules in response to inflammatory stimulations (Kim & Joh, 2006; Kreutzberg, 1996; Tang & Le, 2016). While it is not clear whether neuroinflammation is the initiating trigger in PD pathogenesis, direct injection of endotoxin lipopolysaccharide (LPS) in the SNc causes robust microglial activation and acute DAergic neuron loss in rodents (Liu, Du, & Hong, 2000; Tomás-Camardiel et al., 2004). In addition, systemic administration of LPS by intraperitoneal injection leads to a progressive and selective degeneration of DAergic neurons in several mouse models (Frank-Cannon et al., 2008; Gao et al., 2011; Qin et al., 2007). Moreover, following LPS administration, PD-related human α -synuclein A53T transgenic mice develop persistent neuroinflammation, resulting in degeneration of DAergic neurons and accumulation of Lewy body-like intracellular protein inclusions (Gao et al., 2011). Parkin-deficient mice, a model of PD-related Parkin recessive mutation, also show behavioral deficiency and DAergic neuron loss after prolonged LPS treatment (Qin et al., 2007). These early studies support a potential interplay between genetic and neuroinflammatory factors in the etiopathogenesis of PD (Tansey, McCoy, & Frank-Cannon, 2007; Vlahjinac et al., 2013).

Nuclear receptor related 1 protein (NURR1), also known as NR4A2, is a member of orphan nuclear receptor NR4A subfamily essential for the development and maintenance of midbrain DAergic neurons (Perlmann & Wallén-Mackenzie, 2004). NURR1 expression in the DAergic neurons is reduced during normal aging (Chu, Kompoliti, Cochran, Mufson, & Kordower, 2002) and is further decreased in PD patients' brains and peripheral blood mononuclear cell (Decressac, Volakakis, Björklund, & Perlmann, 2013; Liu et al., 2012). NURR1 regulates the expression of functional DA genes, such as dopamine (DA) synthase tyrosine hydroxylase (TH), DA transporter, aromatic amino acid carboxylase, and vesicular monoamine transporter 2 (Jankovic, Chen, & Le, 2005). In addition to DAergic neurons, NURR1 is also expressed in microglia and peripheral leukocytes and acts as a transcriptional repressor of neurotoxic factors through mediating the turnover of nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) (Saijo et al., 2009; Li et al., 2018). NURR1 has been shown to suppress LPS-induced neuroinflammation and protect against DAergic neuron loss *in vitro* (Saijo et al., 2009). However, it remains to determine that what extent NURR1 exert its anti-inflammatory and neuron protective function via acting on microglia and other immune cells.

Previous studies have shown that *Nurr1* homozygous germline knockout (KO) mice failed to develop midbrain DAergic neurons and died shortly after birth (Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterström et al., 1997). On the other hand, *Nurr1* heterozygous KO mice survive, but display motor impairments associated with reduced DA content in the striatum and increased susceptibility to neurotoxic insults (Jiang et al., 2005; Le, Conneely, He, Jankovic, & Appel, 1999; Le, Conneely, Zou, et al., 1999). Furthermore, *Nurr1* conditional KO mice, which deleted *Nurr1* selectively in DAergic neurons, recapitulate some PD-related pathological and biochemical features in the nigrostriatal pathway (Kadkhodaei et al., 2009, 2013). While these previous *in vivo* studies on NURR1 mainly focused on the DAergic neurons and established an essential physiological function of NURR1 in the development and survival of DAergic neurons, the role of NURR1 in microglia *in vivo* has not been critically evaluated. Whether a conditional deletion of *Nurr1* in microglia renders DAergic neurons more susceptible to inflammatory injury remains to be determined. Toward this direction, we generated and characterized *Nurr1*^{*Cd11bcre*} conditional KO mice in which *Nurr1* was deleted in the microglia. We found that a lack of *Nurr1* increased neuroinflammation and made DAergic neurons loss to LPS-induced cytotoxicity.

Materials and Methods

Animals

Cd11b-Cre [Tg(ITGAM-cre)2781Gkl] transgenic mice were generated to manipulate gene expression in the microglia as previously described (Boillée et al., 2006). *Cd11b* is an integrin expressed exclusively in the myeloid lineage (Wieghofer, Knobloch, & Prinz, 2015). These mice were crossbred with floxed *Nurr1* mice to generate Cre-positive, homozygous floxed *Nurr1* mice, referred as *Nurr1*^{*Cd11bcre*} mice in this study. Littermates of Cre-negative, homozygous floxed *Nurr1* mice were referred as control mice. Mice were housed under standard conditions with controlled 12h light/dark cycles (lights on at 8 AM), room temperature (22 ± 1°C), and humidity (50 ± 10%), with food and water provided ad libitum. All animal care and experimental procedures were performed in compliance with the Animal Ethics Committee guidelines of the First Affiliated Hospital of Dalian Medical University.

Generation of floxed *Nurr1* knock-in mice

The floxed *Nurr1* knock-in mice were generated by Shanghai Model Organisms Center, Inc (Shanghai, China). The FRT-pGK-Neomycine-polyA-FRT-loxp selection cassette was inserted into intron 2 and another copy of loxp was inserted into the intron 3 of *Nurr1* genomic DNA. The gene targeting vector was electroporated into a mouse embryonic stem (ES) cell line CJ7 with 129S1/SvImJ background. After drug selection, the resistant ES clones were identified by long PCR and confirmed by sequencing. Positive ES cell clones were expanded and injected into C57BL/6J blastocysts to generate the chimeric offspring (Liu, 2003). The chimeric mice were mated with C57BL/6J mice to obtain the floxed *Nurr1* heterozygous knock-in mice with the FRT-flanked Neo selection cassette. These mice were then crossbred with FLP mice to remove the FRT-flanked Neo selection cassette and generated the floxed *Nurr1* heterozygous knock-in mice.

Genotyping

Cd11b-cre transgenic mice were identified by PCR screening (2×EasyTaq PCR SuperMix, Transgen Biotech) of tail DNA using an antisense primer, CAGGTATGCTCAGAAAACGCCT, and a sense primer TGGGCCAACCCAAGAAACAAGT, of which the transgene band size is 445bp. The floxed *Nurr1* knock-in mice were identified using AAACAAAACAGGGCAACAGG, and GCCTGTGCTGTAGTTGTCCA. The PCR product size of wild type allele (*Nurr1*^{+/+}) is 662bp and homozygous *Nurr1* flox allele (*Nurr1*^{fl/fl}) is 766bp.

Primary microglia isolation

Primary microglia were isolated from the cortex of postnatal day 1–5 *Nurr1*^{Cd11bcre} pups and littermate controls. After removing meninges, the brains were minced and digested with trypsin and DNase I (TransGen Biotech). Cells were re-suspended in F12 supplemented with 10% fetal calf serum and seeded in 25 cm² flasks. After cells grew to confluence (7–10 days), the flasks were shaken at 180 rpm for 18 hrs at 37°C, and the floating cells were collected and allowed to adhere to a flask for 1 hr before gentle shaking. The cells were collected and used for further experiment.

Systemic LPS administration paradigms

Nurr1^{Cd11bcre} mice at 10–12 months of age and their age-matched littermates were injected intraperitoneally with phosphate buffer saline (PBS) or 0.33 mg/kg LPS (E. coli, serotype 0127: B8, Sigma) twice a week for 1 month, followed by a 3-month rest with no additional intraperitoneal injections (n=5 per group). Their body weights were monitored twice a week. After 3-month rest, mice were subjected to behavioral tests and then sacrificed. The brains were isolated and processed for either biochemical or histological studies.

Behavioral tests

Accelerating rotarod test—Mice were placed on an accelerating rotarod (Life Science Inc) to measure their motor coordination and balance abilities. The speed of rotarod was increased from 5 to 45 revolutions per minute over 8 minutes. The latency to fall from the rotarod was recorded. All animals were pretrained for 5 consecutive days and tested three times and the average time was used for data analysis.

Open-field test—Mice were put in a 46×46 cm² arena with an infrared photo beam activity monitor for 30 min to test their locomotion activity. The arena was illuminated by reflected light and the animal movements were recorded. The arena was cleaned with 75% ethanol after each test.

Y maze—Each mouse was put in the central area of the Y maze and allowed to freely explore the maze for 10 min. The arm entries and total distance travelled by each mouse was recorded by video. The total number of entered arms subtracting 2 was the effective alternative opportunity. Percentage alternation was calculated by dividing the number of trials containing entries into the three arms by the alternation opportunity*100% (Yang, Zhang, Zheng, Shen, & Chen, 2014).

Morris water maze—All animals were trained in a Morris water maze for 4 consecutive days with 3 trials per day. The training goal was to reach the submerged platform within 90s (escape latency). The water was maintained at 26°C and mice were put in a random quadrant to reduce the bias. On the fifth day, we removed the platform and measured the latency in the platform quadrant of each mouse.

Immunostaining

After anesthetizing, mice were transcardially perfused with PBS. The brains were rapidly isolated and post fixed in ice-cold 4% paraformaldehyde and subsequently dehydrated for 24 hrs in 15% and 30% sucrose at 4°C for immunostaining. Brain was sectioned coronally at 10 µm thickness using a cryostat microtome. Frozen slides were preincubated for 1 hr in blocking solution, containing 5% bovine serum albumin, 0.25% Triton X-100, and 0.01% sodium-azide in PBS. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C. After rinsing with PBS, slides were incubated with fluorophore-conjugated secondary antibodies for 1 hr at room temperature and then with Hoechst 33342 (Sigma Aldrich) for 1 minute. Slides were visualized with an Olympus light/fluorescence microscopy. Primary antibodies were as follows: mouse anti-NURR1 (1:1000; R&D systems), rabbit anti-TH (1:1000; Millipore), mouse anti-TH (1:2000; Sigma Aldrich), rabbit anti-IBA1 (1:1000; Wako), rabbit anti-phospho-NFκB-p65 (1:1000; Cell Signaling Technology), rabbit anti-GFAP (1:1000; Sigma Aldrich), rabbit anti-α-synuclein (phospho S129) (1:1000; Abcam). Microglial and astrocytes immunostaining was evaluated in slices within SNc and substantial nigra pars reticulata (SNr) at the same coordinates (at least three non-overlapping areas in each slice). TH-positive cells in SNc were counted in every fifth sections covering the AP-2.7 to AP-4.0 mm at a magnification of 100 by an observer who was blind to animal genotype and treatment. The outline of SNc was delimited according to anatomical landmarks (Baquet, Williams, Brody, & Smeyne, 2009).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction were performed according to protocols described previously (Tang et al., 2014). After perfused with ice-cold PBS, the brain was dissected and frozen at −80°C for biochemical detection. Total RNA was extracted with RNAiso Plus (Total RNA extraction reagent; Takara). Real-time PCR (TransStart Top Green qPCR SuperMix; TransGen Biotech) was performed by Applied Biosystems 7500 Real-time PCR Systems. The primer sequences were synthesized by Invitrogen (Table 1).

Measurement of striatal DA concentration

Brains were rapidly removed, and right striatum were collected using a tissue punch, weighed and kept at −80°C for high-performance liquid chromatography, which was performed as described in detail previously (Xiao, Yang, & Le, 2015).

Experimental design and statistical analysis

The experimental design is provided in Results section for each experiment. Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software Inc., California,

USA). All data were reported as the mean $\pm$ standard error of mean. Student's t test was used to compare two groups. Two-way ANOVA with Tukey's multiple comparisons test was performed for multiple comparisons. Value $p < 0.05$ was considered as differences. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $P < 0.0001$. Image J software (<https://imagej.nih.gov/ij>) was used for quantification of images.

Results

Conditional deletion of *Nurr1* in the microglia of *Nurr1^{Cd11bcre}* mice

To investigate the *in vivo* function of *Nurr1* in microglia, we generated *Nurr1^{Cd11bcre}* by crossbreeding floxed *Nurr1* knock-in (*Nurr1^{fl/fl}*) mice with *Cd11b^{cre/wt}* mice (The targeting vector structure of *Nurr1^{fl/fl}* mice was shown as Fig. 1A). We isolated primary microglia from the cortex of newborn pups to confirm the specific deletion of *Nurr1* in microglia by qRT-PCR. As expected, no *Nurr1* transcripts were detected in the cultured primary microglia isolated from the *Nurr1^{Cd11bcre}* pups (Fig. 1B). In addition, a lack of *Nurr1* did not alter the expression of other NR4A subfamily members, *Nur77* and *Nor1*, in the microglia (Fig. 1B). It is noteworthy that the microglia transcriptome and morphology can be altered under the culture conditions (Bohlen et al., 2017; L. Lin, R. Desai, X. Wang, E. H. Lo, & C. Xing, 2017). Since the expression of *Nurr1* was easily detected in the control *Nurr1^{fl/fl}* microglia cultures under the same condition, the loss of *Nurr1* expression in the cultured *Nurr1^{Cd11bcre}* microglia cannot be attributed to the experimental manipulations.

Nurr1^{Cd11bcre} mice display an abnormal locomotor activity and progressive impairments of motor coordination and balance abilities

We conducted series of behavioral tests to assess motor and cognitive performance in three different age groups of *Nurr1^{Cd11bcre}* and control mice. We assigned the 6- to 10-month-old mice as the young adult mice, the 11- to 15-month-old ones as the adult mice, and the 16- to 20-month-old ones as the aged mice. Our data indicated that *Nurr1^{Cd11bcre}* mice travelled a longer distance than control mice in all age groups in Open-field tests (Fig. 2A). Noticeably, the abnormal locomotor activity of *Nurr1^{Cd11bcre}* mice was more profound in the young adult mice compared to the adult and aged groups (Fig. 2A). On the other hand, the performance of mice in rotarod tests was comparable between *Nurr1^{Cd11bcre}* and control mice in both young adult age and adult age groups (Fig. 2B). However, there was a reduction by 28% of the time on the rotarod in aged *Nurr1^{Cd11bcre}* mice when comparing with their age-matched control littermates (Fig. 2B).

Nurr1^{Cd11bcre} mice show an increased microglial activation in the midbrain regions

A previous *in vitro* study demonstrates that a reduction of *Nurr1* expression in microglia exaggerates neuroinflammation, resulting in death of DAergic neurons (Saijo et al., 2009). To investigate the anti-inflammatory and neuro-protective effects of *Nurr1* in the microglia *in vivo*, we examined the activation of microglia and survival of DAergic neurons in the midbrain of aged *Nurr1^{Cd11bcre}* and control mice. We observed a 19% increase of the number of ionized calcium binding adaptor molecule 1 (IBA1)-positive microglia in the *Nurr1^{Cd11bcre}* mice (Fig. 3A, B). Consistently, a reduction of *Nurr1* expression in the microglia also resulted in increased levels of proinflammatory cytokines *Tnf- α* and *Il-1 β* in

the midbrain regions (Fig. 3C). Meanwhile, we also quantified the expression of anti-inflammatory genes including arginase-1 (Arg-1) and transforming growth factor- β (Tgf- β) in the midbrain, showing that Tgf- β was markedly reduced in the aged *Nurr1^{Cd11bcre}* mice (Fig. 3C). On the other hand, the number of DAergic neurons in the SNc of *Nurr1^{Cd11bcre}* mice only showed a modest reduction, which is not statistically significant (Fig. 3A, D). Similarly, we did not find any substantial reduction of DA content in the striatum of *Nurr1^{Cd11bcre}* mice (Fig. 3E). Taken together, these data suggest that while genetic deletion of *Nurr1* in the microglia and other Cre-expressing leukocytes enhances neuroinflammation, but it is not sufficient to induce substantial degeneration of DAergic neurons *in vivo*.

Chronic LPS exposure induces motor dysfunction in adult *Nurr1^{Cd11bcre}* mice

Since environmental toxins are implicated in the etiopathogenesis of PD and LPS exposure triggers neuroinflammation (Goldman, 2014; Qin et al., 2007), we treated 10- to 12-month-old *Nurr1^{Cd11bcre}* or age-matched control mice with intraperitoneal injection of LPS at 0.33mg/kg body weight or vehicle phosphate-buffered saline (PBS) twice a week for one month. We examined behavioral and pathological abnormalities for three months after LPS treatment. The administration of LPS substantially compromised the performance of *Nurr1^{Cd11bcre}* mice in rotarod tests compared to the control mice (Fig. 4A). As controls, the injection of PBS did not affect the rotarod performance of *Nurr1^{Cd11bcre}* mice (Fig. 4A). Additional LPS treatment induced hyperactivity of *Nurr1^{Cd11bcre}* mice in the Open-field tests (Fig. 4B). On the other hand, LPS treatment did not affect the cognitive performance of *Nurr1^{Cd11bcre}* mice in Y maze and Morris water maze tests (Fig. 4C–E). These results indicate that chronic LPS exposure specifically induces motor dysfunction in *Nurr1^{Cd11bcre}* mice.

Chronic LPS exposure exacerbates neuroinflammation in *Nurr1^{Cd11bcre}* mice

After behavioral tests, we examined the cytotoxic effects of LPS exposure in the midbrain of *Nurr1^{Cd11bcre}* mice. We observed an increased microglial activation, manifested with enlarged cell bodies, retracted processes and increased cell numbers in the midbrain of LPS-treated *Nurr1^{Cd11bcre}* mice compared to those LPS-treated control animals or PBS-treated *Nurr1^{Cd11bcre}* mice (Fig. 5A–C). Moreover, the transcription of *IL-1 β* was nearly doubled in the LPS-treated *Nurr1^{Cd11bcre}* mice when compared with PBS-injected *Nurr1^{Cd11bcre}* mice or LPS-injected control littermates (Fig. 5D). In summary, all these data demonstrate that repeated LPS exposure induces microglial activation and exacerbates neuroinflammation in the midbrain of *Nurr1^{Cd11bcre}* mice.

Astrocytes are hypertrophic in the midbrain of *Nurr1^{Cd11bcre}* mice after chronic LPS exposure

Under the hypothesis that astrocytes activation can be induced and modulated by a range of molecules instructions, such as transmitters, microRNAs, cytokines, steroid hormones, serum proteins and β -amyloid, a neurodegeneration associated molecule (Liddel et al., 2017; Sofroniew, 2014), we further examined the astrocyte marker glial fibrillary acidic protein (GFAP) in the midbrain of LPS or PBS-treated *Nurr1^{Cd11bcre}* and control mice. We found a substantial increase of GFAP staining intensity particularly in the SNr and SNc regions of *Nurr1^{Cd11bcre}* mice compared to the control animals (Fig. 5E, F). These data

suggest that the deletion of *Nurr1* in microglia and peripheral leukocytes enhances LPS-induced astrocyte activation in the *Nurr1^{Cd11bcre}* mice.

Chronic LPS exposure induces DAergic neuron degeneration in the SNc of *Nurr1^{Cd11bcre}* mice

We next counted the TH-positive DAergic neurons in the SNc of *Nurr1^{Cd11bcre}* or control mice treated with LPS or PBS. We found that LPS treatment led to a substantial loss of DAergic neurons in the SNc of *Nurr1^{Cd11bcre}* mice compared to the LPS-treated control or PBS-treated *Nurr1^{Cd11bcre}* mice (Fig. 6A, B). In addition, LPS exposure also led to a reduction of DA content in the striatum of *Nurr1^{Cd11bcre}* mice (Fig. 6C). Together, these results suggest that a lack of *Nurr1* in microglia and peripheral leukocytes potentiates LPS-induced inflammatory insults to the nigrostriatal DAergic neurons.

NF κ B-mediated inflammatory signaling is potentiated in the midbrain of LPS-treated *Nurr1^{Cd11bcre}* mice

Nurr1 exerts an anti-inflammatory function by docking to the phosphorylated NF κ B-p65 (p-NF κ B-p65) and then recruiting the CoREST protein complex to inhibit the NF κ B-mediated inflammatory pathways (Saijo et al., 2009). We therefore examined the p-NF κ B-p65 level in the ventral midbrain of *Nurr1^{Cd11bcre}* mice after LPS stimulation. Immunostaining showed that the level of p-NF κ B-p65 was substantially increased in the LPS-treated *Nurr1^{Cd11bcre}* mice compared to the LPS-treated control or PBS-treated *Nurr1^{Cd11bcre}* mice (Fig. 7A, B). These data demonstrate that the deletion of *Nurr1* in microglia and other Cre-expressing leukocytes potentiates the activation of NF κ B, supporting the notion that NURR1 provides feedback regulation of NF κ B signaling against neuroinflammation.

Chronic LPS exposure induces formation of cytoplasmic α -synuclein inclusions in the DAergic neurons of *Nurr1^{Cd11bcre}* mice

Cytoplasmic inclusion containing abnormal accumulation of α -synuclein are one of main pathological hallmarks of PD (Chu & Kordower, 2007). Previous studies demonstrate that LPS treatment induces aggregation of phosphorylated α -synuclein (pS129) in DAergic neurons of PD-related α -synuclein A53T transgenic mice (Gao et al., 2011). Here we also observed a substantial increase of α -synuclein pS129 in the cytosol of DAergic neurons in SNc of LPS-treated *Nurr1^{Cd11bcre}* mice compared to the LPS-injected control mice or PBS-treated *Nurr1^{Cd11bcre}* mice (Fig. 8). These results suggest that a lack of *Nurr1* in microglia and other Cre-expressing leukocytes renders DAergic neurons more conducive to LPS-induced α -synuclein aggregation.

Discussion

The present study examined the protective function of *Nurr1* in microglia and peripheral leukocytes against neuroinflammation and associated nigrostriatal DAergic neuron loss. We chose *Cd11b-Cre* mice to selectively delete *Nurr1* in the microglia and other immune cells by crossbreeding with floxed *Nurr1* knock-in mice. *Cd11b-Cre* [Tg(ITGAM-cre)2781Gkl] transgenic mice have been used successfully to manipulate gene expression in the microglia for studying the impact of neuroinflammation on neurodegenerative diseases (Boille et al.,

2006). Under the control of human *CD11b* promoter, Cre DNA recombinase is also expressed by peripheral macrophage (Boillée et al., 2006). It has been reported that Nurr1 expresses not only in DAergic neurons and several other phenotypic neurons (Ahn et al., 2018; Castillo et al., 1998; Saucedo-Cardenas et al., 1998; Zetterström et al., 1997; Moon et al., 2019), but also in glia and peripheral leukocytes (Saijo et al., 2009; Li et al., 2018; Jakaria et al., 2019). Therefore, we produced a line of *Nurr1* cKO mice that deleted *Nurr1* in the microglia and other Cre-expressing peripheral leukocytes by crossbreeding floxed *Nurr1* knock-in mice with *Cd11b-Cre* mice. Most of our work was focused on investigating the link between the microglia-induced neuroinflammation and DAergic neurodegeneration in the brain of *Nurr1^{Cd11bcre}* mice. Since Cre DNA recombinase is also expressed by macrophages and other leukocytes (Boillée et al., 2006), we cannot exclude the possibility that *Nurr1* deletion in peripheral Cre-expressing leukocytes is also involved in the degeneration of DAergic neurons. Interestingly, our previous work demonstrate that *Nurr1* expression is decreased in the peripheral monocytes of PD patients, in which the reduction of Nurr1 expression is correlated with the increase of inflammatory cytokines expression, which supports the notion that the reduction of *Nurr1* in the peripheral immune cells, such as monocytes, might also play a role in the PD-related degeneration of DAergic neurons (Le et al., 2008; Li et al., 2018). Future experiments will be required to specifically examine the contribution of Nurr1 in either microglia or peripheral immune cells against PD-related neuropathological abnormalities.

In the present study, we found an increased microglia activation and abnormal motor behavior phenotypes in *Nurr1^{Cd11bcre}* mice. However, these mice did not develop any apparent degeneration of DAergic neurons. By contrast, systemic application of low-dose LPS not only exacerbated neuroinflammation and motor phenotypes, but also caused significant nigrostriatal DAergic neuron loss. Therefore, we provide *in vivo* evidence to demonstrate that genetic deletion of *Nurr1* in microglia exacerbates the environmental toxin-induced inflammatory injury to nigrostriatal DAergic neurons. Our findings thereby establish a critical physiological function of NURR1 in protecting nigrostriatal DAergic neurons against environmental insults, and further support NURR1 as an important molecular target to improve the current treatment of PD.

The *Nurr1^{Cd11bcre}* mice displayed an abnormal locomotor activity and age-dependent impairment of motor coordination and balance. However, the underlying cell-type and neural circuit mechanism of the motor behavioral abnormalities remain to be determined. While PD is clinically manifested with slowness of movement. However, many mouse models with either toxin-induced lesion of nigrostriatal DAergic neurons or PD-related genetic mutations show hyperactivity in the Open-field tests (Sgobio et al., 2014; Unger et al., 2006). The hyperactivity is likely due to the over-compensation of the impaired DA transmission (Unger et al., 2006), although the detailed circuit mechanisms are not known. Besides Open-field test, rotarod test is another commonly used experimental procedure to evaluate the motor coordination, balance and leaning in PD-related rodent models (Karl, Pabst, & von Hörsten, 2003). Impaired rotarod performance has been observed in MPTP and 6-OHDA-lesioned models (Ayton et al., 2013; Monville, Torres, & Dunnett, 2006), as well as *Nurr1* heterozygous KO mice (Jiang et al., 2005). Our recent study also highlights a critical involvement of nigrostriatal DAergic neurons in rotarod motor skill learning (Wu et al.,

2019). Consistent with these previous findings, the rotarod performance of *Nurr1^{Cd11bcre}* mice was decreased in an age-dependent manner. Furthermore, the declined rotarod performance was accelerated by the peripheral injection of LPS. Since it is difficult to correlate behavioral phenotypes with postmortem pathological abnormalities, future studies will need to apply live imaging techniques in behaving mice to investigate how neuroinflammation affects the activity of DAergic neurons in motor control.

A number of studies have demonstrated that systemic LPS stimulation can cause a loss of DAergic neurons and other PD-related pathological abnormalities (Biesmans et al., 2013; Krishna, Dodd, & Filipov, 2016). A single intraperitoneal injection of LPS at 5 mg/kg leads to significant DAergic neuron damage in C57BL/6 mice 7 months after LPS treatment (Qin et al., 2007). When we injected the control mice with 0.33 mg/kg LPS, this low-dose LPS caused only microglia activation, but not DAergic neuron loss. In contrast, the same dosage of LPS treatment exacerbated the microglial and astrocyte activation, which may result in substantial loss of DAergic neurons in the *Nurr1^{Cd11bcre}* mice. Microglia are usually classified into two subtypes according to their functions, pro-inflammatory M1 and immunosuppressive M2, although this classification remains controversial (Ransohoff, 2016; Tang & Le, 2016). We found the pro-inflammatory factor *Il-1 β* was elevated in LPS-*Nurr1^{Cd11bcre}* mice, which supports the possibility of M1 microglia activated. Analogically, A1 astrocytes is postulated to be harmful and has been shown to be induced by activated microglia and abundant in many neurodegenerative diseases including PD (Liddel et al., 2017). NURR1 has been shown to act as a transrepressor of NF κ B p50/p65 subunits-mediated transcriptional activation of proinflammatory genes in the glia cells (Saijo et al., 2009). NURR1 activator C-DIM 12 suppresses the LPS-induced binding of NF κ B p65 subunit to the promoters of proinflammatory genes and concomitantly enhances NURR1 nuclear translocation (De Miranda et al., 2015). In line with these early findings, an increase of NF κ B activity was also detected in LPS-treated *Nurr1^{Cd11bcre}* mice, which correlates with increased expression of the genes encoding proinflammatory cytokines and activation of A1 astrocytes (Liddel et al., 2017). Considering the proinflammatory cytokines, such as *Il-1 β* overexpressed and phosphorylated NF κ B p65 activation, we suspect the astrocytes in LPS-*Nurr1^{Cd11bcre}* mice are more likely to be the A1 type.

Previous studies also reveal genetic interplay between NURR1 and α -synuclein. Overexpression or abnormal accumulation of α -synuclein suppresses *Nurr1* expression and interrupts NURR1 function, while forced expression of *Nurr1* protects DAergic neurons against α -synuclein-induced cytotoxicity (Decressac et al., 2012). Here we found that systemic LPS treatment leads to increased α -synuclein aggregation in the soma of DAergic neurons of *Nurr1^{Cd11bcre}* mice. Substantial evidence has shown that α -synuclein could induce neuroinflammation in the brain (Couch, Alvarez-Erviti, Sibson, Wood, & Anthony, 2011; Shao et al., 2019). On the other hand, neuroinflammation can also enhance α -synuclein post-translational modification and aggregation in PD mouse models (Gao et al., 2011). However, the exact molecular mechanism of the elevated α -synuclein aggregation in DAergic neurons in *Nurr1^{Cd11bcre}* mice remains to be determined.

In summary, our findings support an important physiological function of NURR1 in microglia and other leukocytes in protecting DAergic neurons against inflammatory injury.

The *Nurr1^{Cd11bcre}* mice may serve as a useful preclinical model system to further elucidate how neuroinflammation leads to DAergic neuron loss in PD and test potential therapeutics.

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Main points

LPS treatment could exacerbate neuroinflammation and dopaminergic neuron degeneration in *Nurr1^{Cd11bcre}* conditional knockout mice.

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Figure 1. Construction and conditional *Nurr1* deletion in *Nurr1^{Cd11bcre}* mice

(A) The strategy to insert loxP into the intron 2 and 3 in *Nurr1* genomic DNA. The number shows the exons of *Nurr1*. (B) qRT-PCR analysis of *Nurr1*, *Nur77* and *Nor1* mRNA in primary microglia in *Nurr1^{Cd11bcre}* mice and control (n=3 per each group). Unpaired t test: *Nurr1*, p=0.0015; *Nur77*, p=0.8033; *Nor1*, p=0.75.

Figure 2. Behavioral performance in *Nurr1^{Cd11bcre}* mice

(A) Locomotor test of 6–10 month-old, 11–15 month-old and 16–20 month-old *Nurr1^{Cd11bcre}* mice and their littermate control (n=10–12). *Nurr1^{Cd11bcre}* mice perform a significant increased ambulatory distance than control. Two-way AVOVA: age, $F(2, 60)=12.85$, $p<0.0001$; genotype, $F(1, 60)=68.7$, $p<0.0001$; interaction: $p=0.0002$. Tukey's multiple comparison test: 6–10 months Ctrl versus 6–10 months *Nurr1^{Cd11bcre}*, $p<0.0001$; 11–15 months Ctrl versus 11–15 months *Nurr1^{Cd11bcre}*, $p=0.0251$; 16–20 months Ctrl versus 16–20 months *Nurr1^{Cd11bcre}*, $p=0.0708$. (B) Accelerating rotarod performance of 6–10 month-old, 11–15 month-old and 16–20 month-old *Nurr1^{Cd11bcre}* mice and their littermate control (n=10–12). Two-way AVOVA: age, $F(2, 60)=3.591$, $p=0.0337$; genotype, $F(1, 60)=4.9$, $p=0.0307$.

Figure 4. Behavioral alternations of *Nurr1^{Cd11bcre}* mice after LPS exposure

(A) Accelerating rotarod performance of each group after 12 weeks. After LPS treatment, *Nurr1^{Cd11bcre}* mice (n=5) showed a significant decrease of time on the rotarod when compared to controls (n=5). Two-way ANOVA: treatment, $F(1,16)=6.049$, $p=0.0257$; genotype, $F(1,16)=7.725$, $p=0.0134$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0305$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0473$. (B) Travelled distance of *Nurr1^{Cd11bcre}* mice and aged-matched control mice in locomotor test after LPS administration. Two-way ANOVA: treatment, $F(1,16)=1.363$, $p=0.2601$; genotype, $F(1,16)=18.61$, $p=0.005$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0081$. (C) Alternation rate of *Nurr1^{Cd11bcre}* mice and aged-matched control mice in Y maze test after LPS or PBS administration. Two-way ANOVA: treatment, $F(1,16)=0.0054$, $p=0.9423$; genotype, $F(1,16)=2.638$, $p=0.1238$. (D) The escape latency of the first 4 days in Water Morris test of *Nurr1^{Cd11bcre}* and control mice after LPS or PBS administration. (E) Comparison of the swimming time in platform quadrant of each group mice on the fifth day in Morris water maze test. Two-way ANOVA: treatment, $F(1,16)=3.045$, $p=0.1001$; genotype, $F(1,16)=0.0686$, $p=0.7968$.

Figure 5. Inflammation exaggerated in *Nurr1^{Cd11bcre}* mice after LPS exposure

(A) Double-label immunofluorescence of IBA-1 (green), TH (red) and Hoechst (blue) in SN showed significant increased of activated microglia in *Nurr1^{Cd11bcre}* mice after LPS injection. Microglia of control mice with PBS exhibited resting ramified morphology (as arrows showed). Square boxes are the magnification of microglia that arrows point to. Scale bar = 100μm. (B) There was a significant increase of the number of IBA-1 positive cells in SNc and SNr of *Nurr1^{Cd11bcre}* mice with LPS injection when compared to the control mice with the same administration (n=3 per each group). Two-way ANOVA: treatment, $F(1,8)=15.7$, $p=0.0042$; genotype, $F(1,8)=15.31$, $p=0.0045$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0262$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.025$. (C) Fractal dimensions of microglia in each group (n=3 per each group). Two-way ANOVA: treatment, $F(1,8)=8.249$, $p=0.0208$; genotype, $F(1,8)=8.474$, $p=0.0196$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0196$. (D) IL-1β mRNA expression. Two-way ANOVA: treatment, $F(1,8)=15.7$, $p=0.0042$; genotype, $F(1,8)=15.31$, $p=0.0045$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0262$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.025$. (E) GFAP signal intensity. Two-way ANOVA: treatment, $F(1,8)=15.7$, $p=0.0042$; genotype, $F(1,8)=15.31$, $p=0.0045$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0262$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.025$.

LPS, $p=0.0229$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0238$. (D) qRT-PCR analysis of IL-1 β mRNA in the midbrain of these mice with or without LPS treatment ($n=4-5$ per each group). Two-way ANOVA: treatment, $F(1,15)=7.259$, $p=0.0167$; genotype, $F(1,15)=15.41$, $p=0.0014$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0002$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0010$. (E) Double label immunostaining of TH (red) and GFAP (green) in SN of each phenotype mice after LPS or PBS administration. Astrocytes became hypertrophic in mice after LPS stimulation, especially in *Nurr1^{Cd11bcre}* mice. (F) Comparison of relative fluorescence intensity of GFAP in SNc and SNr of each group ($n=3$ per each group). Two-way ANOVA: treatment, $F(1,8)=63.12$, $p<0.0001$; genotype, $F(1,8)=12.26$, $p=0.0081$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0223$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0006$; Ctrl-PBS versus Ctrl-LPS, $p=0.011$.

Figure 6. Nurr1^{Cd11bcre} mice were more sensitive to LPS mediated neurotoxicity.

(A) Representative brain images with TH immunostaining after LPS injection indicated that prominent DA lesions of SN in *Nurr1^{Cd11bcre}* mice after LPS stimulation. Scale bar is 200 μ m. (B) Quantification of TH positive neurons in SNc. A decline of TH positive neurons was detected in *Nurr1^{Cd11bcre}* mice after LPS treatment when compared to their littermates control mice (n=3 per each group). Two-way ANOVA: treatment, $F(1,8)=15.4$, $p=0.0044$; genotype, $F(1,8)=10.75$, $p=0.0112$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0446$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0237$. (C) HPLC analysis of DA level in striatum in mice after LPS or PBS injection (n=5 per each group). Two-way ANOVA: treatment, $F(1,16)=5.802$, $p=0.0284$; genotype, $F(1,16)=3.5$, $p=0.0798$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.1756$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p=0.0904$.

Figure 7. LPS exposure activated NFκB pathway in *Nurr1^{Cd11bcre}* mice
 (A) Double label immunostaining of TH (red) and phospho NFκB p65 (green) in SN of the mice after LPS or PBS administration. Numerous phospho NFκB were found in SN only in LPS-injected *Nurr1^{Cd11bcre}* mice. Scale bar = 100μm. (B) Relative fluorescence intensity of phospho NFκB p65 in SNc and SNr of each group (n=3 per each group). Two-way ANOVA: treatment, $F(1,8)=120.4$, $p<0.0001$; genotype, $F(1,8)=104.2$, $p<0.0001$. Tukey's multiple comparison test: Ctrl-LPS versus *Nurr1^{Cd11bcre}*-LPS, $p<0.0001$; *Nurr1^{Cd11bcre}*-PBS versus *Nurr1^{Cd11bcre}*-LPS, $p<0.0001$.

Figure 8. Formation of cytoplasmic α -synuclein inclusions in *Nurr1^{Cd11bcre}* mice after LPS stimulation
Double label immunostaining of phosphorylated α -synuclein (pS129) (green) and TH (red) in the SNc area of the mice after PBS or LPS treatment. Aggregation of phosphorylated α -synuclein (pS129) was found in the cytoplasm of TH positive neurons in SNc area of LPS-injected *Nurr1^{Cd11bcre}* mice. Scale bar = 25 μ m.

Table 1

Primer sequences for quantitative polymerase chain reaction (qPCR).

	Forward (5' to 3')	Reverse (5' to 3')
<i>Gapdh</i>	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
<i>Nurr1</i>	ACGGGCTGGATTCCCAATAG	AGGCATGGCTTCAGCAGAGT
<i>Nur77</i>	TGATGTTCCCGCCTTTGC	CAATGCGATTCTGCAGCTCTT
<i>Nor1</i>	GAGGTCGTCTGCCTTCCAAA	TGGTTCAAAGCATTATAGAACT
<i>Tnf-α</i>	GACCCCTTTACTCTGACCCC	AGGCTCCAGTGAATTCGGAA
<i>IL-1β</i>	AAGGGGACATTAGGCAGCAC	ATGAAAGACCTCAGTGCGGG
<i>Arg-1</i>	CTTGCGAGACGTAGACCCTG	TCCATCACCTTGCCAATCCC
<i>Tgf-β</i>	CACTCCCGTGGCTTCTAGTG	CTTCGATGCGCTTCCGTTTC