Identification of the Transcription Factors of CD200R1 and Its Potential Roles in Parkinson's Disease

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Abstract

Background

Neuroinflammation is known to be involved in the pathogenesis of Parkinson's disease (PD). Abnormal activation of microglia plays a key role in this pathological process. CD200R1 is a membrane glycoprotein primarily expressed in microglia in central nervous system responsible for transducing signaling maintaining microglia in stationary status. Our previous studies have demonstrated the dysregulation of CD200R1 and its involvement in PD pathogenesis. The binding of transcription factors with promoter regions is the basic and essential step for the regulation of gene expression. However, little is known about the human CD200R1 promoter region and the mechanism of the dysregulated expression of CD200R1 in PD.

Methods

Luciferase reporter system was initially employed to identify the core region of CD200R1 promoter and figure out its potential transcription factors. Subsequently, we investigated the interaction adopting electrophoretic mobility shift assay and chromatin immunoprecipitation assay. The regulatory function of the detected transcription factors were further proved through its down-regulation and overexpression. We then collected the peripheral blood mononuclear cells from both PD patients and their healthy counterparts with matched age and sex to evaluate whether consistent results existed under clinical setting. Ultimately, the mouse model was established through knocking-out the identified transcription factor and its role in neuroinflammation and pathogenesis of PD was explored.

Results

We defined that the core promoter region of CD200R was located within -482 to -146 bp upstream of the translation initiation site (TIS). In addition, we demonstrated that NFKB1 directly bound to the CD200R1 core promoter region and regulated its transcriptional activity. Besides, the expression of NFKB1 and CD200R1 was significantly correlated in human peripheral blood mononuclear cells and knocking down NFKB1 significantly reduced CD200R1 expression. Moreover, both NFKB1 and CD200R1 were significantly downregulated in samples from PD patients. Furthermore, NFKB1^{-/-} mice exhibited exacerbated microglia activation and dopaminergic neuron loss after MPTP treatment.

Conclusion

Our study provided novel understanding of the transcriptional regulation of CD200R1 and its role in microglia homeostasis in the pathogenesis of PD.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease worldwide and is characterized by dopaminergic neuron loss and α -synuclein aggregation in substantia nigra (SN)[1]. Accumulating evidence has demonstrated that multiple genetic and acquired factors contribute to the loss of dopaminergic cells in SN of PD patients[2, 3]. Among these culprits, neuroinflammation, which is primarily

caused by the activation of microglia and subsequent production of proinflammatory cytokines, plays an important role during the neurodegenerative process of PD[4, 5] .

Microglia are thought to be the macrophages resident in center nerve system (CNS) and the main effector of brain immune function[6]. Autopsy on PD patients revealed overactivated microglia in SN[7, 8]. Large amounts of neuroinflammation-related molecules, such as Interleukin-6, Interleukin-1 β , tumor necrosis factor- α (TNF- α) and interferon-gamma (IFN- γ), have been detected in brain microglia, as well as in the cerebrospinal fluid and serum of PD patients[9]. These previous studies suggest that the persistent activation of microglia is involved in the disease's progression.

For decades, it was presumed that microglia were stationary and inactive immune cells which were the first responders to neuroinflammation or damage and rapidly adapted their phenotype and functions in response to the brain milieu[6]. And any disturbance in this environment would lead to the activation of microglia[10]. The communication between neurons and microglia determines microglia homeostasis in CNS[11]. The molecular pair CD200 and its receptor CD200R1, mainly expressed on the membrane of neurons and microglia, respectively, have attracted our attention due to its powerful immunoregulatory functions[12]. In mice, knocking down either CD200 or CD200R1 can cause microglia overactivation, while overexpressing CD200 or CD200R1 can lead to less microglia assemblies and decreased inflammatory factor level[13-16].

Previous work revealed that disrupting CD200-CD200R1 axis would cause neuroinflammation. For instance, Broderick et al. found that mice lacking CD200 expression had a higher level of activated microglia and presented with severe symptoms of experimental autoimmune encephalomyelitis (EAE)[17]. Blocking CD200R1 using its antibody aggravated EAE in rats by activating macrophages[18]. Furthermore, we previously demonstrated that interfering the interaction between CD200 and CD200R1 by blocking the receptor in 6-OHDA-treated rats, an experimental rat model of PD, could result in higher microglial activation, nigral dopaminergic neurons loss, and enhanced releasing of pro-inflammatory cytokines[19]. These results implied that any factors affecting CD200 and CD200R1 expressions or their interaction may disequilibrate microglia homeostasis and lead to PD.

Studies have reported the dysregulation of CD200R1 expression in neurodegenerative diseases including PD. For instance, viral model of multiple sclerosis showed reduced CD200R1 expression in the spinal cord at the later chronic-phase[20]. Walker et al. demonstrated that the mRNA expression of CD200R1 was significantly reduced in hippocampal and infratemporal gyrus samples from patients with Alzheimer's disease (AD)[21]. An age-dependent downregulation of CD200 and CD200R1 has been found in the brain of MPTP-administered mice, another experimental model of PD, with its expression inversely correlated with the increase of pro-inflammatory factors, including IFN γ , TNF- α and IL-1 β [22]. Moreover, a recent study demonstrated that CD200R1 expression was downregulated at both the early and late stages of the PD model[23]. Significantly, we have previously reported that compared with the peripheral blood mononuclear cells (PBMCs) from young adults, PBMCs from the old and PD patients were found unable to effectively upregulate the expression of CD200R1 in response to inflammatory stimulations[24]. Altogether, these results suggested that the dysregulation of CD200R1 expression was associated with the development of PD and other neurodegenerative diseases. Thus, exploring the regulatory mechanism of CD200R1 expression is of great significance for understanding the pathogenesis of PD as well as other neurodegenerative diseases.

Transcription factor-promoter combination is one of the most important mechanisms regulating gene expression. Previously, two transcription factors of Cd200r1 were identified in mouse. Dentesano's group showed that C/EBPb negatively regulated basal Cd200r1 expression in mouse microglia in vitro[25]. Another report from them implied that the agonist 15d-PGJ2 of the transcription factor PPARg inhibited the reduction of Cd200r1 expression caused by inflammatory stimulus, although it did not change the basal level of Cd200r1 mRNA in mouse microglia[26]. However, we found that the promoter activity of human CD200R1 was not significantly altered when overexpressing C/EBPb or PPARg, and yet little is known about the transcriptional regulation of human CD200R1, as well as its involvement in the pathogenesis of PD.

The present study aimed to identify the core promoter region of CD200R1, to determine the transcription factors decisively responsible for the regulation of CD200R1, and to evaluate their impact on microglia activation and dopaminergic neurons loss in PD.

Materials And Methods

Isolation and culture of PBMCs

Sample collection protocol for this study was approval by the Health Research Ethic Authority of Ruijin Hospital Affiliated to Shanghai Jiaotong University School of Medicine and the written informed consent was signed by each donor. About 5 ml of blood was obtained from each donor using BD Vacutainer tubes containing acid-citrate-dextrose anticoagulant. PBMCs were isolated by Ficoll-Paque gradient centrifugation. For small interfering RNA (siRNA) experiment, PBMCs were cultured in Dulbecco's modified eagle medium (DMEM) medium supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO/Invitrogen, Shanghai, China) and placed in a 37°C humidified incubator with 5% CO₂ for 24 h before transfection. Genomic DNA was extracted using HiPure Tissue DNA Micro kit (Magen, Shanghai, China).

SiRNA and transfection

The Silencer Select siRNA targeting NFKB1, and the corresponding negative controls were purchased from RiboBio (Guangzhou, China). SiRNA transfection was performed on HEK293t cell line and PBMCs using Lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA), following the manufacturer's protocol.

Construction of luciferase reporter gene vectors and luciferase reporter assays

CD200R1 promoter sequences were amplified by PCR using genomic DNA from HEK293T cells as a template. Seven different promoter fragments were amplified using the primers listed in Table 1. PCR was performed following the protocol of PrimeStar (Takara, R045A). The PCR products were digested and cloned into pGL3-basic luciferase plasmids (Promega, Beijing, China) and transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen, Shanghai, China). Transcriptional activity was detected by the Dual-Luciferase Reporter Assay System (Promega, E1980) on a Synergy™ H4 Hybrid Microplate Reader (Biotek, Shanghai, China). Readings were taken in duplicate for each sample. The transcriptional activities are reported as relative luciferase activity, which was calculated as the ratio of firefly luciferase activity to that of Renilla luciferase activity.

Quantitative RT-PCR (qRT-PCR)

RNA was extracted using HiPure RNA Mini kit (Magen, Shanghai, China) and reverse transcription was conducted utilizing PrimeScript PT reagent kit with gDNA Eraser (TAKARA, Dalian, China). SYBR Premix Ex Taq™ (Takara, RR420) and 7500 real time PCT system (Applied Biosystem, USA) were applied for qRT-PCR. Relative expression between groups was calculated using the $2^{-\Delta\Delta C_t}$ method. The primer sequences used in this step were described in Table 1.

Chromatin immunoprecipitation-qPCR

Chromatin immunoprecipitation (ChIP) experiment was performed on PBMCs using the SimpleChIP Plus Sonication ChIP Kit 4C and RT Reagent (CST, USA), following the manufacturer's protocol. Briefly, cells were cross-linked in 37% formaldehyde (Sangon, Shanghai, China) for 10 min at room temperature before stopping it with 0.125mol/L of glycine. Chromatin was fragmented by sonication using Ultrasonic Signal Transmitting Instrument (Xianou, Nanjing, China). For immunoprecipitation, 100μL of sonicated chromatin was incubated at 4°C overnight with ChIP grade anti-NFKB1 antibody (Abcam, Cambridge, MA). Antibody-chromatin complex was then incubated with protein A beads at 4°C for 2 h. Then the beads were washed and incubated with 1μL of proteinase K at 55°C for 60 min. Purified DNA was extracted for qPCR analysis. qPCR was conducted using gene-specific primers (Table 1). Input sample was used for normalization and the percentage value method was used to calculate the relative abundance of CD200R1 fragments in the ChIP assay.

Electrophoretic mobility shift assay (EMSA)

HEK293t cells were transfected with pcDNA3.1 vector or NFKB1 overexpression vector by Lipofectamine 3000 (Invitrogen, Shanghai, China). Total protein was extracted and quantified with BCA Protein Assay kit. EMSA was performed using biotin 5'-end-labelled complementary oligonucleotides synthesized by Biosune company (Shanghai, China). After annealing, the binding reaction of double-stranded oligonucleotides, acting as probes, and proteins was performed in incubation for 30 min at 22°C, according to the instructions of the chemiluminiscence EMSA kit (Beyotime, China). The reaction mixtures were resolved onto a 5% nondenaturing polyacrylamide gel for electrophoresis at 80V for 1 h at 4°C, and the DNA-protein complex was then transferred onto nylon membrane. To detect the DNA-protein combination signals, the membrane was incubated with streptavidin-conjugated horseradish peroxidase and visualized with chemiluminescent substrate, according to the manufacturer's instructions.

Cell-based enzyme-linked immunosorbent assay (ELISA) of CD200R1

CD200R1 expression in PBMC was determined using a cell-based ELISA. 96 well-plates were pretreated with poly-lysine at 37°C for 1 h before PBMCs were seeded. After various treatments, cells were centrifuged at 1,000 rpm for 10 min in a rotor specifically designed for micro-titer plates (Beckman Coulter, Microplus Carrier for rotor GH 3.8). The supernatants were removed, and cells were fixed with 4% paraformaldehyde for 30 min at room temperature. After washing with phosphate-buffered saline (PBS), the non-specific binding sites were blocked by 5% BSA at room temperature for 1 h, followed by incubation with CD200R1 antibody (AF3414 R&D system) at 4°C overnight. After washing by PBS for three times, horse radish peroxidase (HRP)-conjugated secondary antibody was added and incubated at room temperature for 1 h. The plate was then

washed and added with the substrate TMB (3,3',5,5'-tetramethyl benzidine) for reaction. After incubation at room temperature for 15 min, the reaction was stopped by H_2SO_4 . The absorbance at 450 nm was detected using an ELISA plate reader (Softmax 190, Molecular Devices). After first reading, fixed cells were washed with PBS for 30 min and stained with crystal violet. The reading for absorbance at 598 nm was recorded. The expression level of CD200R1 was represented by the ratio of 450 nm/598 nm.

Animals and treatments

All animal experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals. The Shanghai Jiao Tong University School of Medicine Animal Care and Use Committee approved all animals and procedures used in the studies. Eight-week-old homozygous $\text{p50}^{-/-}$ and $\text{p50}^{+/+}$ (B6,129Nfkb1tm1Bal and B6,129 2/J; Jackson Laboratory, Bar Harbor, ME) mice were used in the experiments. Animals were caged in groups of three or four given ad libitum. The animals were kept in a temperature-controlled environment at $22 \pm 2^\circ\text{C}$ on a 12:12 light-dark cycle.

Mice were randomly divided into four groups, (i) Saline/NFkB1 $^{+/+}$ group: 0.2 ml saline intraperitoneal (IP) injection of NFkB1 $^{+/+}$ mice; (ii) Saline/NFkB1 $^{-/-}$ group: 0.2 ml saline IP injection of NFkB1 $^{-/-}$ mice; (iii) MPTP/NFkB1 $^{+/+}$ group: 20 mg/kg MPTP IP injection of NFkB1 $^{+/+}$ mice; (iv) MPTP/NFkB1 $^{-/-}$ group: 20 mg/kg MPTP IP injection of NFkB1 $^{-/-}$ mice. Animals were treated once-daily for five consecutive days at 8:00-9:00 in the morning. Motor function was evaluated with multiple paradigms, including rotarod test, swim test and traction test. Each group had 12 mice. After behavioral test, mice were killed with an overdose of anesthesia (10% Chloral hydrate intraperitoneal i.p., 0.4 mL/100 g). Six mice of each group were used for immunohistochemistry as indicated in the part of "Tissue preparation".

Tissue preparation

At 7 d post last MPTP-injection, the animals were deeply anesthetized with pentobarbital (100 mg / kg, i.p.) and perfused with 150 ml of 0.9% saline and then fixed by 250 ml of 4% paraformaldehyde in phosphate buffer (PB). Brains were then dissected out (3-4 mm in thickness) and fixed with paraformaldehyde in 100 mM PB for 24 h before placed into 30% sucrose solution in PBS at 4°C for 72 h. Brains were then cryosectioned coronally on a Leica1650 cryostat (cut thickness: 25 μm) with a random start, and sections before and after the two anatomical regions were used to confirm that the entire structure had been quantified. Sections were collected serially throughout the SN and placed into PBS for further experiments.

Immunohistochemistry

Free-floating sections were pretreated with 0.3% H_2O_2 in 0.1 M PBS (pH 7.2-7.5) for 10 min at RT (60 rpm) to block endogenous peroxidase activity, and washed with 0.1 M PBS for 3 times. The tissue was then blocked with diluted blocking serum (Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) for 20 min at room temperature. Sections were then incubated with the primary antibody against TH (mouse anti-TH, 1:4000, Sigma) or CD11b (mouse anti-CD11b, 1:1000, Serotec) overnight at 4°C . Then the sections were washed and incubated with diluted biotinylated secondary antibody (Vector laboratories) for 30 min, followed by avidin-biotin complex (Vector laboratories) for 30 min at room temperature. Finally, the sections were developed with 3,3'-diaminobenzidine tetra-hydrochloride (Vector Laboratories), mounted onto glass slides for drying

overnight, and dehydrated in a gradient of ethyl alcohol and xylene the next day. The slides were sealed with permount mounting medium.

Stereological estimation of TH-positive neurons and microglia

To estimate the number of nigral dopaminergic neurons or microglia, stereological counts were performed and every sixth sections was selected between levels 2.80 and 3.80 mm from the bregma. After delineation of the SN pars compacta with a 4x objective, counts were calculated at $\times 60$ magnification in ImageJ with the following parameters: an optical dissector height of 8 mm, a counting frame of $50 \times 50 \mu\text{m}$, and an area of a grid of $100 \times 100 \mu\text{m}$. A coefficient of error of < 0.10 was acceptable.

Behavioral test

The traction test was used to measure muscle strength and equilibrium. It was performed on the fifteenth day after the last MPTP injection. Mice forepaws were placed on a rope (diameter 5 mm) and the placement of its hind limbs was scored from 1 to 3, with a lower score indicating severer deficiency. The score was assessed according to the following criteria: both hind limbs seizing the rope scored 3, one limb seizing the rope scored 2, and no limb seizing the rope scored 1. Mice were allowed to hang upside down. The animals were allowed to seize the rope for 30 s and the time of falling was recorded.

The rotarod test was performed for eight consecutive days after the IP injection. This test evaluates motor coordination and balance ability in PD mouse model (Liu et al., 2015a, Rial et al., 2014). An apparatus (YLS-4C, Academy of medical sciences in Shandong, China) consisting of a five-lane rotating rod (diameter 7.5 cm.) was able to simultaneously test five mice. The mice were made familiar with the rotarod for three consecutive days before each MPTP injection to assess sensorimotor coordination. In the evaluating session, the mice were placed on a rotating axis that smoothly accelerated from 5 to 20 rpm for a maximum of 2 min. The length of time that each animal was able to stay on the rod was recorded as the latency period to fall. The experiment was repeated three times for each animal at 10 min rest intervals to prevent stress and fatigue. The mean endurance performance was determined by calculating the mean time of three independent experiments at the given speed.

Swim test was performed for eight consecutive days in a water tub (length, 40 cm; width, 25 cm; height, 25 cm), which was filled to a depth of 15 cm with warm water at a temperature of $27 \pm 2^\circ\text{C}$ to avoid hypothermia. The mice stayed in the water tub twice daily for acclimatization for three consecutive days prior to experimentation. The score system was detailed as follows: 0, hind part sinks with head floating; 1, occasional swimming using hind limbs while floating on one side; 2, occasional floating, mostly swimming; 3, continuous swimming (Haobam et al., 2005, Kavitha et al., 2013). The experiment was repeated three times for each animal and the average of the three trials was calculated for statistical analysis.

Statistical analysis

All data are presented as the means $\pm$ standard deviations (means $\pm$ SDs). Statistical analysis was performed with GraphPad Prism 8 software. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was used to test differences between groups at specific times. Pearson correlation analysis was used to

find CD200R1 expression related to NFKB1 expression. All differences were considered statistically significant at the level of $p < 0.05$.

Results

Defining the promoter region of CD200R1

We previously identified that the promoter region of human CD200R1 was located at -876 to 146 bp upstream of CD200R1 translation initiation site (TIS)[12]. In order to find the decisive transcription factors, the minimal essential core promoter region of CD200R1 needs to be identified. We therefore constructed seven different deletion constructs and transfected those constructs as well as other two constructs integrating -1989~-146 and -876~-146, respectively, as reported in our previous study into HEK293T cells. Then, luciferase activity was measured. The results (Figure 1A and 1B) demonstrated that deletion of the distal promoter fragment from -1989 to -482 bp did not decrease the promoter activity significantly. However, the deletion of -482 to -365 bp and -365 to -266 bp fragments, rather than -266 to -146 bp, led to a statistically significant decrease of promoter activity ($p < 0.0001$ and $p < 0.0001$, respectively), revealing that a region within -482 to -267 bp incorporated the core promoter region of CD200R. For further confirmation, we deleted the -482~-267 bp from the long fragment of -1989 to -146 bp, and the results showed that the strong promoter activity of the -1989 to -146 bp fragment disappeared after deleting the -482 to -267 bp fragment (Figure 1C and 1D). To summarize, the 482 to 267 bp upstream of the TIS forms the core region of the CD200R1 promoter and is sufficient to drive its transcription activity.

NFKB1 binds to the promoter of CD200R1 and regulates its transcription activity

Transcription factors with a functional DNA-binding domain can be recruited to the promoter regions in complex with co-activators. To identify the transcription factors involved in CD200R1 regulation, five strategies were used: i) in silico analysis identifying the putative binding sites for transcriptional factors within CD200R1 core promoter sequence using two different programs, UCSC CHIP-seq databases (<http://genome.ucsc.edu/>) and JARSPAR (<http://jaspar.genereg.net/>); ii) excluding transcription factors that are not expressed in the brain thorough the Human Protein atlas website (<https://www.proteinatlas.org/>); iii) A total of six shared TFs by JARSPAR and UCSC chip-seq were showed in Figure 2A and evaluated in further studies; iv) the other 11 transcription factors in relation with either inflammation or neural functions were also enrolled for further analysis (Figure 2A and 2B); and v) the reported two transcription factors of Cd200r1 in mouse, CEBPB and PPARG, were also evaluated in this study. Totally, 17 candidate transcription factors were included.

The functional relevance of these transcription factors in regulating CD200R1 promoter activity was assessed by overexpression and knockdown experiments. Through overexpressing these candidate transcription factors in HEK293T, respectively, we found significant increases in CD200R1 promoter activity with NFKB1 ($p < 0.0001$), CREB1 ($p < 0.0001$) and RELA ($p < 0.0001$) (Figure 2C). Whereas in the monocyte cell line U937 and the microglial cell line BV2, only NFKB1 was found to significantly elevate the CD200R1 promoter activity (both $p < 0.0001$) (Figure 2D and 2E).

Consistently, knocking down NFKB1 reduced the activity of CD200R1 promoter ($p < 0.0001$) (Figure 3A). To confirm their specific interaction, ChIP assay was performed and the CD200R1 promoter sequence was detected in the DNA-protein complex immunoprecipitated by anti-NFKB1 antibody (Figure 3B).

We further performed EMSA experiments to examine the direct binding of NFKB1 to the CD200R1 promoter. Total proteins were extracted and incubated with biotin labeled probes: a positive control oligonucleotide formerly confirmed to bind with NFKB1, a negative control oligonucleotide with unknown combination, and the four indicated experimental oligonucleotides (Table 2), respectively. The results revealed that probes 3 (-425~-365) and 4 (-482~-420) showed the strongest binding capacity with NFKB1 when overexpressing NFKB1 (Figure 3C), in consistence with the prediction results. Taken together, we conclude that NFKB1 can directly bind to the CD200R1 promoter region.

NFKB1 positively regulates CD200R1 expression

Primary microglia from NFKB1+/+, NFKB1+/- and NFKB1-/- mice were isolated to evaluate the regulatory role of NFKB on CD200R1. Compared with the NFKB+/+ group, a 20% reduction of CD200R1 mRNA expression level in NFKB1 -/- group and a 40% elevation in NFKB1 +/- mice were observed ($p < 0.0001$, Figure 3D). Furthermore, the mRNA expression of CD200R1 and NFKB1 was assessed in PBMCs from ten healthy individuals. PBMCs were selected because of their similarity with monocytes to some extent, as mimics of microglia. Sequential Spearman correlation analysis showed a strong positive correlation between CD200R1 and NFKB1 expressions ($r = 0.89091$, $p = 0.00054$) (Figure 3E). In addition, silencing NFKB1 using siRNA significantly downregulated CD200R1 mRNA levels ($p < 0.05$, Figure 3F); and cell-based ELISA suggested that the protein expression of CD200R1 was also significantly reduced (Figure 3G).

Reduced NFKB1 and CD200R1 expression in PD patients

Given that CD200R1 was significantly regulated by NFKB1, we examined the mRNA expression of NFKB1 and CD200R1 in PBMCs from 44 PD patients and 45 age- and sex-matched healthy controls (Table 3). All these patients had no familial history of PD. Both CD200R1 and NFKB1 expressions were significantly reduced in PD patients ($P < 0.0001$) (Figure 4A and 4B). Spearman correlation analysis showed a strong positive correlation between CD200R1 and NFKB1 expressions ($r = 0.801$, $p < 0.0001$) (Figure 4C). These results further validated the regulatory role of NFKB1 on CD200R1 expression and their relevance in the development of PD.

Knockout of NFKB1 exacerbates MPTP-induced neurodegeneration, microglia activation and PD-related motor impairment

Our previous *in vivo* study has demonstrated that impairment of CD200-CD200R1 signaling would cause abnormal dopaminergic neurons loss and activation of microglia *in vivo*, participating in the pathogenesis of PD[19]. To evaluate the role of NFKB1 and CD200R1 in dopaminergic neuron loss and microglia activation, we stained the midbrain coronal sections of MPTP-induced PD mouse model with an antibody against tyrosine hydroxylase (TH) and counted the TH-immunoreactive (TH-ir) neurons in SN. Specifically, we applied a dose of 30mg/kg of MPTP to yield an animal model of PD[22, 27], and found that it could cause the loss of most of the dopaminergic neurons in SN (>60%) at 1week post-lesion. Therefore, in the present study, we used a sub-dose of MPTP (20mg/kg) that was reported to result in moderate dopaminergic neurodegeneration in

SN. This is also the sub-toxic dose of MPTP suggested by several groups [28, 29]. As expected, we observed that the sub-toxic dose of MPTP was able to induce moderate dopaminergic neurodegeneration in SN in NFKB+/+ mice (Figure 5A and 5B). However, the TH-ir neurons were significantly reduced in NFKB1-/- mice (Figure 5A and 5B). These results indicate an exacerbating effect of NFKB1 knockout on the degeneration of dopaminergic neurons (Figure 5A and 5B).

Microglial activation was also evaluated. Staining of a microglia-specific marker CD11b (a constitutive marker of microglia) showed the morphological changes of microglia. Besides, the total number of CD11b-positive cells in SN in MPTP/NFKB1-/- mice was significantly increased, compared with that in MPTP/NFKB1+/+ mice (Figure 5C and 5D).

To evaluate whether the PD phenotype of MPTP/NFKB1-/- mice is consistent with the dopaminergic neuron loss and microglia activation in SN, we conducted swim test, traction test and rotarod test, which are routine tests evaluating PD phenotype to estimate the motor coordination. In swim test (Figure 4E), several mice in the MPTP/NFKB1+/+ group exhibited a slight reduction of swim score in comparison with the untreated NFKB+/+ mice. However, the swim score in the MPTP/NFKB1-/- group was significantly lower than that of the untreated NFKB1-/- group and MPTP NFKB1+/+ group ($P < 0.001$, respectively). Results from traction test (Figure 5F) suggested that MPTP/NFKB1-/- mice had a decreased strength as the hanging time was shorter than the MPTP/NFKB1+/+ group. Rotarod test (Figure 5G) suggested that compared with the MPTP/NFKB1+/+ mice, MPTP +/- NFKB1-/- mice had a shorter dropping latency. These results indicate that NFKB1 knockout can exacerbate the motor impairments of MPTP.

Discussion

CD200-CD200R1 signaling functions in maintaining the quiescent state of microglia. CD200R1 plays a key role in the interaction since it is responsible for transducing the downstream signals inhibiting microglia activation. Either the dysregulation of CD200-CD200R1 interaction or the abnormal expression of CD200R1 has been reported to be involved in the pathogenesis of PD. The abnormal expression of CD200R1 has been identified in PD; however, the mechanisms that regulate human CD200R1 expression under physiological and pathological conditions are largely unknown.

The binding of transcription factors to promoter regions is the basic and essential step for gene expression. To identify the transcription factors of CD200R1, we firstly defined that nucleotides between -482 to -267 bp upstream of TIS constituted the core promoter region of CD200R1. Further in silico studies in combination with overexpression experiment in multiple cell lines revealed that CD200R1 promoter region presented with potential binding sites for transcription factor NFKB1. Then we confirmed that NFKB1 could definitely bind to the promoter region of CD200R1 and promote its transcriptional activity. Additionally, a strong correlation between CD200R1 and NFKB1 expression was shown in PBMCs from either healthy individuals or PD patients. And their expressions were significantly reduced in PBMCs of PD patients. Downregulation of CD200R1 could result in microglia activation and dopaminergic neurons loss in PD mouse model. Our results showed that a lack of NFKB1 expression could accelerate the sub-dose MPTP-induced microglia activation, neurodegeneration and motor impairment. As a result, we concluded that the dysregulation of CD200R1 in PD is mainly or partly attributed to the downregulation of NFKB1.

Studies have reported that reduced expression of NFKB1 is associated with neurodegeneration. Yu and his group[30] reported that mice lacking NFKB1 exhibited enhanced loss of hippocampal pyramidal neuron after receiving kainic acid excitotoxin, compared to the wild-type mice. Another research group demonstrated that the lack of NFKB1 exacerbated the death of hippocampal neurons in response to chemical exposure[31], implying that NFKB1 can protect neurons from damage by excitatory toxins. In addition, Bernal's group demonstrated that deletion of NFKB1 contributed to reduced apoptosis, increased cellular senescence and accelerated aging[32]. Recently, Edward et al. reported that *nfk1b*^{-/-} mice exhibited increased inflammation, increased frequency of senescence and aging phenotypes in multiple organs and early mortality[33]. Furthermore, the decreased NFKB1 expression was observed during the neurodegenerative process under both experimental and clinical conditions. Saldaña et al. reported that the NFKB1 expression was decreased in SN in the brain of patients with Lewy body dementia[34]. In another study, Saldaña et al. found that NFKB1 levels were reduced in experimental models of dopaminergic neurodegeneration[35]. Here, we demonstrated that NFKB1 expression was reduced in PBMCs from PD patients. The above studies and our results imply that NFKB1 plays an important role in anti-inflammation and neuroprotection. Our data provided supportive evidence for the neuroprotective role of NFKB1 in PD.

Conclusion

In summary, our present work identified the core promoter region of CD200R1 and its transcription factor NFKB1, and revealed their contributions to CD200R1-associated microglia homeostasis. Our work is an important supplement to the mechanism underlying CD200R1 regulation. Our findings also provided novel insight into the development of treatment strategies for PD. However, despite the identification of NFKB1 as a transcription factor regulating CD200R1 transcription, other regulatory mechanisms, acting on the enhancer and silencer that might also contribute to the dysregulation of CD200R1 in PD, remain to be explored. Further studies are warranted to uncover the mystery of the decreased CD200R1 expression in PD.

Abbreviations

TIS: Translation initiation site

PD: Parkinson's disease

SN: Substantia nigra

CNS: Center nerve system

TNF- α : Tumor necrosis factor-alpha

IFN- γ : Interferon-gamma

EAE: Experimental autoimmune encephalomyelitis

AD: Alzheimer's disease

PBMCs: Peripheral blood mononuclear cells

siRNA: Small interfering RNA

DMEM: Dulbecco's modified eagle medium

FBS: Fetal Bovine Serum

ChIP: Chromatin immunoprecipitation

EMSA: Electrophoretic mobility shift assay

ELISA: Cell-based enzyme-linked immunosorbent assay

PBS: Phosphate-buffered saline

Declarations

Ethics approval and consent to participate

All the procedures in this study were approved by the ethics committees of Shanghai Ruijin hospital affiliated to Shanghai Jiaotong University School of Medicine, and written informed consent was obtained from all patients.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JD designed and supervised the study. JD, SL and RS wrote the manuscript. SL and RS performed experiments and analyzed data. LH, YW, WX and YW was involved in the data analysis. SL and FF generated cell lines transfected with the core promoter region. HP and XW performed bioinformatics analysis. XZ, LH, YZ, YQ, XS and YS recruited participants and collected blood samples.

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Tables

Table 1 Primers list^a

Primer	Sequence
CD200R1p-1989 F	5'-TGCACAACAGAGACTTAACCC-3'
CD200R1p-704 F	5'-CCATTTTCAGACTTCTGATCACC-3'
CD200R1p-583 F	5'-CTCCATGCCCTCAAGGATCTG-3'
CD200R1p-482 F	5'-TGCACAACAGAGACTTAACCC-3'
CD200R1p-315 F	5'-GAGAAAAGCTTCTGTTCG -3'
CD200R1p-266 R	5'-CCCTTAGGTTTTTTCTGGATAAATC-3'
CD200R1p deletion-482 to -267 F	5'-CCAACACCTTGATTTTAGTCCGTCTTTGCTTCAGGGCTTC -3'
CD200R1p deletion-482 to -267 R	5'-GAAGCCCTGAAGCAAAGACGGACTAAAATCAAGGTGTTGG -3'
CD200R1 p-146 R	5'-CTAGCCCCTTCCTTCACGTC-3'
CD200R1 qPCR for CHIP F	5'-TACAGGAAATCTATTGCTGT-3'
CD200R1 qPCR for CHIP R	5'-CTAGCCCCTTCCTTCACGTC-3'
CD200R1 qPCR for mRNA F	5' -TGCTGTGTCAAGTTCCAGAG- 3'
CD200R1 qPCR for mRNA R	5' -CTTCCTTCTAGCCCCTTCCT- 3'
Cd200r1 qPCR for mRNA F	5' -TACGAGTGAATCACACAGCA- 3'
Cd200r1 qPCR for mRNA R	5' -CAGGACACATCAGACACATTG- 3'
NFKB1 qPCR for mRNA F	5'- GGACCAGCAAAGGTTATTGTT- 3'
NFKB1 qPCR for mRNA R	5' -AGATCCCATCCTCACAGTGTT- 3'
Nfkb1 qPCR for mRNA F	5' -AGAGGATTTGCTGAGGGTTG- 3'
Nfkb1 qPCR for mRNA R	5' -TGCTGAGGATTCTGTCTGTG- 3'

^a The numbers after the symbol "-" indicate the distance from the CD200R1 translation start site; F, forward primers; R, reverse primers; p, plasmid.

Table 2. 5'-end-labelled complementary oligonucleotides for EMSA^a.

Name	Sequence	Position ^b	
probe 1	AGAGGAGAACCAATGAGCTGAGAGATAATTCAATCAATCTGCTTCCTC	-482	-428
probe 2	TATCTTGGACACGTTCAATGCCTTACTGTAAAGTTTGTCTGTGTACAGCAGAAGTG	-427	-371
probe 3	GCCAAAGTAGATCAATATTCTGTTTTACTTTTTGGTGCAAGTTTAGACAAATCTGTT	-370	-315
probe 4	TCAACCTGGAGAAAGCCTCTAAAAGTCATCATAATTGGGAAATTTACAAGGATGG	-314	-267

^a EMSA, Electrophoretic mobility shift assay.

^bPosition, distance from translation initiation site.

Table 3. Demographic and clinical characteristics of controls and PD patients^a.

Value	Control	PD	p value ^b
	N=45	N = 44	
Sex			0.608
Male, n (%)	22(48.9)	19(43.2)	
Female, n (%)	23(51.1)	25(56.8)	
Age, mean±SD	64.70±9.41	64.13±9.78	0.441
Age of onset, mean±SD	n.a.	61.05±8.95	n.a.
Duration [year], n (%)			n.a.
≤ 2yr	n.a.	18(40.9)	
2-5yr	n.a.	17(38.6)	
5-10yr	n.a.	8(18.2)	
> 10yr	n.a.	1(2.3)	
Hoehn-Yahr stage, Median (IQR)	n.a.	1.77(0.81)	n.a.

^a Attributes data are presented as mean ± SD; variables data are presented as numbers (%)

^b the p-value of sex based on Chi-Squared Test and p-value of age based on student's t-test.

Figures

Figure 1

Core promoter sequence identification of CD200R1. (A) Schematic representation of dual-luciferase reporter constructs containing different fragments within -1989 to -146 upstream of the translation initiation site. (B) Dual-luciferase assay results in human HEK293T cells transfected with different luciferase reporter vectors as indicated in (A). (C) Schematic representation of dual-luciferase reporter construct containing the long

promoter region -1989 to -146 and the construct with the sequence of -482 to -267 deleted. (D) Dual-luciferase assay results in human HEK293T cells transfected with luciferase reporter vectors containing the two sequences listed in C. One-way ANOVA was used for statistical analysis. ** P<0.01, **** P<0.0001.

Figure 2

NFKB1 is a potential transcription factor of CD200R1 and regulates its transcriptional activity. (A) A schematic representation of the CD200R1 core promoter sequence. The sequence spans the nucleotides -482 to -146 upstream of the CD200R1 translation initiation site. (B) In silico programs prediction results. (C) Dual-

luciferase assay results in human HEK293T cells co-transfected with the core promoter vector and indicated transcription factors. (D) Dual-luciferase assay results in human U937 cells co-transfected with the core promoter vector and RELA, CREB1 and NFKB1, respectively. (E) Dual-luciferase assay results in microglia cell line BV2 transfected with the core promoter vector and NFKB1. One-way ANOVA was used for comparison. **P<0.01, **** P<0.0001.

Figure 3

NFKB1 binds to CD200R1 promoter and regulates CD200R1 expression at mRNA and protein levels. (A) Dual-luciferase assay in HEK 293T cells transfected with siRNA targeting NFKB1 and the core promoter vector. (B) PBMC chromatin was immunoprecipitated with antibodies against CD200R1 and quantified by RT-PCR, using a primer set specific for the core region (-482 to -146 bp). (C) EMSA assay after indicated treatment. (D) RT-PCR was performed to detect CD200R1 mRNA expression in primary microglia from NFKB1+/+ and NFKB1-/- mice. (E) Spearman correlation analysis of NFKB1 and CD200R1 mRNA expressions in PBMCs from healthy individuals (n=10). (F) Comparison of CD200R1 mRNA level in PBMCs transfected with a non-targeting siRNA or NFKB1 siRNAs. (G) Protein expression of CD200R1 in PBMCs transfected with a non-targeting siRNA or NFKB1 was detected by ELISA. Unpaired student test was used for statistical analysis. **P<0.01, ****P<0.0001.

Figure 4

Expression levels of NFKB1 and CD200R1 in PD patients. NFKB1 and CD200R1 mRNA levels were assessed in PBMCs from PD patients (n=44) and age- and sex-matched healthy controls (n=45) by RT-PCR. (A) CD200R1 mRNA expression in healthy control vs. PD groups. (B) NFKB1 mRNA expression in healthy control vs. PD groups. Non-parameter analysis was used for comparison between groups (C) Spearman correlation analysis between CD200R1 and NFKB1 mRNA expressions.

Figure 5

Lack of NFKB1 exacerbates phenotypes in MPTP-induced PD mouse model. (A). Animals (6 mice per group) were sacrificed on day 7 post MPTP injection and the loss of dopaminergic neurons in SN was characterized. Representative sections (~24 sections per mouse) of SN were immunostained with an antibody against tyrosine hydroxylase (TH). (B) Stereological cell counts of TH-ir neurons in SN were shown (n = 6/group). All the results were obtained in a double-blinded procedure. Data are presented as mean $\pm$ S.E.M. (C) Animals (6 mice per group) were sacrificed on day 7 post MPTP injection and the microglia activation in SN was

characterize. Representative sections (~24 sections per mouse) of SN were immunostained with an antibody against CD11b. (D) Total stereological cell counts of CD11b-positive neurons in SN were shown (n = 6/group). All the results were obtained in a double-blinded procedure. Data are presented as mean $\pm$ S.E.M. (E-G) Behavioral impairments in NFKB1 knockout mice administered with 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP). The swim score of swim test (E) and the hanging time of traction test (F) were compared 7 days after MPTP treatment. Rotarod test (G) was performed prior to MPTP injection, and at days 1, 2, 3, 4, 5, 6 post-injection. N=12 in each group. Data are presented as mean $\pm$ standard error; Differences were analyzed by one-way analysis of variance followed by least significant difference post hoc test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.