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NEW evidences for fractalkine/CX3CL1 involved in substantia nigral microglial activation and behavioral changes in a rat model of Parkinson's disease

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Abstract

Activated microglia are instrumental to neurodegeneration in Parkinson's disease (PD). Fractalkine, as an exclusive ligand for CX3CR1 expressed on microglia, has recently been reported to be released out by neurons, and induce microglial activation as a neuron-to-glia signal in the spinal cord. However, the role of fractalkine-induced microglial activation in PD remains unknown. In our study, we injected 1-methyl-4-phenylpyridinium (MPP^+) into unilateral substantia nigra (SN) which induced ipsilateral endogenous fractalkine expression on neuron and observe the increase of CX3CR1 expression in response to MPP^+ by Western blotting analysis. Moreover, pre-administration of anti-CX3CR1 neutralizing antibody which potentially blocked microglial activation can promote rotation behaviors. To further investigate the role of fractalkine in PD, we injected exogenous fractalkine in unilateral SN, and observed microglial activation, dopaminergic cell depletion, and motor dysfunction. All these effects can be totally abolished by cerebroventricular administration of anti-CX3CR1. Intracerebroventricular administration of minocycline, a selective microglia inhibitor, can prevent fractalkine-induced rotation behaviors, and inhibit dopaminergic neurons from degeneration in the way of dose-dependent. Our studies demonstrate that fractalkine-induced microglial activation plays an important role in the development of PD, and provide an evidence of fractalkine and CX3CR1 as new therapeutic targets for PD treatment.

Keywords: Parkinson's disease; Microglia; Fractalkine; CX3CR1; Rat; Rotation behaviors; Substantia nigra; MPP^+

1. Introduction

Parkinson's disease (PD) is one of the most common age-related neurodegenerative diseases, which strikes 1–2% of the aged ("over 50") population (de Lau and Breteler, 2006; de Rijk et al., 1995; Shastry, 2001). PD symptoms include tremor, rigidity, and bradykinesia. It is pathologically characterized by the progressive degeneration of dopaminergic neurons, which are mainly located in the substantia nigra pars compacta (SNpc). Although the underlying mechanisms for degeneration in PD are unknown, several etiological factors are proposed to contribute to the disease, such as mitochondrial dysfunction, oxidative stress proteasomal dysfunction and neuroinflammation are proposed as contributing factors.

Increased attention has recently been drawn to the neuroinflammatory process in PD etiology since reactive microglia were first found in the SN of PD patients in 1988 (McGeer et al., 1988; Teismann et al., 2003). Further studies indicated

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that the activated microglia also exist in the SN of several PD animal models (Cicchetti et al., 2002; Kohutnicka et al., 1998; Sloane et al., 1999; Yu et al., 2002; Sugama et al., 2003; Mastroeni et al., 2008), for instance, the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model. MPTP and its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), are widely used to model environmental cause of PD in nonhuman primates and rodents, in which they induce a selective loss of dopaminergic neurons in SN (Eberhardt and Schulz, 2003; Smeyne and Jackson-Lewis, 2005). A brief exposure of young human adults to this neurotoxin resulted in a progressive Parkinsonian features, as well as clear microglial activation and dopaminergic cell loss in SN by postmortem examination (Langston et al., 1983, 1999; Ovadia et al., 1995). These findings not only indicate ongoing nerve cell loss after a time-limited insult, but also suggest that activated microglia may perpetuate neuronal degeneration (Hirsch et al., 2005). However, the pathway that leads to microglial activation in PD animal models or in human PD is still unknown.

Fractalkine is the sole member of the CX3C chemokine class (Asensio and Campbell, 1999; Combadiere et al., 1998; Hughes et al., 2002). In the central nervous system (CNS), fractalkine is a unique chemokine, which acts exclusively on the fractalkine receptor (CX3CR1) (Bajetto et al., 2002; Chapman et al., 2000), and this receptor binds only fractalkine (Hughes et al., 2002; Jung et al., 2000). Intriguingly, fractalkine is the only chemokine that is expressed extracellularly on neurons (Asensio and Campbell, 1999; Hatori et al., 2002) while CX3CR1 is expressed predominantly by microglia. This suggests fractalkine, as a neuron-to-glia signal, might prime microglia to activate in response to injury or inflammation (Lindia et al., 2005; Sun et al., 2007; Verge et al., 2004). Although fractalkine/CX3CR1 pathway has been implicated to mediate inflammatory response in several neurological disorders (Cotter et al., 2002; Sato et al., 2006; Soriano et al., 2002; Sunnemark et al., 2005), their exact roles in dopaminergic neurons and their surrounding glia of the SN are still enigmatic.

In the present study, we have observed that endogenous or exogenous fractalkine can both induce microglial activation and cause dopaminergic neurons depletion. In contrast, inhibition of microglial activation by pre-administration anti-CX3CR1 or minocycline can abolish dopaminergic neurons depletion. This raised new evidence for microglia activation involved in PD development.

2. Materials and methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College of Fudan University, China) weighing 250–280 g. Rats were housed in groups of two in suspended cages and maintained on a 12:12 h light-dark cycle and a constant room temperature of 21 °C with free access to food and water. Prior to experimental manipulation, animals were handled daily at least for 3 days. All experimental protocols and animal handling procedures were approved by Animal Care and Use Committee (ACUC) of Fudan University, and were consistent with the National Institutes of Healthy Guide for the Care and Use of laboratory animals. All efforts were made to minimize the number of animals used and their suffering.

2.2. Intracerebral surgery

All animals were securely placed into a sterotaxic device (Stoelting, USA) under chloral hydrate (400 mg/kg, intraperitoneal, i.p.; SCR Co., Shanghai, China) anesthesia with bregma and lambda at a horizontal level. For an intranigral microinjection, 26-gauge stainless steel cannulae were unilaterally implanted 4 mm above the nigral to minimize the injury to the rats' brain according to the following coordinates: anteroposterior (AP): −5.0 mm from bregma; mediolateral (ML): 2.1 mm from midline in the left side; dorsoventral (DV): −7.7 mm from skull, adapted from Paxinos and Watson (1997). A guide cannula for an intracerebroventricular (i.c.v.) injection on the left side was stereotaxically implanted 1.5 mm above the lateral ventricle (AP = −1 mm, ML = 1.5 mm, DV = 3.5 mm from bregma), as Paxinos and Watson (1997) indicated. After surgery the animals were left in a temperature-controlled chamber until they recovered from anesthesia, as they returned to their home cage. They were housed individually and allowed to recover for 3 days before the experiment began.

2.3. Administration of drugs

Animals were pretreated with the norepinephrine uptake inhibitor desipramine (25 mg/kg, i.p.) (Sigma–Aldrich, USA). Endotoxin-free goat anti-CX3CR1 (Santa Cruz Biotechnology, CA, USA) or IgG protein (Santa Cruz Biotechnology, CA, USA) was diluted in 0.01 M sterile PBS. Endotoxin-free rat recombinant fractalkine (chemokine domain amino acid residues 25–100; R&D systems) or IgG protein was diluted in 0.01 M sterile PBS containing 0.1% bovine serum albumin (BAS, SERVA). 1-Methyl-4-phenylpyridinium (MPP^+, Sigma–Aldrich, USA) and minocycline hydrochloride (Sigma–Aldrich, USA) was dissolved in sterile saline.

For an intranigral microinjection, a 5 μl Hamilton syringe was inserted via the guide cannula and protruded 4 mm from the tip of the guide cannulae. On day 0, a total volume of 2 μl of vehicle, MPP^+ (20 μg) or fractalkine (30 ng) (we used 30 ng of fractalkine in all the cases, since rats failed to elicit rotational bias when treated with a lower dose of 3 ng, or immediately died with a higher dose of 300 ng in our preliminary experiment) was infused into SN during 5 min. The doses of MPP^+ and fractalkine were chosen in accordance

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with previous studies (Barc et al., 2002; Sun et al., 2007). The injection syringe was left in place for an additional 5 min to minimize spread of the drug along the injection track. At the end of the behavioral test, the animals received a 2 μl infusion of 4% methylene blue to verify the location of the injection site and the extent of infusion.

For an intracerebroventricular (i.c.v.) microinjection, a 25-μl Hamilton syringe filled with solution of drugs or vehicle was inserted via the guide cannula and protruded 2 mm to approach the lateral ventricle. Drugs were injected slowly in 5 min at a volume of 15 μl and left the injection syringe in place for 1 min. Rats received i.c.v. injection of vehicle, neutralizing anti-CX3CR1 antibody (15 μl) or minocycline (4 μl) according to each experiment respectively. The doses with minocycline or anti-CX3CR1 neutralizing antibody were chosen in view of our previous studies (Kofman et al., 1993; Sun et al., 2007). At the end of each experiment, cannula placements were examined. Those rats, which cannula placements were outside of the lateral ventricle, were eliminated from the data analysis.

2.4. Drug-induced rotations

Rats were placed in an iron bowl, and the rotations were counted for all the groups after apomorphine administration. Rotations (360°, in short axis) ipsilateral and contralateral to the side of infusion were counted by individuals trained in behavioral observation. The ipsilateral rotations were defined as positive (+), while contralateral rotations as negative (−). Rats were tested following response to apomorphine (0.5 mg/kg, s.c.; Sigma–Aldrich, USA) on the assigned days following MPP^+ or fractalkine infusion. The number of ipsi- and contralateral rotations elicited was recorded from the initiation of rotational bias for 30 min (Ungerstedt, 1971).

2.5. Western blotting analysis

After defined survival time, rats were killed by overdose of urethane (2 g/kg, i.p., SCR Co., Shanghai, China). The midbrain of naive, sham surgery (vehicle infusion), or MPP^+ infusion rats were rapidly removed. The SNs were dissected according to the shape of the hippocampus, and then split into a left (ipsilateral to MPP^+ infused side) and right (contralateral) half from the ventral midline. After dissection, all tissues were rapidly frozen in liquid nitrogen and stored at −70 °C until further processing. In order to assess the development of PD, rats were tested for apomorphine-induced rotations before sacrificed.

Frozen SN tissues were directly homogenized in a lysis buffer (10 μl/mg) containing a cocktail of protease inhibitor and PMSF (Sigma–Aldrich). Supernatant, after 10,600 rpm centrifugation for 15 min at 4 °C, was used for Western blotting analysis.

Equal amount of protein (40 μg) was loaded on each lane and separated by 12% SDS-PAGE for the CX3CR1 detection. The resolved proteins were transferred onto PVDF membranes. The membranes were blocked in 10% nonfat dry milk for 2 h at room temperature (RT), and incubated overnight at 4 °C with goat anti-CX3CR1 (1:2000, Santa Cruz Biotechnology) or mouse anti-Tubulin (1:10,000, Sigma–Aldrich) primary antibody. The blots were incubated for 2 h at RT with horseradish peroxidase (HRP)-conjugated donkey anti-goat (1:5000, Santa Cruz Biotechnology) or donkey anti-mouse secondary antibody (1:1000, Santa Cruz Biotechnology). Signals were finally visualized using enhanced chemiluminescence (ECL, Pierce) and exposed onto X-films for 1–10 min. Pre-absorption of the primary antibodies with the blocking peptide (Santa Cruz Biotechnology) served as specificity control. All Western blotting analysis was performed at least three times, and parallel results were obtained. X-ray films with blotting bands for each sample from different rats were scanned, and the density of band area was quantified with a method described by Zhuang et al. (2005). The same size square was drawn around and each band was subtracted. Tubulin expression was used as loading control for protein expression, and CX3CR1 level was normalized against tubulin level. The expression level for protein is an average of densities per band area from different treated rats.

2.6. Immunohistochemistry

After defined survival times, rats were given an overdose of urethane (2 g/kg, i.p.) and perfused intracardially with 120 ml 0.9% saline followed by 400 ml cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Brains were removed and post-fixed in the same fixative for 2–4 h at 4 °C, and immersed into gradient sucrose from 10% to 30% in PB for 24–48 h at 4 °C before cryoprotection. Coronal brain sections (free-floating, 30 μm) were cut by a freezing microtome (Leica 1900, German) and processed for immunofluorescence. All the sections were blocked with 10% normal donkey serum in 0.01 M phosphate buffered saline (PBS, pH 7.4) with 0.3% Triton-X-100 for 1 h at room temperature (RT) and incubated over night at 4 °C with primary antibody (details in Table 1). The sections were then incubated for 2 h at 4 °C with fluorescein isothiocyanate (FITC) or rhodamine-conjugated donkey secondary antibody or with 4,6-diamidino-2-phenylindole (DAPI, 1:800, Sigma–Aldrich). Omission of primary antibody served as negative control. The stained sections were examined with a Leica fluorescence microscope (German), and images were captured with a CCD spot camera. TH-positive neurons were counted in number. However, because the morphology of microglia is complex and the immunoreactive (IR) staining includes both cell bodies and their processes, cell counts may not sufficiently quantify activation (as is indicated in Fig. 2B). Therefore, the integrated densities (pixelwise integration of density) of OX-42-IR within the SN were measured with the Leica Qwin 500 image analysis system (Germany) at six randomly selected sections for each animal. The relative density of images was determined by subtracting the background density in each image (Zhuang et al., 2005; Sun et al., 2007).

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Table 1
Characteristics of antibodies used in the study.

	Raised in	Dilution	Vendor	Catalog
CX3CR1	Goat	1:2000 (for WB)	Santa Cruz Biotechnology	sc-20432
CX3CR1	Rabbit	1:1000	Abcam	AB7200
CX3CL1	Goat	1:1000	R&D system	AF537
OX-42	Mouse	1:3000	Serotec	MCA275GA
GFAP	Mouse	1:3000	Sigma–Aldrich	G3893
NeuN	Mouse	1:3000	Chemicon	MAB377
TH	Rabbit	1:3000	Sigma–Aldrich	T8700

2.7. TdT-mediated fluorescein FragEL™ DNA fragmentation detection

The transverse sections of midbrain segments from sham rats ($n=2$) and rats of 7 days following intranigral administration of fractalkine ($n=2$) or fractalkine with anti-CX3CR1 neutralizing antibody ($n=2$) were stained with fluorescein FragEL™ DNA fragmentation detection kit (QIA39, CalBiochem). Specifically, the sections were incubated with proteinase K for 10 min at RT and with terminal deoxynucleotidyl transferase (TdT) equilibrium buffer for 15 min at RT, and then with a TdT labeling reaction mixture at 37 °C for 1–1.5 h in a humidified chamber. The positive control was made using HL-60 cells. The specific protocol was the same as before except that the specimen was first incubated with 1 mg/ml DNase I in 1× TBS (20 mM Tris, pH 7.6; 140 mM NaCl) containing 1 mM MgSO4 at RT for 20 min and then directly with TdT equilibrium buffer. The same specimens were examined with different filters. The total cell population was visualized using a filter for 4,6-diamidino-2-phenylindole (DAPI; 330–380 nm). The fluorescein-labeled nuclei were analyzed using a standard fluorescein filter 465–495 nm. These images were examined with Leica microscopy (Wang et al., 2006).

2.8. Statistical analysis

Data were expressed as mean ± S.E.M. All statistical comparisons were computed with Sigmastat 3.1 for the Macintosh. Pre-surgery baseline of behavior measures were analyzed by one-way analysis of variance (ANOVA). Post-drug time-course measures were analyzed by repeated measures Two ANOVA followed by Newman–Keuls post hoc tests, where appropriate. Western blotting analysis and immunohistochemical analysis were performed by Student's *t*-test or one-way ANOVA, $p<0.05$ was considered statistically significant.

2.9. Experimental procedures

2.9.1. Experiment 1: CX3CR1 and fractalkine expression in the SN after MPP^+ administration

After baseline behavioral assessments, rats received intranigral injection of 20 μg/2 μl MPP^+ (day 0). On days 3, 7, 14, and 28 after surgery, rats were sacrificed and the SNs were removed for Western blotting analysis. Sham rats received an intranigral injection of sterile NS (2 μl) and were killed on day 7 after NS injection. All rats could move freely in their home cages before they were killed. In order to assess the development of PD, rats' behaviors were tested before sacrifice.

According to the results of Western blotting analysis, we examined the SN with morphological changes in sham rats ($n=4$) and the rats on the 14th day following MPP^+ injury ($n=4$) treated with NS or anti-CX3CR1 neutralizing antibody. Detailed double staining was described in Table 2.

2.9.2. Experiment 2: effects of i.c.v. anti-CX3CR1 neutralizing antibody on behaviors of MPP^+ rat

After baseline behavioral assessments, rats received i.c.v. injections of CX3CR1 neutralizing antibody (0.3 μg/15 μl or 3 μg/15 μl) or IgG (3 μg/15 μl) once daily either from 1 h

Table 2
Double staining used in experiments.

FITC	Rhodamine	DAPI	Rat groups	Experiment
Fractalkine	TH		Sham, MPP, MPP + anti-CX3 (day 14)	Experiment 2
Fractalkine	NeuN		Sham, MPP, MPP + anti-CX3 (day 14)	Experiment 2
Fractalkine	GFAP		Sham, MPP (day 14)	Experiment 2
Fractalkine	OX-42		Sham, MPP (day 14)	Experiment 2
CX3CR1	OX-42		Sham, fract + NS, fract + 10 μg Mino, fract + 50 μg Mino, MPP, MPP + anti-CX3 (day 14)	Experiment 2/7
GFAP	CX3CR1		Sham, MPP (day 14)	Experiment 2
NeuN	CX3CR1		Sham, MPP (day 14)	Experiment 2
OX-42	TH		Sham, fract, fract + NS, fract + 10 μg Mino, fract + 50 μg Mino (day 14)	Experiment 7
TdT		DAPI	Sham, fract + NS, fract + 10 μg Mino, fract + 50 μg Mino (day 14)	Experiment 8
	TH	DAPI	Sham, fract, fract + 3 μg anti-CX3 (day 14) and postive control	Experiment 8

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prior to MPP^+ administration or from day 14 after MPP^+ administration. Behaviors were tested on days 3, 7, 14, 21, and 28.

2.9.3. Experiment 3: effects of intranigral fractalkine on behavioral response to apomorphine

After baseline behavioral assessments, rats received an intranigral injection of 30 ng/2 μl either fractalkine or IgG (day 0). Apomorphine-induced rotation behaviors were assessed 1 and 12 h later to assess PD development. Other behavioral assessments were performed on days 1, 2, 3, 5, 7 and 14. On the last day of assessments, the rotations were recorded every 5 min in an hour after apomorphine treatment.

2.9.4. Experiment 4: effects of levodopa on fractalkine-induced Parkinsonian behaviors

On day 8 after fractalkine injection, rats from Experiment 3 received an intraperitoneal injection of levodopa (25 mg/kg) (Carey et al., 1995) 5 min after apomorphine (0.5 mg/kg, s.c.) administration. Rotations were counted continuously from L-DA injection for 30 min. The data were collected and divided into three parts for analysis, 5–15, 15–25 and 25–35 min.

2.9.5. Experiment 5: effects of co-administration of fractalkine with anti-CX3CR1 neutralizing antibody on behaviors

After baseline behavioral assessments, rats received i.c.v. injections of CX3CR1 neutralizing antibody (3 μg/15 μl) either at 6 and 1 h prior to fractalkine injection or twice 3 days later after fractalkine injection. Behaviors were tested at 1, 12 h and on days 1–14 later. IgG (3 μg/15 μl) was given to the control group.

2.9.6. Experiment 6: effects of co-administration of fractalkine with minocycline on behaviors

After baseline behavior assessments, rats received i.c.v. injections of either minocycline (50 μg/4 μl or 10 μg/4 μl) (Kofman et al., 1993) or sterile NS (4 μl) at 1 h prior to intranigral injection of fractalkine, and once each day after injection for another 2 days. Behaviors were tested 1, 12 h and 1–14 days later.

2.9.7. Experiment 7: effects of intranigral injection of fractalkine or co-administration with minocycline on microglia and TH-positive neurons in SN

After behavioral assessments, rats from Experiments 3, 5 and 6 were perfused for OX-42 and TH immunohistochemical analysis on day 14 after fractalkine injection. Meanwhile, CX3CR1 positive cells double stained with OX-42 and TH neuron together with DAPI were also examined.

2.9.8. Experiment 8: neurotoxicity of fractalkine in SN

To examine whether fractalkine administration can produce cell in SN apoptosis, we use Tunnel staining to confirm the results in sham, fractalkine and fractalkine together with CX3CR1 treated rats.

3. Results

3.1. MPP^+ induced up-regulation of CX3CR1 expression in the SN

Following intranigral injection of MPP^+, Western blotting analysis revealed a dramatically increase in the level of CX3CR1 on the lesion side of SN.

In the ipsilateral SN, CX3CR1 levels were significantly increased at all time points examined after PD ($F_{5,18} = 79.459$, $p < 0.01$), especially on days 7 and 14, then mildly decreased on day 28; while on the contralateral SN, there was no evidence that up-regulation of CX3CR1 level occurred from days 3 to 28 after MPP^+ lesion (Fig. 1A and B). According to the results above, we investigated the expression of CX3CL1/fractalkine and CX3CR1 in the ipsilateral SN on day 14 after MPP^+ injury.

3.1.1. Activation of fractalkine in SN neurons after MPP^+ injury, while it weakly expressed in sham animal

A specific anti-fractalkine antibody was used to study the changes in fractalkine activation in MPP^+ and sham animals. A few fractalkine immunoreactive (IR) neurons were found in the sham group (Fig. 1Ca and Da), with low level of fractalkine expression on cell membrane. MPP^+ induced a robust activation of fractalkine in the midbrain. Numerous fractalkine-IR cells were found in the injured side of SN (Fig. 1Cb and Db). In the sham group, about 38% of TH-positive neurons (TH, a dopaminergic neuron marker) expressed a low level of fractalkine, while in the MPP^+ lesion group, much more fractalkine positive neurons were found in the ipsilateral SN, with enhanced immunoreactivity. However, there came a dramatic decline in TH-positive neurons. The ratio of fractalkine-IR to TH-IR neurons was significantly different between groups (one-way ANOVA, $F_{2,9} = 15.275$, $p = 0.001$). A Student–Newman–Keuls post hoc analysis determined that the anti-CX3CR1 neutralizing antibody treatment obviously decreased the decline of dopaminergic neurons and the ratios ($p = 0.002$ vs. MPP^+ group).

The co-localization of fractalkine with NeuN, a nuclear protein, indicates that the fractalkine is localized in the neurons in SN. The ratio of fractalkine-IR to NeuN-IR neurons was significantly different between groups (one-way ANOVA, $F_{2,9} = 1071.275$, $p < 0.001$). A Student–Newman–Keuls post hoc analysis determined that the anti-CX3CR1 neutralizing antibody treatment did not affect the ratios ($p = 0.475$ vs. MPP^+ control animals).

Referred to the ratios of fractalkine/TH and fractalkine/NeuN, we compared with TH and NeuN by unifying the fractalkine-IR positive cells. One-way ANOVA revealed significant effect of intracerebral injection of CX3CR1 neutralizing antibody (3 μg) treatment on MPP^+ elicited decline of TH/NeuN ratios ($F_{2,9} = 15.455$, $p = 0.001$) (Fig. 1Ec).

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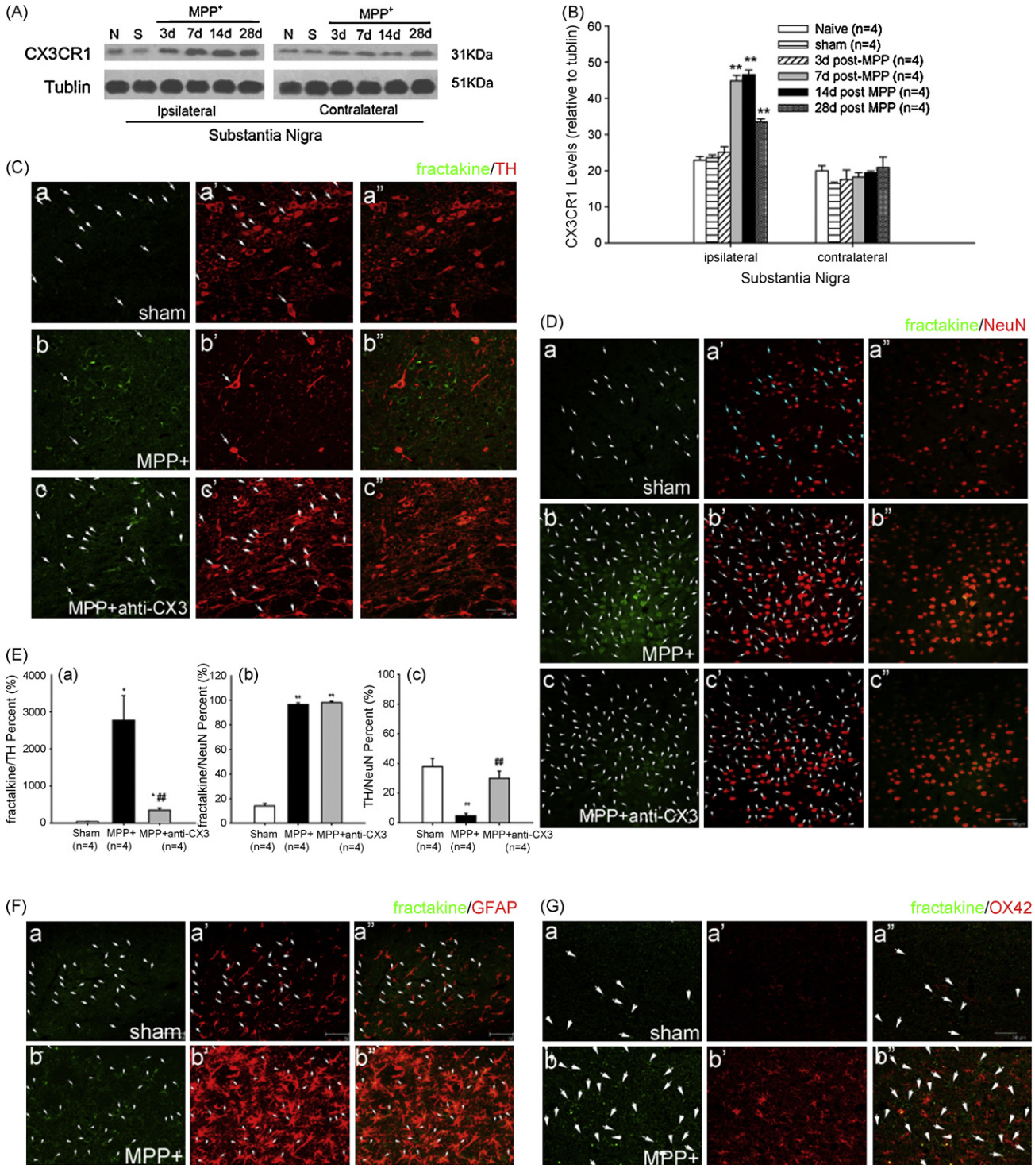

Fig. 1. (A and B) Time course of CX3CR1 expression in the SN following intranigral administration of MPP+: Western blotting analysis showed an increase in the level of CX3CR1 in the ipsilateral SN of MPP+-induced PD rats model, while there were no changes in the contralateral side. Tublin served as a loading control (A). Quantification of CX3CR1 level in both sides of SN $*p<0.05$, $**p<0.001$ vs. sham group (B). (C–G) Double staining of fractalkine with neuron or glia on day 14th following injury: MPP+ induced obvious fractalkine activation (Cb, Db, Fb, Gb), TH-positive neurons degeneration (Cb′), and NeuN-IR (Db′) enhancement, as well as astrocytes (Fb′) and microglia (Gb′) activation in ipsilateral SN, compared with sham (Ca/b, Da/b, Fa/b, Ga/b). CX3CR1 neutralizing antibody treatment did not alter the fractalkine expression level after MPP+ administration (Cc and Dc). Fractalkine is co-localized with TH, a dopaminergic neuron marker (Ca′–c′, red), and NeuN, a neuronal marker (Da′–c′), neither with GFAP, an astrocytic marker (Fa′–b′), nor OX-42, a microglia marker (Ga′–b′) in the SN. Arrows in C–G indicate fractalkine-positive neurons. Notice that the morphology of OX-42-IR and GFAP-IR is quite different from that of fractalkine-IR cells (scale, 50 μm, 40×). Ratios of fractalkine-IR to TH-IR (Ea), fractalkine-IR to NeuN-IR (Eb), and TH-IR to NeuN-IR (Ec) is quantified in ipsilateral SN. $*P<0.05$, $**P<0.001$, one-way ANOVA, compared to sham ($n=4$); $^{\#\#}P<0.001$, one-way ANOVA, compared to MPP+ ($n=4$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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Double immunofluorescence showed that fractalkine did not co-localize with GFAP (Fig. 1F), an astroglial marker, or OX-42 (Fig. 1G), a microglial marker, although OX-42-IR or GFAP-IR cells were positioned in closed contact with fractalkine-IR cells with the overlapping processes.

3.1.2. *CX3CR1 was activated in microglia, not in neurons or astrocytes*

Immunohistochemistry showed a substantial increase in the number and intensity of CX3CR1 cells in the ipsilateral SN when examined 14 days after MPP^+ administration, compared to the sterile saline group. Double immunofluorescence showed that almost all CX3CR1-IR cells in the SN 14 days post-lesion express OX-42 (CD11b), with very few CX3CR1-IR cells expressing NeuN or GFAP (Fig. 2).

OX-42 immunoreactive cells exhibited distinct morphologies in resting microglia with small cell body and multiple thin, long processes and activated microglia with enlarged amoeboid morphology and much fewer and shorter processes (Fig. 2B). The sham group had a preponderance of resting microglia, while much more activated microglia were detected in the MPP^+ injected rats.

3.2. *Intracerebroventricular administration of anti-CX3CR1 neutralizing antibody inhibited MPP^+-induced Parkinsonian behaviors*

Baseline measurements of behaviors to apomorphine-induced rotations (one-way ANOVA, $F_{5,35} = 0.522$, $p = 0.758$) did not differ prior to MPP^+ injection.

To address the role of CX3CR1 in the development of MPP^+-induced Parkinsonian behaviors, anti-CX3CR1

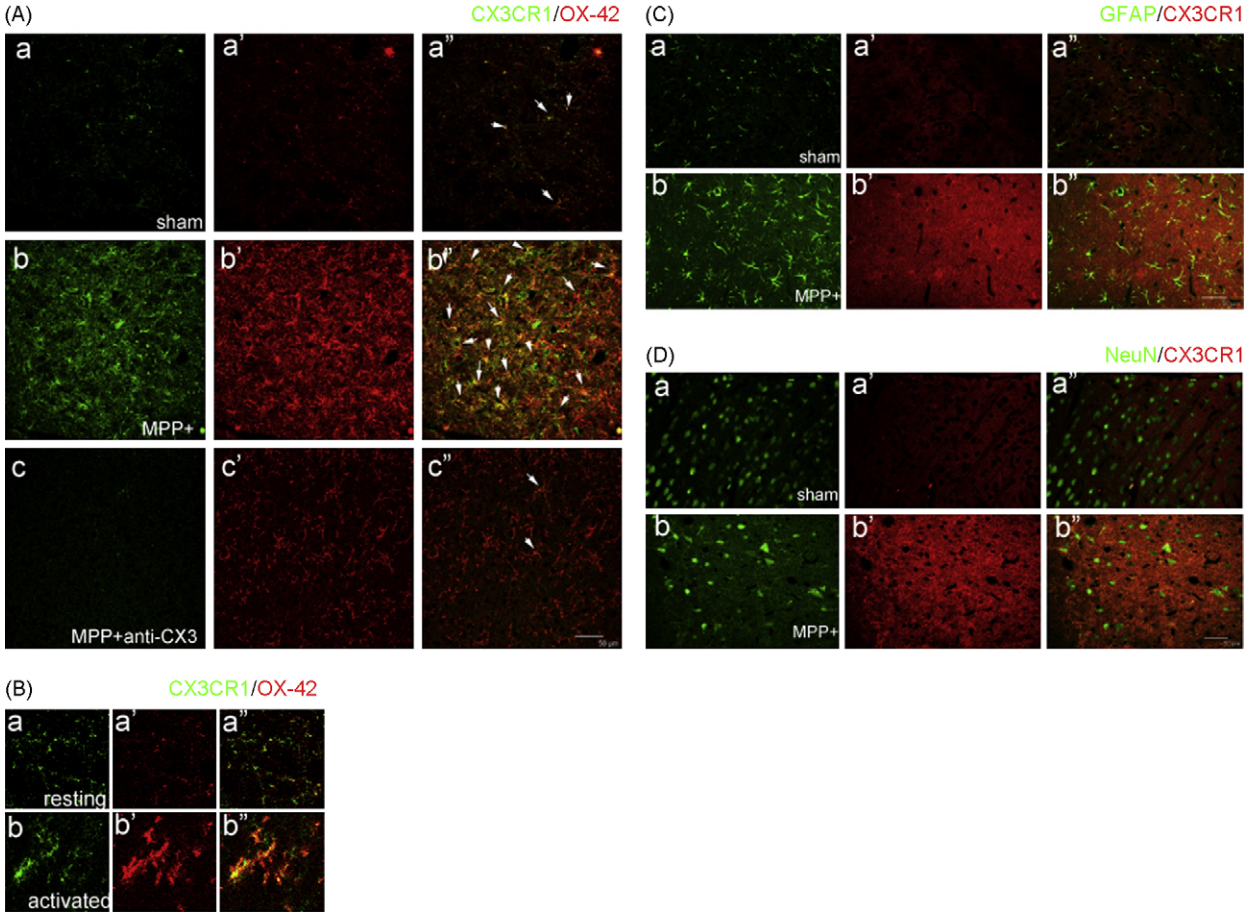

Fig. 2. Double staining of CX3CR1 with neuron or glia in SN: CX3CR1-IR was up-regulated after fractalkine or MPP^+ injection (Ab, Bb, Cb′, Db′) compared with the sham group (Aa, Ba, Ca′, Da'), while it was decreased by minocycline or CX3CR1 neutralizing antibody (Ac, f). Minocycline dose-dependently inhibited CX3CR1-IR, and the higher dosage did better effect (Ac, d). CX3CR1 is only co-localized with OX-42 (Aa″–c″, Ba″–b″), neither with GFAP (Ca′–b′), nor with NeuN (Da″–b″) in the SN. Arrows in A indicate double-labeled cells. Distinct morphologies of OX-42 immunoreactive microglia was detected in SN under high-magnification including resting microglia with small cell body and multiple thin, long processes (Ba), and activated microglia with enlarged amoeboid morphology and much fewer, shorter processes (Bb). Notice that in the same area, it is different numbers between resting and activated microglia. There are several (more than 10, not very clear) resting microglia and processes detected in Ba, while only 2 or 3 activated microglia in Bb. Therefore, counting the number of microglia may not be an accurate. Scale for A, C–D, 50 μm, 40×; for B, 10 μm, 100×. MPP, MPP^+; Fract, fractalkine; anti-CX3, CX3CR1 neutralizing antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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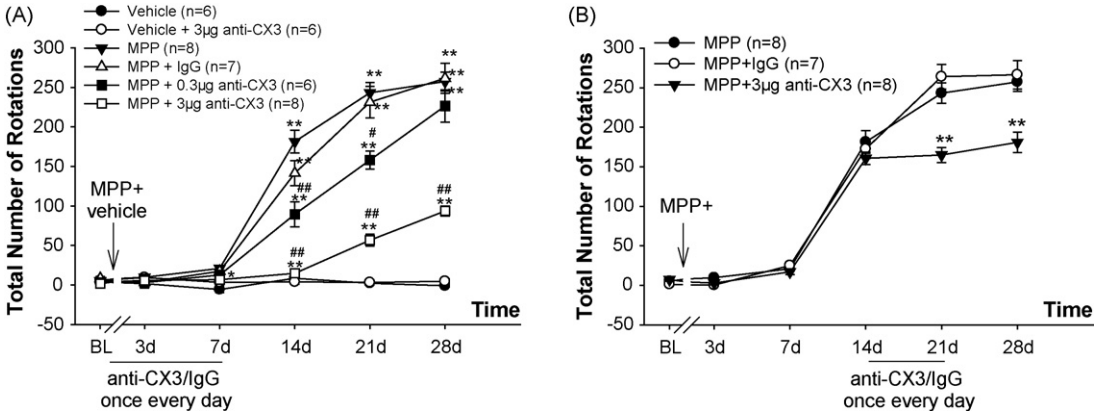

Fig. 3. Effects of i.c.v. CX3CR1 neutralizing antibody on behaviors of MPP^+ rat: i.c.v pre-administration with anti-CX3CR1 neutralizing antibody (3 μg) not only blocked the onset of rotation behaviors in the MPP^+ rats (A), but also lightened the established behaviors (B). The rotation behaviors elicit by apomorphine was improved in a dose-dependent manner, and the higher dose, 3 μg did better effect. Anti-CX3CR1 neutralizing antibody or control IgG was given once every day for a session of 7 days either from 1 h prior to MPP^+ administration or from 7 days post-injection. $^*P<0.05$, $^{**}P<0.001$, two-way ANOVA, compared to IgG control group ($n=6$), or $^{\#\#}P<0.001$, two-way ANOVA, compared to MPP + 0.3 μg anti-CX3 group ($n=6$). MPP, MPP^+; Fract, fractalkine; anti-CX3, CX3CR1 neutralizing antibody.

neutralizing antibody (0.3 or 3 μg) or control IgG (3 μg) was intracerebroventricular administrated once daily after MPP^+ injected for a week. After MPP^+ injection, rats rotated ipsilaterally to the injured side. Anti-CX3CR1 dose-dependently blocked MPP^+-induced rotation behaviors. The higher the dose of CX3CR1 neutralizing antibody (3 μg) was, better the effect of inhibition on rotation behavior development was. Two-way ANOVA revealed significant effect of intracerebroventricular administration of CX3CR1 neutralizing antibody (3 μg) treatment on rotation behaviors ($F_{1,78}=217.482$, $p<0.001$) and interaction between CX3CR1 antibody treatment and time ($F_{5,78}=40.007$, $p<0.001$). Rats of control group (intranigral vehicle) were not affected by intracerebroventricular administration of CX3CR1 antibody. Control IgG (3 μg) did not alter apomorphine-induced rotation behaviors of the fractalkine treated rats (Fig. 3A).

To investigate whether CX3CR1 was involved in the maintenance of the MPP^+-induced Parkinsonian behaviors, the effects of intracerebroventricular administration of CX3CR1 neutralizing antibody (3 μg) on the apomorphine-induced rotation behaviors were examined 14 days after intranigral MPP^+ when it stably occurred, and CX3CR1 neutralizing antibody was intracerebroventricular administrated for a session of a week, as mentioned before. Intracerebroventricular administration of CX3CR1 antibody (3 μg) inhibited established rotation behaviors mildly. Two-way ANOVA revealed significant effect of intracerebroventricular administration of CX3CR1 antibody treatment (apo-induced rotation behaviors: $F_{1,78}=38.134$, $p<0.001$) and interaction between CX3CR1 antibody treatment and time (apo-induced rotation behaviors: $F_{5,78}=13.039$, $p<0.001$). Control IgG (3 μg) had no effect on observed behaviors (Fig. 3B).

3.3. Rotation behaviors induced by fractalkine in unilateral SN were completely blocked by intracerebroventricular administration of CX3CR1 neutralizing antibody

The premise of Experiment 3 was that, if endogenous fractalkine is a neuron-to-glia signal that triggers microglial activation and consequent induced PD, then exogenous fractalkine should be sufficient to induce changes in behavioral responses. This was indeed the result we observed. Prior to fractalkine administration, baseline measures did not differ between groups, for the apomorphine-induced rotation behaviors ($F_{1,12}=0.261$, $p=0.619$). Intranigral fractalkine produced rotation behaviors ($F_{1,108}=442.805$, $p<0.001$) (Fig. 4A) compared with vehicle controls.

To determine whether the neutralizing antibody for CX3CR1 blocks CX3CR1-mediated biological actions of fractalkine, we injected anti-CX3CR1 neutralizing antibody (3 μg) into cerebral ventricle 6 and 1 h prior to fractalkine administration. Pretreatment with anti-CX3CR1 neutralizing antibody completely prevented fractalkine-induced behavior abnormalities. Neither IgG nor post-treated with anti-CX3CR1 did. Thus, fractalkine-induced Parkinsonian syndromes are mediated via activation of the fractalkine receptor, CX3CR1 (Fig. 4B and C).

3.4. Levodopa decreased the rotation behaviors induced by fractalkine

In the first 10 min after L-dopa or vehicle injected, groups had no noticeable difference between their responses to apomorphine-induced rotation behaviors ($F_{1,6}=0.692$, $p=0.437$), while in the next 10 min, groups showed significant difference in behaviors ($F_{1,6}=25.633$, $p=0.002$). Consequently, in the last 10 min, the behavioral abnormality

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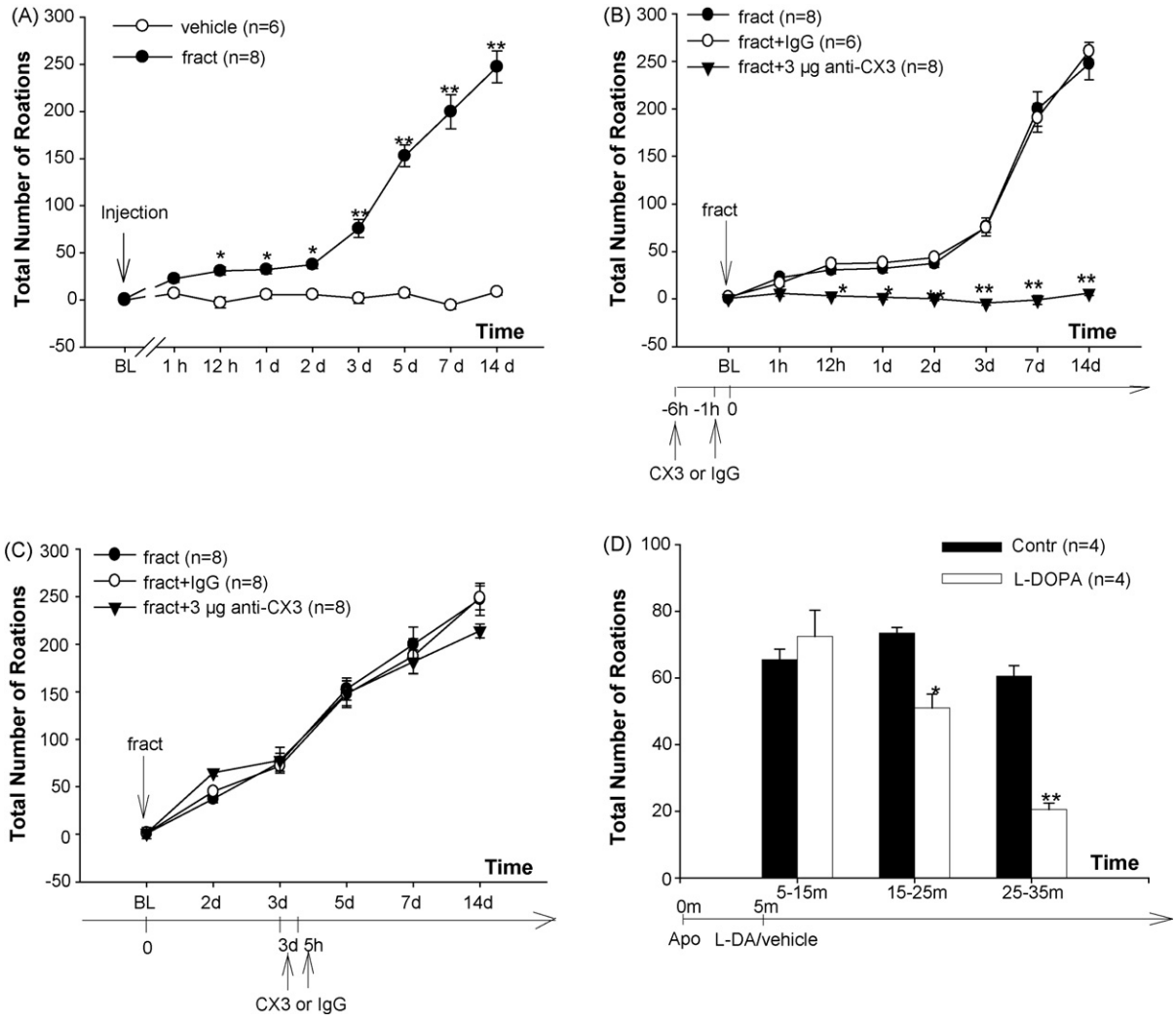

Fig. 4. Apomorphine-induced rotation behaviors were investigated before and after fractalkine or vehicle administration (A). *$P<0.05$, **$P<0.001$, two-way ANOVA, compared with vehicle group ($n=6$). I.c.v. pre-administration with CX3CR1 neutralizing antibody (3 μg) blocked the onset of rotation behaviors induced by apomorphine in the fractalkine group (B), but did not alter the established rotation behaviors (C). CX3CR1 neutralizing antibody or IgG was administrated either at 6 and 1 h prior to or twice 3 days later postfractalkine injection. The antibody did no effect on the sham (vehicle injected) rats. So did IgG on the MPP^+ rats. *$P<0.05$, **$P<0.001$, two-way ANOVA, compared to IgG control group ($n=6$). Intraperitoneal injection of L-dopa improved rotation behaviors induced by apomorphine on day 8 in fractalkine-injected rats. L-dopa or vehicle was given 5 min after apomorphine administration (D). *$P<0.05$, **$P<0.001$, *t*-test, compared to control ($n=4$).

was robustly improved by L-dopa ($F_{1,6}=117.073$, $p<0.001$) compared with control groups (Fig. 4D).

3.5. Intracerebroventricular administration of minocycline inhibited rotation behaviors induced by fractalkine

To examine whether inhibiting microglia prevent fractalkine-induced Parkinsonian behaviors, minocycline was intracerebroventricular administrated 1 h prior to fractalkine injection and once daily for a session of 3 days.

The ipsilateral rotations were recorded every 5 min in 60 min after apomorphine treatment on day 14. Compared with sham group, the ipsilateral rotations were immediately enhanced after apomorphine injection and lasted throughout the recording period. However, the apomorphine-induced rotations were dose-dependently decreased by i.c.v. MPP^+ treatment (Fig. 5A). The dose of 50 μg/4 μl dramatically reversed the rotation behaviors induced by apomorphine, as shown in Fig. 5A. Two-way ANOVA revealed that significant effect of i.c.v. of minocycline (50 μg) treatment on rotation behaviors ($F_{1,96}=629.729$, $p<0.001$) and interaction between minocycline treatment and time ($F_{7,96}=118.848$, $p<0.001$). Rats of sham group (vehicle instead of MPP^+) were not affected by i.c.v. minocycline administration. i.c.v. sterile saline did not alter apomorphine-induced rotation behaviors in fractalkine rats (Fig. 5B).

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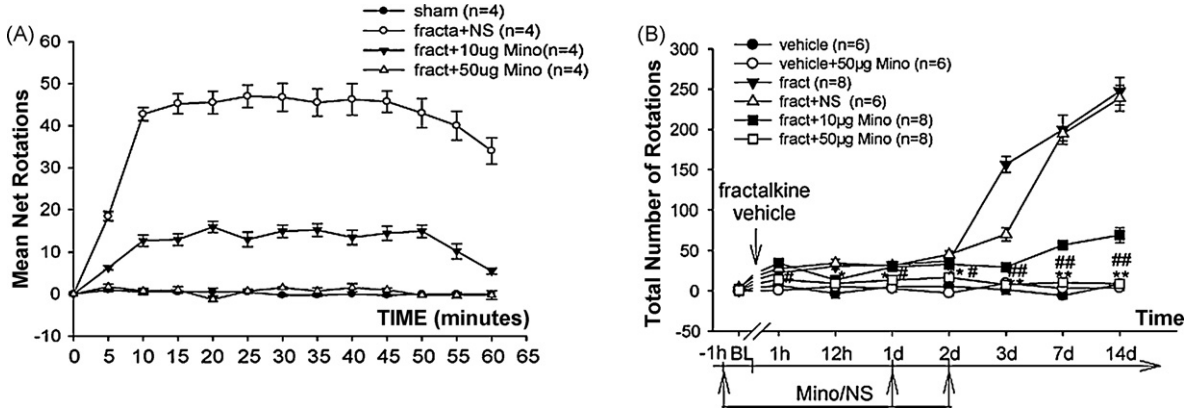

Fig. 5. Numbers of ipsiversive (positive scale) and contraversive turns (negative scale) countered over 1 h at intervals of 5 min on day 14 (A) ($n = 4$ animals per group). i.c.v. pre-administration with minocycline (50 μg) blocked the onset of rotation behaviors in the fractalkine injected rats (B). Minocycline or NS was given once every day for a consequent 3 days from 1 h prior to intranigral injection of fractalkine. $^{*}P < 0.05$, $^{**}P < 0.001$, two-way ANOVA, compared to vehicle group ($n = 6$); or $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, two-way ANOVA, compared to frac + 10 μg Mino ($n = 6$). Fract, fractalkine; Apo, apomorphine; L-DA, L-dopa; contr, contralateral side; anti-CX3, CX3CR1 neutralizing antibody; Mino, minocycline; NS, normal sterile saline.

3.6. I.c.v. minocycline prevented fractalkine-induced glial activation and TH-positive neurons loss

To examine the roles of exogenous fractalkine and minocycline play in SN, we assessed immunoreactivities of OX-42 and TH in the ipsilateral SN of sham, fractalkine, fractalkine + NS and fractalkine + minocycline (10 and 50 μg) rats. As expected, unilateral SN injection of fractalkine produced both a robust increase in the expression of OX-42-IR and a dramatic decline in the number of TH-positive neurons in the ipsilateral side, compared with vehicle group. However, pre-administrated minocycline before fractalkine injection not only suppressed OX-42-IR, but also prevented TH depletion in a dose-dependent manner and the higher dose (50 μg) did better effect on day 14 post fractalkine injection. Pre-administered with sterile saline failed to affect fractalkine-induced microglial activation and dopaminergic neurons loss (Fig. 6A and B).

A double staining of fractalkine-receptor, CX3CR1, and OX-42 has also been provided in all groups of fractalkine rats. Weak immunoreactivity for CX3CR1 was found in microglia in SN in sham rats. However, 14 days after fractalkine injection, the increased CX3CR1-IR was detectable on the ipsilateral side of SN. The CX3CR1-IR was decreased by minocycline administration. As shown in co-localization of CX3CR1 with OX-42, CX3CR1 was only expressed in microglia, neither in neuron nor astrocytes. Not all microglial cells were positive for CX3CR1, but all CX3CR1-IR cells are positive for OX-42 (Fig. 6C).

3.7. Neurotoxicity of fractalkine in SN

Moreover, to clarify whether cells containing inclusions display apoptotic death, DAPI and TUNEL staining were performed (Fig. 7). The double fluorescence with anti-TH antibody confirmed its co-localization with DAPI and dopaminergic neuron staining in the SN (Fig. 7A). The ratio of TH-positive neurons to DAPI was significantly different between groups ($F_{3,12} = 109.297$, $p < 0.001$). A Student–Newman–Keuls post hoc analysis determined that the 10 or 50 μg minocycline obviously decreased the decline of dopaminergic neurons ($p = 0.004$ and $p < 0.001$ vs. NS treated fractalkine animals). Morphological examinations of certain DAPI-labeled cells in fractalkine groups, concomitant with inclusions, revealed marked changes in the nuclear structure with fragmented and/or condensed chromatin, consistent with apoptosis (i.e. apoptotic nuclei). However, when cells were co-localized with TH in sham or fractalkine + 50 μg Mino animals, chromatin of these healthy cells exhibited an intact feature, with low density in the nuclear.

Furthermore, considering that SN delivery of fractalkine may result neurotoxicity in SNpc, whether cells were damaged was examined using TdT-mediated fluorescein FragEL™ DNA fragmentation detection kit. Few fluorescein-labeled apoptotic cell nuclei were seen on the ipsilateral SN in the sham rats (Fig. 7C) or rats following fractalkine with anti-CX3CR1 neutralizing antibody, while significant apoptotic cell signals were seen in the fractalkine administration rats. Fig. 7C shows the positive control specimen, in which almost all HL-60 cells were fluorescein-labeled (green), indicating complete apoptosis of all HL-60 cells. The results suggested the involvement of neurotoxicity in the effects produced in the SN by fractalkine.

4. Discussion

As is widely accepted, the known mechanism for MPTP/MPP$^+$ neurotoxicity is that MPP$^+$, the active compound of MPTP, crosses the neuronal membrane by a specific uptake mechanism. Once inside the cells, MPP$^+$ leads to a major inhibition of the respiratory chain but also to oxidative

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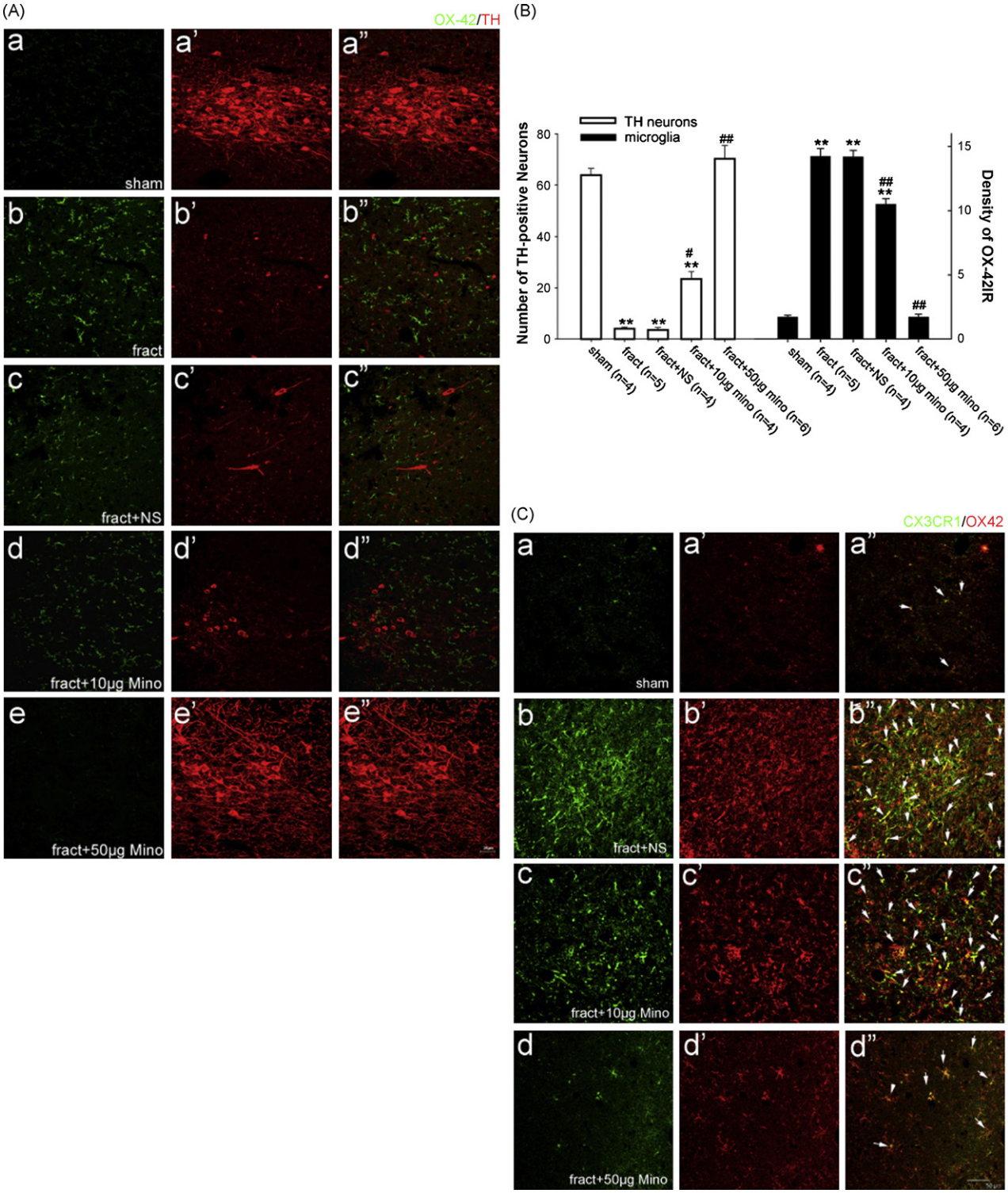

Fig. 6. Photomicrographs showing TH (dopaminergic marker) and OX-42 (microglial marker) IR in the ipsilateral SN on day 14 after fractalkine injection. (A) Ipsilateral side of sham (Aa, a′, a″); fractalkine 7 days (Ab, b′, b″), fractalkine + NS (Ac, c′, c″); fractalkine + 10 μg minocycline (Ad, d′, d″) and fractalkine + 50 μg minocycline (Ae, e′, e″); 40×. (Aa–e) OX-42; (Aa′–e′) TH; (Aa″–Ae″) merged. (B) Quantification of OX-42 and TH immunoreactivity in SN: $^{*}P < 0.05$, $^{**}P < 0.001$, one-way ANOVA, compared to sham group ($n = 4$); or $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, one-way ANOVA, compared to fract + NS group ($n = 5$). (C) Double staining of CX3CR1 with OX-42 in SN: minocycline dose dependently not only inhibited fractalkine-induced microglial activation but also decreased CX3CR1-IR. Arrows indicate double-labeled cells. It is clear that all the CX3CR1 positive cells were co-localized with OX-42 positive cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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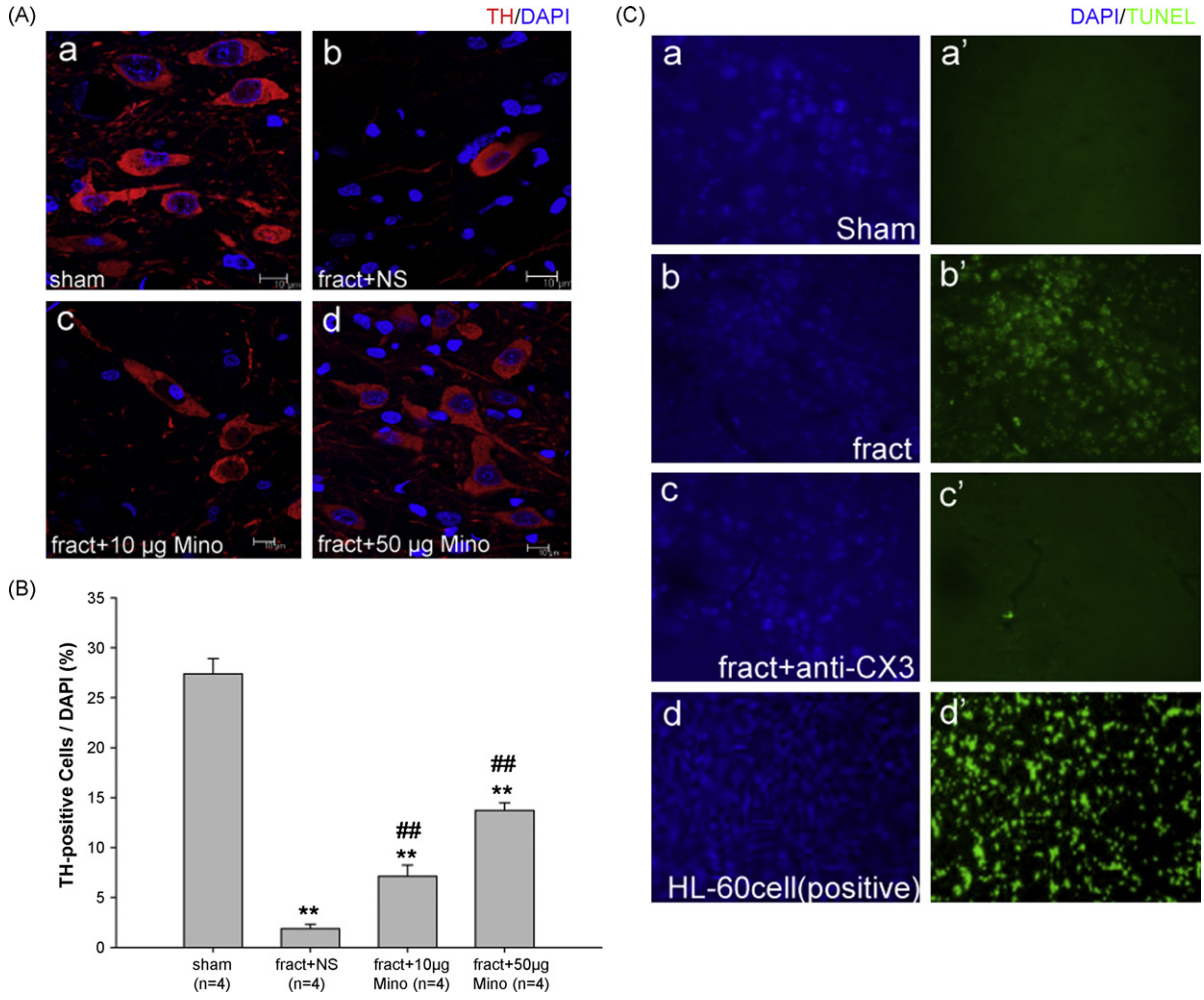

Fig. 7. Double staining of TH with DAPI in SN (A). The DAPI-labelled cells in fractalkine groups, concomitant with inclusions, revealed marked changes in the nuclear structure with fragmented and/or condensed chromatin, consistent with apoptotic nuclei (Ab and c). However, when cells were co-localized with TH in sham (Aa) or fractalkine + 50 μg Mino (Ad) animals, chromatin of these healthy cells exhibited an intact feature, with low density in the nuclear. (B) Quantification of ratios of TH to DAPI: $^{*}P < 0.05$, $^{**}P < 0.001$, one-way ANOVA, compared to sham group ($n = 4$); or $^{\#\#}P < 0.001$, one-way ANOVA, compared to frac + NS group ($n = 4$). Representative photographs showing fluorescein-labeled apoptotic cells (green) in SN (Ca–c) and the positive control HL-60 cells (Cd). The same specimens were detected with different filters. The whole cell population (blue) was visualized using a filter for DAPI. The fluorescein-labeled apoptotic nuclei were analyzed using a standard fluorescein filter 465–495 nm: (Ca and a′) sham rats, (Cb and b′) fractalkine rats, (Cc and c′) rats following fractalkine administration with i.c.v. CX3CR1 neutralizing antibody, and (Cc and c′) rats following NS instead of fractalkine administration. Scale bar indicates 50 μm. Fract, fractalkine; Mino, minocycline; NS, normal sterile saline. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

stress, both triggering cell death (Blum et al., 2001). However, our study presents an additional view for the neurotoxicity of MPTP/MPP$^+$ that fractalkine/CX3CR1 may be involved in the development of PD.

There are at least three major new findings in our study. In brief, first, increased level of CX3CR1 was observed in the ipsilateral midbrain of MPP$^+$-induced rat. Second, i.c.v. injection of neutralizing antibody against CX3R1 completely blocked Parkinsonian syndromes induced by exogenous fractalkine. Finally, activated microglia participated in dopaminergic cell loss caused by fractalkine. It is based on the evidence that minocycline, a selective microglia inhibitor, could totally abolish those defects insulted by fractalkine.

4.1. MPP$^+$ up-regulated endogenous fractalkine level in the ipsilateral SN and CX3CR1 neutralizing antibody partially blocked MPP$^+$-induced rotation behaviors

As mentioned in Section 1, fractalkine is a chemokine that is tethered to the extracellular surface of neurons and can be released upon injury or diseased conditions, as forming a diffusible signal (Chapman et al., 2000). Its exclusive receptor, CX3CR1, is expressed by microglia (Cardona et al., 2006; Lindia et al., 2005; Re and Przedborski, 2006; Schall, 1997; Verge et al., 2004). Prior works have led to the concept that fractalkine may play a role in signaling between neuron and microglia (Harrison et al., 1998; Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998). In vitro exposure

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of neuronal mixed cultures and brain slices to chemicals such as glutamate, tumor necrosis factor or interferon gamma is sufficient to release fractalkine (Chapman et al., 2000; Erichsen et al., 2003). In a conclusion, fractalkine can exert a pro-inflammatory role by inducing microglia migration and activation (Ransohoff et al., 2007; Streit et al., 2005).

According to the evidence above, we presume that, in response to MPP^+ insult, fractalkine can be cleaved from the neuronal membranes by metalloproteinases and secrete to form a fractalkine gradient extends from the neuron to quiescent microglia (Leonardi-Essmann et al., 2005; Re and Przedborski, 2006; Schulte et al., 2007). Then the released fractalkine, as a bioactive chemoattractant peptide, attracts CX3CR1-expressing microglia and recruits them to accumulate in the infected area (Chapman et al., 2000; Schulte et al., 2007; Tarozzo et al., 2002). This may explain why CX3CR1-IR level is up-regulated after MPP^+ insult. Our data on increased level of CX3CR1-IR after MPP^+ injection are broadly in accordance with those (Harrison et al., 1998) who detected an increase in the CX3CR1 concomitant with the proliferation of resident microglia after facial motor nerve axotomy in the rat, and with the study of those who observed an elevated CX3CR1 expression on microglia in a prior model of chronic neurodegeneration and inflammation (Hughes et al., 2002). It should also be noted that the time course of CX3CR1 up-regulation followed by intranigral injection of MPP^+ paralleled the microglial activation and the initiation of dopaminergic cell loss, further supporting the notion that the increased CX3CR1 may be enrolled in the dopaminergic neurodegeneration process.

Furthermore, blockade of CX3CR1 receptor in MPP^+ rats by its neutralizing antibody, can partially prevent the microglial activation, thus dampening dopaminergic cell death. It is worth mentioning that CX3CR1 neutralizing antibody only partially blocked the activation of microglia and dopaminergic cell death in MPP^+-induced PD animals, suggesting that other mechanisms may also be involved in, including aggregated α-synuclein released from dying or dead dopaminergic neurons could activate microglia in a NADPH oxidase (PHOX) dependent manner (Austin et al., 2006; Klegeris et al., 2008; Su et al., 2007; van Muiswinkel et al., 2004; Wakamatsu et al., 2008; Zhang et al., 2005, 2007); prostaglandin E2(PGE2), a catalytic product of COX-2, may activate microglia through EP2 receptor (Jin et al., 2007; Shie et al., 2005; Vijitruth et al., 2006). Although this possibility cannot be confirmed with certainty, our observation that a blockade of CX3CR1 by its neutralizing antibody suppressed only a percentage of reactive microglia rather support the view that microglia are activated through more than one mechanism.

4.2. *Exogenous fractalkine promoted SN microglial activation and dopaminergic neuron degeneration*

It is speculated that the overactivity of fractalkine/CX3CR1 system may set in motion a neuroinflammatory process by activating microglia and cause dopaminergic cell to demise. To further understanding fractalkine/CX3CR1 system in regulating microglia-mediated dopaminergic neurotoxicity, recombinant fractalkine was microinjected directly into unilateral SN of rats. As a neuron-to-glia signal (Tarozzo et al., 2003), exogenous fractalkine binds to the unique membrane receptor CX3CR1 expressed by microglia, activates microglia, then leads to the release of glial-excitatory substances which induce astrocytes activation (Milligan et al., 2004; Verge et al., 2004). Activated microglia and astrocytes release a variety of neuro- and glial-excitatory substances that have each been implicated in dopaminergic neurotoxicity (Bal-Price and Brown, 2001; Kim et al., 2004; Kim and Joh, 2006; Minghetti and Levi, 1998; Nagatsu and Sawada, 2005; Raghavendra et al., 2004; Tikka and Koistinaho, 2001), which might contribute to the pathogenesis of PD. Mounting evidence indicates that dopaminergic neurons in the SN are particularly vulnerable to inflammation (Kim and Joh, 2006) and neurotoxin released by reactive microglia, such as TNF-α (Sriram et al., 2002, 2006), nitric oxide (Bal-Price and Brown, 2001; Minghetti and Levi, 1998) and reactive oxygen species (McNaught and Jenner, 1999; Stokes et al., 1999). Therefore, following fractalkine injection, we observed resident microglial activation, dopaminergic cells death, motor disabilities, and behaviors response to levodopa, all of which were in accordance with those seen in classic toxin-induced rat models of PD (Goralski and Renton, 2004; Schober, 2004; Sindhu et al., 2006; Yazdani et al., 2006). As far as we know, this is the first demonstration that intranigral fractalkine infusion can result in PD-like neuropathology and disability. However, blockade of CX3CR1 by its neutralizing antibody, can totally prevent the accumulation of CX3CR1-expressing microglia to the injured area, thus abolished neuroinflammation and dopaminergic neuron death. This confirmed that fractalkine-to-CX3CR1 signal is the only way that fractalkine activates microglia.

A notable result in our study is that at 12 h after fractalkine injected, there comes a significant difference between fractalkine and vehicle in apomorphine-induced rotation behaviors. How did dopaminergic neurons dysfunction? We do not think they were dead in such a short term. Then what causes the motor disability? Can microglia itself contribute to this abnormal behavior? More extensive research is needed to be undertaken to gain more detailed insights into function of activated microglia.

4.3. *Dopaminergic cell loss induced by fractalkine mediated by activated microglia*

Another intriguing result in current study is that when pretreated rats with minocycline, minocycline dose-dependently blocked the fractalkine-induced microglial activation and dopaminergic neuron loss, and the higher dose completely abolished fractalkine effects. The same contribution as CX3CR1 neutralizing antibody did. Based on many in

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vivo and in vitro studies, minocycline inhibits microglial activation under various conditions without affecting neurons, astroglia, or oligodendroglial progenitors (Owolabi and Saab, 2006; Raghavendra et al., 2003, 2004). Our results by pretreatment of minocycline strongly supported the proposal that the neurotoxin of exogenous fractalkine is exerted mainly though activation of microglia. It is true that CX3CR1 is also expressed by T lymphocyte subpopulations and specific leukocyte subsets in peripheral tissues (Foussat et al., 2000; Ramos et al., 2007; Sans et al., 2007), and intranigral exogenous fractalkine might recruit T lymphocyte and/or leukocyte to the infected area through chemoattraction, however, studies by Hughes et al. (2002) have already excluded this possibility. After sterotaxically injected recombinant CX3CL1 into the rat striatum, only microglia were activated, no overt breakdown of blood–brain barrier (BBB) was found and no neutrophils or T cells were recruited to the brain. Thus, the interpretation that exogenous fractalkine-induced dopaminergic neurotoxin is almost completely mediated by reactive microglia is supported not only by the aforementioned study by Hughes et al., but also by our demonstration that minocycline, a selective microglia inhibitor, could totally abolish the dopaminergic cell death and behavioral defects. Our study of minocycline on fractalkine-induced neuropathology is in line with previous reports in other neurological animal models (Milligan et al., 2004, 2005). Therefore, the usage of minocycline has provided important insights into the role of fractalkine and might be served as a potential drug to treat fractalkine related neurological disorders in future studies.

In our preliminary experiment, we have tried several methods of minocycline administration, including intragastric (oral) administration, intraperitoneal injection, intranigral and intracerebroventricular injection. Oral administration seemed useless in improving motor disabilities, while intranigral produced much more damage to the midbrain. And intraperitoneal injection did have some effects; however, it needed a large dose to get an effective blood drug level, which would cost much. Comparing all these methods, we finally decided to choose i.c.v. administration, because it is better than others in both drug effect and cost.

In conclusion, the present study demonstrated for the first time that CX3CR1 are involved in the neuroinflammatory process in MPP^+ rat model of PD. Moreover, microinjection of fractalkine into SN of the rats, through activation of microglia, exhibited most of the characteristics of the experimental models of PD, including dopaminergic cell death, glial activation, behavioral defects and the response to levodopa. Collectively, the data presented herein are consistent with the importance of microglia in the process that leads to the progressive dopaminergic cell loss in PD and sheds light on further study of fractalkine/CX3CR1 system, and possibly other chemokines, in the pathogenesis of PD.

Conflicts of interest

None.

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