Histamine-4 receptor antagonist JNJ7777120 inhibits pro-inflammatory microglia and prevents the progression of Parkinson-like behaviour and pathology in a rat model

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Abstract

Activation of microglial cells is presumed to play a key role in the pathogenesis of Parkinson's disease (PD). The activity of microglia is regulated by the histamine-4 receptor (H_4R), thus providing a novel target to prevent the progression of PD. However, this putative mechanism has so far not been validated. In our previous post-mortem study, we found that mRNA expression of H_4R was upregulated in the basal ganglia of PD patients. In the present study, we found indeed an upregulation of microglia associated inflammation markers from microarray data of the substantia nigra pars compacta (SNpc) of PD patients. We validated the mechanism underlying our human PD results using the rotenone-induced PD rat model, in which the expression of H_4R and microglial markers mRNA were significantly increased in the SNpc. Inhibition of H_4R in rotenone-induced rats by infusion of the specific H_4R antagonist JNJ7777120 into the left lateral ventricle blocked microglial activation, reduced apomorphine-induced rotational behaviour, and prevented dopaminergic neuron degeneration and associated decreases in striatal dopamine levels. These changes were accompanied by a reduction of Lewy body-like neuropathology. Our results provide first proof of the efficacy of an H_4R antagonist in a commonly used

PD rat model, and provides a lead for a promising therapeutic strategy aimed at modifying H_4R activation to clinically tackle microglial activation and thereby the progression of PD.

Keywords: Parkinson's disease, Rotenone, Histamine 4 receptor antagonist, Microglia, α-synuclein

1. Introduction

Parkinson's disease (PD) is the second most prevalent age-related neurodegenerative disorder, affecting approximately 1% of the population above the age of 65, and is mainly characterized by the symptoms tremor, rigidity and postural abnormalities. The dopaminergic neurons are selectively affected in PD, showing a progressive loss in the substantia nigra pars compacta (SNpc) (Hirsch et al., 1988, Damier et al., 1999). In multiple brain areas, a neuropathological ~~hall mark~~hallmark of the disease is the presence of Lewy bodies and Lewy neurites of which the protein α-synuclein is the main component (Braak et al., 2003, Shan et al., 2012c).

Accumulating evidence suggests an important role for inflammation in the pathogenesis of PD, despite the fact that the exact primary cause of the disease is unknown. A number of genes that can either directly cause PD or contribute to its risk are implicated in inflammatory pathways including the major histocompatibility complex alleles (Deleidi and Gasser, 2013, Dzamko et al., 2015). Inflammatory

cytokines are increased in the brain and cerebral spinal fluid (CSF) of PD patients (Orr et al., 2002, Kannarkat et al., 2013). The primary inflammatory cells in the brain, microglia, are activated and play a pivotal role in dopaminergic neurotoxicity (Gao et al., 2002, Imamura et al., 2003, Shao et al., 2013, Doorn et al., 2014, Liddelow et al., 2017). Recently a study showed that a defined set of peptides that are derived from α-synuclein, acted as antigenic epitopes displayed by the major histocompatibility complex alleles and drive helper and cytotoxic T cell responses in patients with PD (Sulzer et al., 2017). The carbohydrate-binding protein, galectin-3/lgals-3, is an immune modulator that plays a crucial role in the α-synuclein-induced activation of microglia (Boza-Serrano et al., 2014, Belloli et al., 2017). An *in vitro* study demonstrated a prominent inflammatory inhibition of microglia cells following ~~by~~ genetic down-regulation or pharmacological suppression of galectin-3 or by using galectin-3 knockout primary microglia (Boza-Serrano et al., 2014).

In the central nervous system, histamine functions as a neurotransmitter (Haas and Panula, 2003) and immune response mediator (Shan et al., 2015b) which also modulates microglial activity that interferes with dopamine neuron survival in the SNpc of PD (Rocha et al., 2014, Rocha et al., 2016). Earlier post-mortem studies have shown that histamine levels (Rinne et al., 2002) and innervation (Anichtchik et al., 2000) are increased in the SNpc in PD. In our previous animal experimental PD model observations, endogenous histamine was shown to aggravate 6-hydroxydopamine (6-OHDA) induced degenerations of dopaminergic neurons in

the SNpc (Liu et al., 2007). Moreover, mRNA expression of the histamine receptor-4 (H_4R) was robustly (4.2-6.3 fold) increased in the basal ganglia of PD patients (Shan et al., 2012a).

Microglia can take on a range of phenotypes *in vivo* and can be pro-inflammatory (M1) or neuroprotective (M2) (reviewed in (Lan et al., 2017)). M1 subtype, a classical activation type, is associated with massive inflammatory response releasing interleukin-1beta (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Benson et al., 2015, Popiolek-Barczyk et al., 2015, Su et al., 2015). In contrast, M2 subtype represents the states of both an alternative activation and acquired deactivation with an anti-inflammatory response releasing cluster of differentiation-206 (CD206) and arginase-1 (Arg1) (Benson et al., 2015, Popiolek-Barczyk et al., 2015, Su et al., 2015). Brain H_4R is mainly expressed by microglia (Schneider and Seifert, 2016). The effects of H_4R on microglia *in vitro* studies were, however, divergent. A H_4R agonist inhibited ~~the~~ lipopolysaccharide (LPS)-induced pro-inflammatory cytokine IL-1β release in both N9 microglia cell line and hippocampal organotypic slice cultures (Ferreira et al., 2012). Conversely, other reports showed that H_4R activation has a pro-inflammatory effect. Dong *et al*. showed that activation was mediated by both the H1 and the H4 receptors and led to the production of the inflammatory cytokines IL-6, and TNF-α (Dong et al., 2014). This observation is in agreement with *in vivo* data showing that intracerebroventricular (ICV) infusion of an H_4R agonist increased the total microglia cell number as

indicated ~~marked~~ by the marker ionized calcium binding adaptor moleculei1 (Iba1) in wild-type mouse brains (Frick et al., 2016). The same study also showed that a H_4R antagonist blocked the effects of histamine on microglial cells (Frick et al., 2016).

Although these lines of evidence raise the importance to evaluate the efficacy of H_4R antagonists in PD, there is no ~~little~~ information regarding the effects of H_4R antagonist targeting in animal models of PD-like pathology and behaviour impairments. In the present study, we set out to define the microglia-mediated inflammation responses in human post-mortem PD brain using data mining of the microarray data from our group and studied the response to the H_4R antagonist JNJ7777120 in a rat model of PD with regards to inflammatory responses and the expression of H_4R- mRNA.

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LS: Yes no information. Hope we are the first one.

There is a single study, Rocha 2016 they injected histamine into SNpc to see the degenerations. Then apply H1R and H4R antagonists on it. Only H1R antagonist showed protective effects.

We think it is not relevant with current work for two reasons 1) that is not a PD model. 2) H4R antagonist doesn't show effects.

We provide evidence for a strong activation of a microglia-related inflammatory response in the PD rat model, which is associated with H_4R-mRNA up-regulation. In addition, we show proof-of-target efficacy of the H_4R antagonist JNJ7777120, which inhibits microglial inflammation and largely prevents the pathological progression of PD-like pathology and motor dysfunction. These findings indicate that the H_4R is a promising novel therapeutic target for PD.

2. Materials and methods

You said: In the present study, we set out to define the microglia-mediated inflammation responses **in human post-mortem PD brain** and studied the

response to the H_4R antagonist JNJ7777120 in a rat model of PD with regards to inflammatory responses and the expression of H_4R- mRNA. Why don't you start then with human material in materials and methods and results?

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2.1. Animals

PD is 1.5 times more prevalent in men than in women (Mayeux et al., 1992, Hirsch et al., 2016). Therefore, we only included adult male Sprague-Dawley (SD) rats (n = 63) from Dalian Medical University Laboratory Animal Centre, weighing 200 to 320 g) in the present study. All animal experiments were conducted according to the institutional guidelines for the care and use of animals. The animal protocol was designed to minimize pain or discomfort to the animals. The ethical standards of experiments were in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals. All animals had *ad libitum* access to food and water. A 12-hr light-dark cycle was maintained, with lights on at 08:00 a.m.. The experiments were designed to include control groups for all experiments, randomized procedures and apply blinded analysis whenever possible. The quantitative PCR (qPCR) experiments were replicated in a separated batch.

2.2. Experimental protocol

Animal surgery procedures have been described in detail in our previous studies and were used with minor modifications (Liu et al., 2007, Liu et al., 2008). In short, rats were anaesthetized with chloral-hydrate (360 mg/kg, i.p.) and placed in a stereotaxic frame (Stoelting, Wood Dale, IL, USA). According to previous studies (Saravanan et al., 2005, Sindhu et al., 2005), 12 μg rotenone (R8875, Sigma-Aldrich, St. Louis, MO,

USA) (Rot, 6 μg/μl, dissolved in 50% DMSO + 50% PEG400) was infused into the right SNpc (Bregma point: lateral (L) = 1.6mm; antero-posterior (AP) = 4.8mm; and dorso-ventral (DV) = 8.2mm) at a flow rate of 1 μl/min by a micro-infusion apparatus (Harvard, Holliston, MA, USA), while sham-operated rats received an identical volume of the 50% DMSO + 50% PEG400 vehicle only. A fixed cannula was implanted into the left lateral ventricle (LV) (L = 1.5mm; AP = 1.0mm; DV = 3.8mm) for intracerebroventricular administration of H_4R/H_3R antagonists and M1 subtype microglia inhibitor.

The H_4R antagonist JNJ7777120 was reported to have a functional antagonism with at least 1000-fold selectivity over the histamine H1, H2, or H3 receptors and no cross-reactivity against 50 other targets including dopaminergic and serotonergic receptors (Thurmond et al., 2004). To confirm our previous study in PD patients (Shan et al., 2012a), a study on H_4R-mRNA in ventral midbrain of PD rats were performed first. After unilateral lesion of SN by rotentone, nineteen rats were randomly divided into three groups: vehicle + saline group (n = 6), rotenone + saline group (n = 6), and rotenone + JNJ7777120 (JNJ, J3770, Sigma-Aldrich St. Louis, MO, USA) group (n = 7). After surgery, JNJ7777120 (ICV, 5 μg/day) was infused into the ventricles via the permanent cannula during the animal's recovery. After three weeks of continuous treatment, the ventral midbrain was free hand dissected, snap frozen on top of dry ice, and stored at -80°C until RNA extractions were performed. The expression of H_4R, M1 subtype microglia marker and one of its released factors:

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cluster of differentiation-86 (CD86) and interleukin-1β (IL-1β), M2 subtype microglia marker and one of its released factors: cluster of differentiation-206 (CD206) and arginase-1 (Arg1) were measured by qPCR in a triplicate fashion.

A second batch of twenty-six rats was randomly divided after surgery into four groups: rotenone + saline group (n = 6), rotenone + H_4R antagonist JNJ7777120 (ICV, 5 μg/day) group (n = 7), rotenone + Don (ICV, 5 μg/day, D6821, Sigma-Aldrich St. Louis, MO, USA) group (n = 7) and rotenone + Car (ICV, 5 μg/day, SML0329, Sigma-Aldrich St. Louis, MO, USA) group (n = 6). To determine the effect of JNJ7777120 on microglia and the specificity of H_4R antagonist on dopaminergic neurons in SNpc, a M1 subtype microglia inhibitor (Donepezil hydrochloride monohydrate, Don) and the well characterized H_3R antagonist Carcinine dihydrochloride (Car), which did not have any effect on dopaminergic neurons in SN in our pilot study, were used as controls (Chen et al., 2004). After three weeks of treatment, all animals were deeply anesthetized with chloral hydrate (360 mg/kg, i.p.) and transcardially perfused with 4% paraformaldehyde (PF) in phosphate buffer (PB, 0.1M, pH 7.4). Then, brain tissues were harvested, coded and stored in 4% PF. Before cutting the brains using a freezing microtone, the brain was immersed into phosphate-buffered saline (PBS, 0.01M, pH7.4) with 30% sucrose solution for cryoprotection.

The third batch of eighteen rats consisted of a vehicle + saline group (n = 6), a rotenone + saline group (n = 6) and a rotenone + H_4R antagonist JNJ7777120 group

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(n = 6), which were tested for apomorphine (National institutes for food and drug control, Beijing, China) induced rotation behaviour every week up to three weeks (here reference to your earlier work?). After three weeks of treatment, animals were decapitated and the striatum was freehand dissected. This tissue was snap frozen on top of dry ice and stored at -80℃. The level of dopamine was measured by high performance liquid chromatography-mass spectrometry (HPLC-MS/MS).

2.3. **RNA isolation, cDNA synthesis, and qPCR**

RNA isolation and qPCR were described into detail in our previous articles (Shan et al., 2012b, McGregor et al., 2017). In short, total RNA was extracted by TRIzol (15596-026, Invitrogen Carlsbad, CA, USA) reagent according to the manufacturer's protocols. RNA concentration and quality was determined with NanoDrop 2000 spectrophotometer (Thermo Fisher, Carlsbad, CA, USA). Firstly, cDNA was synthesized from 1μg RNA by the *TransScript*® One-Step gDNA (AQ131, Transgen Biotech, Beijing, China) and cDNA synthesis SuperMix (AT311, Transgen Biotech, Beijing, China).

Reactions were performed in 20μl volume containing cDNA diluted 1:10, 10μl ×*TransStart*® Top Green qPCR SuperMix (AQ131, Transgen Biotech, Beijing, China), a mixture of 0.2 μM forward and reverse primers. GenBank accession number and sequence for each primer pair are provided in Table 1. Real time PCR was run in a TP800 Thermal Cycler Dice Real Time system (Takara, Shiga, Japan) under the

following conditions: pre-denaturizing at 95°C for 30s, denaturizing at 95°C for 5s, annealing at 55°C for 30s, 40 cycles in total. At the end of each experiment, a melting curve analysis was included, to confirm primer specificity. Samples were normalized to the reference gene β-actin (ACTB) that was stable among the groups (Livak and Schmittgen, 2001). All qPCR data were calculated using the $2^{-\Delta\Delta Ct}$ method. The PCR products were analysed on a 2 % agarose gel.

2.4. **PCR and agarose gel electrophoresis**

Reactions were performed in 50μl volume containing cDNA diluted at 1:25, 25μl 2×The EasyTaq® PCR SuperMix (AS111, Transgen Biotech, Beijing, China), and a mixture of 0.2 μM forward and reverse H_4R primers. GenBank accession number and sequence for each primer pair are provided in Table 1. PCR was run in a T100 Thermal Cycler Dice system (Bio-Rad, Hercules, CA, USA) under the following conditions: pre-denaturizing at 94°C for 3 min, denaturizing at 94°C for 30 s, annealing at 50°C for 30s (SD rats) / 52°C (SH-SY5Y cells) for 30s, and extending at 72°C for 45 s, 40 cycles in total; extending at 72°C for 10 min, and preserving at 4°C. The PCR product was analysed with gel electrophoresis by 2% agarose, EB staining observation, and a BioSpectrum Imaging System (UVP, Upland, CA) scanning.

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2.5. **Immunohistochemical staining and cell counting**

The mesencephalic SN was cut coronally on a frozen sliding microtome (Leica, Wetzlar, Germany) at 30 μm. Slices were collected free floating in PBS and processed for immunohistochemistry. Each brain (located approximately 4.8-6 mm from

Bregma) was cut into about 35-40 slices. All immunohistochemical and cell counting procedures have been published previously (Liu et al., 2007, Liu et al., 2010, McGregor et al., 2017).

In brief, the brain slices were incubated overnight with primary antibody at 4℃ in PBS with 0.25% Triton X-100 (PBST). The next day, all the brain slices were ~~rewarmed~~ brought to room temperature for 60 min. They were followed by incubation in corresponding biotinylated secondary antibody for 30 min and a streptavidin-biotin system for 30 min (KIT-9720; Ultrasensitive TM S-P, Maixin Biotech, Inc., Fuzhou, China) and developed with the diaminobenzidine tetrahydrochloride (DAB) method.

Tyrosine hydroxylase (TH), the rate-limiting enzyme of dopamine synthesis, was used as a marker for dopaminergic neurons (Hirsch et al., 1988). ~~Ionized calcium-binding adaptor molecule 1 (~~Iba1~~)~~ is a microglia/macrophage-specific calcium-binding protein (Ohsawa et al., 2004). The protein α-synuclein is the main component for Lewy body and Lewy neurites (Braak et al., 2003). The following primary antibodies were therefore used for marker detection: anti-TH antibody (ab6211, Abcam, Cambridge, MA, USA) (1:30.000), anti-Iba-1 antibody (019-19741, Wako Catalog, Osaka, Japan) (1:3.000), anti-α-synuclein antibody (10842-1-AP, Proteintech, Chicago, IL, USA) (1:2.000).

The degree of SN lesion was determined by counting the number of TH-immunoreactive (TH-ir) neurons on the ipsilateral (lesioned) and the contralateral

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(unlesioned) side of each section, respectively. An Olympus IX-71 microscope with three axis motorized stage, video camera and Image J (National Institutes of Health, Bethesda, MD, USA) was used for imagine analyses. Seven-Eight sections of each rat (one out of six slices distributed from rostral to caudal throughout the SN for staining TH，IBA-1 and α-synuclein, respectively) were taken for counting, matched for level as closely as possible from animal to animal. The mean for all the brain sections is presented for each rat in Figure 1. To get the same amount of light, the intensity of light was adjusted for unstained control areas in the same section. The collected α-synuclein staining images were transformed into optical density (OD) images by use of a standard transformation curve, as described in a previous own publication in detail (Shan et al., 2018). The ratio of positive cells between ipsilateral side against the contralateral side was used to evaluate the treatment effects.

2.6. **Apomorphine-induced rotations**

The animals were habituated to the environment for 10 min. Then, apomorphine (0.5 mg/kg, subcutaneous injection) was injected to induce ipsilateral rotation behaviour (Sindhu et al., 2005). Consistent with our previous standard (Liu et al., 2008), each 360° circle rotation was counted. The recording continued for 60 min or until the rotation stopped.

2.7. **Analysis of brain dopamine content**

The amount of dopamine in the striatum was measured by HPLC-MS/MS. The striatal tissue was homogenized in the 100nM formic acid solution. The homogenates of striatal tissue were centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatants were collected and 4 times volume of acetonitrile was added into the supernatant. After vortexing and centrifuging, the supernatant was collected and ultra-filtered, then injected (5 μl) onto the HPLC-MS/MS system by an auto sampler for subsequent analysis.

The HPLC-MS/MS system used in the analysis consisted of a 1200 Series HPLC (Agilent, Santa Clara, CA, USA) coupled to a API 3200 LC-MS/MS system (AB Sciex, Foster City, CA, USA) with an electrospray ion (ESI) source. The measurement conditions of MS system were as follows: source temperature, 250℃; ESI source voltage, 3500 V; ion source gas (Gas1), 20 psi; ion source gas (Gas2), 20 psi; and curtain gas (CUR), 20 psi. Chromatographic separation was conducted on a reversed-phase analytical column (Shim-pack C18 column: 2.1 mm × 150 mm, 5 μm; Shimadzu, Kyoto, Japan). The mobile phase (solvent A: 0.1% aqueous formic acid; solvent B: acetonitrile) was used for a gradient elution at a flow rate of 0.2 ml/min. The HPLC elution program was as follows: 98% A (0 min) → 90% A (linear increase in 5 min) → 0% A (5 min) → 98% A (5 min). The column temperature was maintained at 50℃. The MS/MS detection was performed using multiple reaction monitoring (MRM), in positive mode. The mass transitions of the protonated precursor/product ion pairs that were used to record the selected ion mass

chromatograms of dopamine was m/z 154 → 137 (collision energy, 6 eV; declustering potential, 22 V). Data acquisition and processing were performed using Analyst supplied by AB Sciex.

2.8. **Cell culture** experiments ~~and treatment~~

Human SH-SY5Y neuroblastoma cells express multiple markers characteristic for SN dopaminergic neurons *in vivo*, and produce high levels of dopamine and show high histaminergic gene expression (Korecka et al., 2013). The rotenone induced SH-SY5Y cell is a widely used cellular model of PD (Chiu et al., 2015, Zhang et al., 2016). Cells were obtained from The Cell Bank of Chinese Academy of Sciences. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) /F12 (DMEM/F12, SH30023.01B, Logan, UT, USA) without L-glutamine supplemented with medium containing 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin-streptomycin at 37 °C in 5% CO_2 in incubator (Thermo Fisher, Carlsbad, CA, USA). The culture medium was refreshed every 2 to 3 days. The cells were digested (0.5% trypsin) and passaged when reaching 80-90% confluence at the bottom of the culture flasks. Experiments were using logarithmic growth phase cells. Cells were exposed to rotenone or rotenone + H_4R antagonist JNJ7777120. The cell viability was tested by Cell Counting Kit (CCK) and the expression of α-synuclein was detected by immunocytochemistry and western blot, respectively.

2.9. **Cell viability assay**

Cells were seeded in 96-well plates (1×10^4 cells/well) and incubated for 24 h. Subsequently, cells were exposed to rotenone (50 nM, 100 nM, 250 nM, 500 nM, 1μM) for an additional period of 48 or 72h, respectively. H_4R antagonist JNJ7777120 (10 nM, 100 nM, 500 nM, 1 μM, 10 μM) was added to the cultures 2 h prior to rotenone, once a certain concentration and time point of rotenone was selected to establish PD cell model.

Cell viability was determined by *TransDetect*® CCK assay (FC101, Transgen Biotech, Beijing, China) according to the manufacturer's instructions. Briefly, after treatment, the culture medium was discarded and 100 μl fresh culture medium was added. Then, 10 μl CCK solution was added to each well and incubated at 37 °C for 2 h. After that, the absorbance of formazan dye was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

The cell viability of the control group was 100%, and the cell viability of the other groups was calculated according to the formula: cell viability = (absorbance of each experimental group / absorbance of control group) × 100%.

2.10. **Immunocytochemical staining**

SH-SY5Y cells were cultured in 24-well plates and divided into four group: control group (only cell medium), rotenone model group, rotenone + 100 nM JNJ7777120 group, rotenone + 500 nM JNJ7777120 group. After adhering to the plate, the method of administration was the same as that of the cell viability assay. After administration

of 72h, immunocytochemical staining was performed to detect the expression of α-synuclein. First, the cells were washed two times with PBS, and then the cells were fixed with 4% PF for 15min. Then we applied the standard immunohistochemical staining steps we mentioned previously. Cells immersed in PBS in 24-well were analyzed under an inverted microscope (IX-71, Olympus, Tokyo, Japan).

2.11. **Western blot**

SH-SY5Y cells were cultured in 6-well plates (2.5×10^5 cells/well). There were four groups: control group (only cell medium), rotenone model group, rotenone + 100 nM JNJ7777120 group, and the rotenone + 500 nM JNJ7777120 group. After 72h of treatment, total protein was extracted from cells for western-blotting. The total protein of SH-SY5Y cells was extracted by a total protein extraction kit (KGP250, KeyGEN BioTECH, Nanjing, China). The protein concentration was then determined by a BCA protein concentration kit (BL521A, Biosharp, Hefei, China).

Samples were mixed with 6×Protein Loading buffer (DL101, Transgen Biotech, Beijing, China), equal amounts of protein were resolved by 12% SDS–PAGE and subsequently electrotransferred onto a PVDF membrane by electrophoresis (Millipore, MA, USA). Non-specific protein binding-site on membranes was blocked by incubation for 2 hours in 5% non-fat milk in TBST prior to incubation with primary antibodies (α-synuclein: 1:500, β-actin: 1:1000, TA-09, ZSGB-Bio, Beijing, China) for overnight at 4°C. After TBST washing, the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody

(Sheep-anti-rabbit antibody: 1:5000, Sheep-anti-mouse antibody: 1:1000) for 2h at room temperature. Immunoreactive proteins were detected using ECL western blotting substrate (Abbkine, CA, USA). Blots were scanned and analyzed using the ImageQuant™ LAS 500 system(GE Healthcare, Buckinghamshire, UK)and Odyssey 2.1 scanning software (Timezero, Barcelona, Spain). β-actin signal was used to normalize the final protein quantifications.

2.12. **Statistical analysis**

Statistical analyses were carried out using SPSS Statistics 21.0 (SPSS Inc, Chicago, IL). Mann–Whitney U tests were performed to assess post-mortem SN microarray data between PD and control groups. The data between control and PD model group were analysed by the independent t-test, since they were distributed normally. Multiple groups were analyzed by one-way ANOVA followed by a Fisher's least significant difference (LSD) post-hoc correction. Four time points of JNJ7777120 treatment effect were analyzed based on time points x treatments two-way (repeated measures) ANOVA. Significant ANOVA was followed by LSD post-hoc correction. The figures were made by GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). P values lower than 0.05 were considered as statistically significant. Values are presented as mean ± standard error of the mean (S.E.M.). Percentage-changes of mRNA levels or cell densities were calculated using the mean values.

~~3.~~ Results

See earlier:In the present study, we set out to define the microglia-mediated inflammation responses in human post-mortem PD brain using data mining of the microarray data from our group

~~3.1.~~3. *Decrease in dopaminergic neurons in the SNpc in rotentone-lesioned rats*

After three weeks of unilateral rotenone administration, the number of TH positive cells from the ipsilateral (lesioned) side was reduced by 43% compared to those on the contralateral (unlesioned) side (Figure 1A-D) (776.61±7.8 vs 18079.9±16.4 $T_{(10)}$=5.688, P <0.01**)(Figure 1E).

~~3.2.~~3.1. *Validation of* H_4R *changes and the effect of JNJ7777120 on the changes in unilateral rotenone-lesioned animal model*

H_4R- mRNA was expressed in the ventral midbrain of SD rats (Figure 2A). One-way ANOVA revealed altered H_4R-mRNA levels in the ventral midbrain of vehicle + saline, rotenone-lesioned PD rats and JNJ7777120 + rotenone treated PD rats (H_4R, $F_{(2,18)}$= 3.89, P < 0.05*). Post-hoc comparison showed that H_4R-mRNA levels in the ventral midbrain of rotenone-lesioned PD rats are significantly increased compared with the control group (H_4R, 4.2±1.39 vs 1.0±0.35, P <0.05*) (Figure 2B). Compared to the rotenone-lesion, the co-administration of H_4R antagonist JNJ7777120 significantly down-regulated H_4R-expression (H_4R, 4.2±1.3 vs 1.5±0.7, P<0.01*)

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(Figure 2B). In the H_4R antagonist JNJ7777120 treatment group H_4R-mRNA returned to control level (H_4R, 1.5±0.7 v s1.0±0.4, P=0.70) (Figure 2B).

~~3.3.~~3.2. *H_4R-, but not H_3R-, antagonist treatment protects TH positive neurons in the SNpc from loss in rotenone-lesioned PD rats*

One-way ANOVA revealed that there were significant changes in the ratio of TH positive cell numbers between the lesioned side compared to the non-lesioned contralateral side under H_3R- or H_4R- antagonists' treatment ($F_{(2,17)}$ =15.92, P <0.01**). Subsequent post-hoc analysis of the treatment effects indicated that there was a unilateral degeneration of TH positive cells of about 42.5% at the ipsilateral side compared to the contralateral side (1.00±0.09 vs 0.43±0.01, P<0.01**) (Figure 3A, D, G). H_4R-antagonist JNJ7777120 had a protective effect on rotenone induced TH positive neuron degeneration (0.69±0.03 vs 0.43±0.01, P <0.01**) (Figure 3B, E, G). In contrast, H_3R-antagonist Car administration did not influence the cell degeneration caused by rotentone treatment (0.48±0.07 vs 0.43±0.01, P=0.42) (Figure 3C, F, G).

~~3.4.~~3.3. *The H_4R-antagonist JNJ7777120 increases dopamine content in the striatum of rotentone-lesioned PD rats*

One-way ANOVA revealed unchanged dopamine concentration in the contralateral side of the striatum ($F_{(2,17)}$=0.47, P=0.64), whereas the ipsilateral side exhibited strong changes in dopamine levels ($F_{(2,17)}$=25.03, P<0.01**). Subsequent post-hoc analyses

for the ipsilateral side demonstrated that the amount of dopamine in the striatum of the rotentone group was significantly lower compared to the vehicle + saline treated control groups (35.4%, 75.5±8.6 vs 213.4±22.2, P<0.01**)(Figure 4A). The amount of dopamine in the striatum was significantly increased (61.6%, 122.6±12.9 vs 75.5±8.6, P=0.04*) in the JNJ7777120 treated group compared to the rotenone-lesioned group (Figure 4A). The level of dopamine in the JNJ7777120 treated group was 57.4% of the vehicle and saline treatment control groups (122.6±12.9 vs 213.4±22.2, P<0.01**) (Figure 4A).

~~3.5.~~3.4. *The H_4R-antagonist JNJ7777120 ameliorates apomorphine-induced rotational behaviour*

A significant treatment × weeks of treatment interaction was observed for apomorphine-induced rotation ($F_{(3,30)}$=69.92, P<0.01**)(Figure 4B), with a main treatment effect ($F_{(3,30)}$=15.24, P<0.01**)(Figure 4B). Subsequent analysis of the four time points x two treatments interaction indicated that rotenone + JNJ7777120 rats showed significantly less of apomorphine-induced rotation behaviour during the 1st week 57. 4% (63. 7±10.53 vs 148.1±13.0, $T_{(10)}$=4.43, P < 0.01**)(Figure 4B) and the 3ird week 70.6% (78.9±10.8 vs 268.4±19.9, $T_{(10)}$=5.98, P < 0.01*)(Figure 4B) of treatment, and a trend during the 2nd week 41.16% (133.2±14.8 vs 226. 5±31.7,$T_{(10)}$=2.11, P = 0.06) compared to their rotentone counterparts (Figure 4B).

~~3.6.~~3.5. *The expression of microglial makers is inhibited by the H_4R antagonist JNJ7777120 in the SNpc of rotenone-lesioned PD rats*

One-way ANOVA revealed altered CD86-, IL1b-, CD206-, Arg1- and galectin-3-mRNA levels in the ventral midbrain of vehicle + saline, rotenone-lesioned PD rats and Rotenone+JNJ7777120 treated PD rats (CD86, $F_{(2,18)}$=6.41, P<0.01**; IL-1β, $F_{(2,8)}$=30.95, P<0.00**; CD206, $F_{(2,18)}$=3.67, P<0.05*; Arg1, $F_{(2,8)}$=9.18, P<0.05*; galectin-3, $F_{(2,18)}$=4.59, P<0.05*).

CD86- and IL-1β-mRNA levels were significantly up-regulated in the ventral midbrain of rotentone-lesioned PD rats compared to controls (CD86, 2.9±0.5 vs 1.0±0.2, P<0.01**; IL-1β, 6.6±0.7 vs 1.1±0.6, P<0.01**)(Figure 5A and Supplementary Figure 1A). However, when compared to the rotenone lesion, the H_4R antagonist JNJ7777120 treatment significantly down-regulated CD86- and IL-1β-mRNA levels (CD86, 2.9±0.5 vs 1.8±0.4, P<0.05*; IL-1β, 6.6±0.7 vs 4.4±0.2, P<0.01**) (Figure 5A and Supplementary Figure 1A). Furthermore, H_4R antagonist treatment did not influence CD86-mRNA expression compared to controls (CD86, 1.8±0.4 vs 1.0±0.2, P=0.14) (Figure 5A), but significantly increased the expression of IL-1β-mRNA compared to the control group (IL-1β, 4.3±0.2 vs 1.1±0.6, P<0.05*) (Supplementary Figure 1A).

The rotenone lesion slightly up-regulated CD206-mRNA and significantly augmented Arg1-mRNA expression compared to their controls (CD206, 2.0±0.3 vs 1.0±0.2, P=0.11; Arg1, 10.4±2.6 vs 1.0±0.1, P<0.01**)(Figure 5B and Supplementary Figure 1B). The H_4R antagonist treatment significantly up-regulated mRNA levels of CD206

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(CD206, 2.5±0.5 vs 1.0±0.2, P<0.05*)(Figure 5B), as well as Arg1 (Arg1, 9.4±1.5 vs 1.0±0.1, P<0.01**) (Supplementary Figure 1B) in comparison with their control groups.

The galectin-3-mRNA was significantly more augmented in the rotenone lesion group than in the control group (Galectin-3 14.9±4.9 vs 1.00±0.4, P<0.01**). In response to the H_4R antagonist JNJ7777120 treatment this level was less significant (Galectin-3 11.0±3.0 vs 1.00±0.4, P<0.05*) (Figure 5C).

~~3.7.~~3.6. *The H_4R antagonist JNJ7777120 and the M1 inhibitor Don inhibit microglia marker Iba-1 in the SNpc of rotenone-lesioned PD rats*

Rotenone increased both microglia number and density of ramifications in the lesion side compared to the unlesioned side (Figure 6 A, D and G). The number of Iba-1 positive cells between the rorenone lesioned side vs unlesioned side in the SNpc was greatly increased (123.2 ± 28.8 vs 59.1 ± 13.3, P < 0.01**).

The ratio of Iba-1 positive cells between the lesioned and unlesioned side in the SNpc was significantly changed among the rotenone, H_4R antagonist JNJ7777120, and M1 subtype microglia inhibitor Don groups ($F_{(2,19)}$=26.60, P<0.01**) (Figure 6G). Compared with the rotenone group (Representive Figure 6A and D), the H_4R antagonist (Representive Figure 6 B, E) significantly lowered this ratio (1.3 ± 0.1 vs 2.1 ± 0.1, P < 0.01**) (Figure 6G). The M1 subtype microglia inhibitor Don (Representive Figure 6 C, F) significantly reduced the ratio too (1.1 ± 0.1 vs 2.1 ± 0.1,

P < 0.01**) (Figure 6G). The ratio was stable between the JNJ7777120 and Don treatment groups (1.3 ± 0.1 vs 1.1 ± 0.1, P=0.156) (Figure 6G). Additionally, the morphology of Iba-1 positive cells was with more irregular processes in the rotenone-lesioned group than rotenone+ JNJ7777120 group (Figure 6 E and F).

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~~3.8.~~3.7. *The H_4R antagonist JNJ7777120 reduce Lewy Body-like formation in the SN of rotenone-lesioned PD rats*

A small number of dark brown α-synuclein positive bodies (Lewy Body-like structures) was found in the SNpc of the unlesioned side of rats of Rot group (Figure 7A) and a great amount of α-synuclein positive bodies were found in the SN of the lesioned side (Figure 7B). Compared with the unlesioned side, the number of α-synuclein positive bodies in the SNpc in the lesioned side was 219% higher than in the unlesioned side (2.2±0.1 vs 1.0 ± 0.03, P<0.01**). A significant treatment effect of JNJ7777120 was observed on the number of α-synuclein positive bodies in the SNpc ($F_{(2,18)} = 64.43$, P < 0.01**) (Figure 7D). Subsequent post-hoc analysis showed that the H_4R antagonist JNJ7777120 significantly reduced the ratio of α-synuclein inclusion in lesioned side versus the unlesioned side, in comparison with the rotenone-lesioned ratio (62%, 1.4±0.1 vs 2.2±0.1, P < 0.01**) (Figure 7C and D).

~~3.9.~~3.8. *No protective effect of histamine H_4R antagonist JNJ7777120 on rotenone-lesioned SH-SY5Y cells*

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L.S. It is indirectly showing H4R antagonist JNJ doesn't act via dopamine neurons for their survival.

H_4R-mRNA was also expressed in SH-SY5Y cells (Figure 8A). As shown in Figure 8B, rotenone inhibited the activity of SH-SY5Y cells in a dose and time-dependent

manner. To induce cell loss at a comparable level as observed in the *in vitro* experiment, we decided to use 72h of 100nM rotenone treatment for SH-SY5Y cells.

As re-presentative Figure 8C-a shows, SH-SY5Y cells were epithelioid and polygonal, arranged in a neat uniform way, with weak and homogeneous α-synuclein staining. The 100nM Rot significantly reduced the number of cells after 72h (0.70 ± 0.02, P < 0.01**) compared to control group, and some of the cells became irregular, with more dark brown stained α-synuclein inclusions (Figure 8C-b). Quantitatively, one-way ANOVA revealed a significant treatment effect on cell viability ($F_{(6,20)}$ = 20.67, P<0.01**). The subsequent test demonstrated that compared to the rotenone group, the JNJ7777120 concentration up to 10μM (0.45 ± 0.04, P < 0.01**) (Figure 8D) showed cell toxicity. Lack of significant protective effect of the JNJ7777120 was observed on cell viability with other lower concentrations (10nM (0.66 ± 0.04, P=0.44), 100nM (0.69 ± 0.03, P = 0.76)(Figure 8C-c), 500nM (0.65 ± 0.05, P = 0.34) (Figure 8C-d), 1μM (0.61 ± 0.04, P =0.10)).

Following up with western-blotting, 100nM and 500nM of H_4R antagonist JNJ7777120 had no significant effect on the expression of α-synuclein protein in comparison with the rotenone group ($F_{(2,8)}$ = 1.44, P=0.31) (Figure 8E). The SH-SY5Y cells of the control group expressed a small amount of α-synuclein (Figure 8E). Upon 100nM rotenone treatment for 72h, the expression of α-synuclein in SH-SY5Y cells was 169% higher than in the control group (Figure 8E). Compared to

the 100nM rotenone treatment group, the 500nM histamine H_4R antagonist JNJ7777120 (added to the cultures 2 h prior to 100 nM rotenone) did not significantly change the expression of α-synuclein ($F_{(2,16)}$= 2.78, P=0.10, Figure 8E).

4. Discussion

Activated microglia cells contribute to immune deregulation and neuroinflammation and are associated with PD (Lucin and Wyss-Coray, 2009, Liddelow et al., 2017). Firstly, microarray data from our group confirmed that two microglia mediated inflammatory genes in the SNpc of PD patients are significantly changed (Table 2). Here, we provide evidence for an increased microglia associated inflammatory response in the SNpc in an animal model for human PD that was associated with H_4R-mRNA up-regulation. The H_4R antagonist JNJ7777120 suppressed the pro-inflammatory response of microglia by inhibiting the over-activation of M1 subtype microglia and activated neuroprotective M2 subtype microglia in the rotenone-induced PD rat model, associated with prevention of dopaminergic neuron degeneration and extracellular level of dopamine diminishment, improvement of motor impairment and reduction of Lewy body-like formation pathology (Summarized in Figure 9). Our study is, to the best of our knowledge, the first to indicate that pharmacological inhibition of H_4R suppresses neuroinflammation in the SNpc of the rotenone-induced PD rodent model. Thereby, we provide a new strategy to target the microglia mediated immune response in PD.

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There is no info on the human SN in PD (what is n?). There is also no reference to Koen's paper.

If you are so unhappy with/ ambivalent about these data why not simply leave them out?

If you are happy with them, why not a REAL place in the Materials and methods and Results?

If you want to keep the human data Joost should become co-author.

H_4R antagonists have proven to be effective in several preclinical models of human diseases including asthma, LPS–induced inflammation, dermatitis, collagen-induced arthritis, neuropathic pain (Hsieh et al., 2010), colitis and histamine-induced pruritus (Thurmond et al., 2008). Based on successful preclinical data, some were translating into clinical trials for pruritus and atopic dermatitis (reviewed in (Thurmond et al., 2008, Thurmond et al., 2017)). However, ~~very limited~~ no information is available on the ability of H_4R antagonists to treat neurological disorders such as PD. In the present study we tested the possible effect of H_4R antagonists as a novel therapeutic strategy for PD by acting on microglial activation in a PD animal model that has close fidelity to PD patients (Shan et al., 2015a).

4.1. The rotenone-lesion rat model has close fidelity to PD patients

Reproducing the anatomical, neuropathological, neurochemical and behavioural features of PD, rotenone-treated rats are characterized by loss of dopaminergic neurons, display an increased number of Lewy body-like inclusions in the SNpc, a lower level of dopamine in the striatum and more ipsilateral rotation behaviour in response to an apomorphine challenge (Betarbet et al., 2000, Saravanan et al., 2005, Sindhu et al., 2005, Cicchetti et al., 2009). Importantly, in this model we also found a strong augmentation of H_4R-mRNA levels, as we observed in our post-mortem study (Shan et al., 2012a). This important validation paved the way for a translational treatment using the H_4R antagonist. It should be noted that commercialized H_4R antibodies lack specificity (Beermann et al., 2012), which currently hamper

immunocytochemical localization experiments on brain tissues (Beermann et al., 2012).

Interestingly, we observed activation of microglial-mediated inflammatory responses in our current rotenone-lesion rats, which is pointing into the same direction as we observed in the human post-mortem tissue. The microarray data sets previously published (Bossers et al., 2009) was mined to detect transcriptional alterations of microglial-mediated inflammation responses in the SNpc (Table 2). The M1 subtype microglia released pro-inflammatory chemokine, C-C motif chemokine ligand 20 (CCL20) (Guedj et al., 2014) exhibited a trend towards up-regulation (127% versus matched controls). Furthermore, the M2 subtype microglia released anti-inflammatory chemokine, C-C motif chemokine ligand 22 (CCL22) (Peferoen et al., 2015) showed a significant down regulation (87% versus matched controls). CCL20 recruits immune cells while CCL22 recruits anti-inflammatory cell types, dendritic cells T_H2 cells and regulatory T cells (Lan et al., 2017). At the gene expression level, we also found an elevated expression of galectin-3/lgals-3 (178% versus matched controls), which is an immune modulator that is positively associated with α-synuclein-induced activation of microglia (Boza-Serrano et al., 2014, Belloli et al., 2017). This is in line with our observations in rotenone-treated rats. However, it should be noted that different inflammatory markers up/down-regulated at a time dependent-manner (Lan et al., 2017) which may explain at least partly, the various and not fully overlapping markers we observed in the human material and the preclinical animal model.

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4.2. Translational treatment

To the best of our knowledge, this is the first time that it has been demonstrated that the H_4R antagonist JNJ7777120 significantly reduced PD-related alterations in a validated rodent PD model. Three weeks of continuous JNJ7777120 administration protected about 30% of TH positive neurons from Rotenone degeneration. Accordingly, the JNJ7777120 treatment group had 61.2% more striatal dopamine compared to the rotenone lesioned group. Under influence of an apomorphine challenge, ipsilateral rotation behaviour was significantly reduced by 57%, 41%, and 70% after the first, second and third week of treatment, respectively. Furthermore, the main neuropathological hallmark of PD, the presence of Lewy body-like structures, was significantly reduced by JNJ7777120 treatment as well.

4.3. Mechanism of action

H_4R antagonist JNJ7777120 treatment in the rotenone animal model directly suppressed H_4R- expression to control levels. Our previous observations in PD animal models showed that endogenous histamine aggravated 6-OHDA induced degeneration of dopaminergic neurons in the SNpc (Liu et al., 2007). Since histamine directly regulates microglial activation via H_4R (Vizuete et al., 2000, Ferreira et al., 2012, Rocha et al., 2014, Schneider et al., 2015, Frick et al., 2016, Rocha et al., 2016), we tested here the hypothesis that the H_4R-mRNA down-regulation and positive

treatment effects of the H_4R antagonist were mediated through inhibition of microglia activation.

A previous *in vitro* study demonstrated that JNJ7777120 suppressed microglia activation, and simultaneously inhibited pro-inflammatory cytokines IL-6 and tumor TNF-alpha release (Dong et al., 2014). We showed that the H_4R antagonist JNJ7777120 largely inhibited the mRNA levels of M1 subtype microglia associated genes CD68- and IL-1β-, but not of the anti-inflammatory cytokine Arg1. The effect of H_4R antagonist JNJ7777120 administration was similar to that of the M1 subtype microglia inhibitor Don, as both directly suppressed the number of Iba-1 positive cells and reduced Lewy body-like structures in the SNpc *in vivo*.

We further tested the possibility that the H_4R antagonist JNJ7777120 had a direct effect on dopaminergic neurons. The H_4R gene was expressed in SH-SY5Y cells, and rotenone treatment indeed induced toxicity. α-Synuclein protein, the main component of Lewy body-like inclusions, was significantly upregulated under rotenone treatment. However, unlike the *in vivo* results, JNJ7777120 had no effect, even not when applied at high concentration, in this cellular PD model. Moreover, the JNJ7777120 treatment did not modulate the α-synuclein expression as revealed by immunocytochemistry and western blot. Together with the previous experiments, the *in vitro* study shows that JNJ7777120 does not prevent PD-like pathology by acting directly on the dopaminergic neurons.

In summary, the present data show that H_4R activation leads to an increased pro-inflammatory response in microglia in the SNpc of rats with PD-like symptoms. In the PD rat model the selective H_4R antagonist JNJ7777120 was found to reduce the pro-inflammatory microglia response, prevent dopaminergic neuron degeneration and dopamine level diminishment, improve motor impairment, and reduce Lewy body-like formation pathology (summarized in Figure 9). We provide thus support for the efficacy of H_4R antagonist strategies in the treatment of PD-like pathology to reduce microglia numbers and potential damaging effects of neuro-inflammation. This underlines the importance to test H_4R antagonists in clinical trials for PD as a next step.

Acknowledgments

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in 2015 and 2017, respectively. Funding organizations had no further role in the design of the study, in the collection, analysis and interpretation of data.

Contributors

L Shan, CQ Liu, JR Homberg and DF Swaab designed the study. P Zhou, JQ Wang，WZ Li, QY Fang and Y Luan and P Liao acquired and analysed the data. L Shan, CQ Liu, JR Homberg and DF Swaab wrote the article, which all authors reviewed and approved for publication.

Competing interests

All authors report no potential conflicts of interest.

Legend

Figure 1. Representative image of a large number of dark brown Tyrosine hydroxylase (TH) positive cells and fibers in the substantia nigra pars compacta (SNpc) at ~~of~~ the unlesioned side of the rotenone (Rot)-lesioned rats (A,C). The number of TH positive cells and fibers were significantly reduced after three weeks of rotenone treatment (12 µg, ICV) (B,D). (E) The average number of TH positive cells at the unlesioned side was significantly higher than that at lesioned side. Data represent the mean ± S.E.M. The scatter dots plot the data of each sample (n=6 animals /group, each point is the mean of 7-8 sections per animal). $^{**}P < 0.01$. Scale bar = 200 µm in A and B. Scale bar = 100 µm in C and D.

Figure 2. Agarose gel electrophoresis of RT-PCR amplification product showsed that the H_4R gene was expressed in the ventral midbrain of control rats (A). (B) shows that H_4R-mRNA levels were significantly upregulated in response to Rotenone exposure, while the H_4R antagonist JNJ7777120 (JNJ, ICV, 5 µg/day for 3 weeks) downregulated the expression of this gene. Data represent the mean ± S.E.M. (n=6-7 animals/group). $^{**}P < 0.01$.

Figure 3. Representative image of TH positive neurons in the SNpc of rotenone exposed rats (ICV, 12 µg)(A, D n=6 animals), H_4R antagonist JNJ7777120 (JNJ) treated rats (ICV, 5 µg/day for 3 weeks) (B, E n=7 animals), and the negative

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Answer from Qing: No, we counted the number of TH-positive cells. Please see C and D, the brown cells. A and B are the profile of SN in rats.

DFS 1). So you should mention that in eth M&M, and talk not about the number of cells, but the number of cell profiles when you give quantitative data (change also in RESULTS).

From Qing:： Everyone counts the number. Maybe we shouldn't mention the profile. Profile change just is the result of decreased number.

DFS: volume changes affect the number of profiles, not the number of cells when you use nucleoli as marker

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control H_3R antagonist carcinine dihydrochloride (Car, ICV, 5 µg/day for 3 weeks) (C, F n=6 animals)). The image in (A, B, C) is zoomed in (D, E, F), showing less TH positive cells in Rot treatment (A, D) and Rot+Car treatment (C, F) compared to the Rot+JNJ group (B, E). The ratio of TH positive cell between the lesioned side and unlesioned side is significantly increased by JNJ but not following Car administration (G) Bar plots show the mean, S.E.M., and individual data points. $^{**}P < 0.01$ Scale bar=200 µm.

Figure 4. (A) Striatal dopamine (DA) levels were significantly lower in the rotenone (Rot) treatment versus vehicle + saline treatment group. Compared with Rot treatment, the H_4R antagonist JNJ7777120 (JNJ) treatment significantly increased the level of DA. (B) Apomorphine (APO)-induced rotational behaviour was observed within 1 hour after APO injection. JNJ (ICV, 5 µg/day for 3 weeks) was infused into the left lateral ventricle for 1, 2 and 3 weeks after rotenone lesion and reduced rotational behaviour. Results are presented as mean ± SEM. $^{**}P < 0.01$ (n=6 animals/group).

Figure 5. Transcript levels of cluster of differentiation 86 (CD86) of M1 subtype microglia marker (A), cluster of differentiation 206 (CD206) of M2 subtype microglia marker (B), and galectin-3/lgals-3(C) as determined by qPCR in the SNpc in vehicle + saline, rotenone (Rot)-lesioned PD rats and JNJ7777120+Rot (ICV, 5 µg/day for 3 weeks) treated PD rats, respectively. Bar plots show the mean, S.E.M. and the individual data points. $^{**}P < 0.01$ and * P <0.05 (n=6-7 animals/group). Note that… (give brief explanation)

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Figure 6. Representative images of microglial activation in the SNpc, as stained for Ionized calcium binding adaptor molecule 1 (Iba1). Compared to the rotenone (Rot) (12 µg, intranigra injection) lesion (A, D, n=6 animals) induced a greatly increased number of Iba1 positive cells compared to the unlesioned side. The M1 subtype microglia inhibitor Donepezil hydrochloride monohydrate (Don) (ICV, 5 µg/day for three weeks) treatment in Rot rats led to a significant lower number of Iba1 positive cells at the lesioned sides (C, F n=7 animals/group). The H_4R antagonist JNJ7777120 (JNJ) (ICV, 5 µg/day for three weeks) treatment was also associated with less Iba1 positive cells at the rotenone lesioned side (D, F n=7 animals). The images in (E) and (F) are zoomed in (B) and (C), showing less Iba-1 positive and ramified cells compared to the rotenone group (A and D). The ratio of Iba-1 positive cell profiles between the lesioned side and unlesioned side was significantly decreased by JNJ and Don treatment (G). The ratio is similar in the JNJ and Don treatment groups (G). Bar plots show the mean, S.E.M. of the lesioned side/unlesioned side ratio, and individual data points. $^{**}P < 0.01$, Scale bar=200 µm for A, B, C. Scale bar of =100 µm for D, E, F.

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From Qing; we keep 100 and 200 times amplication

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Answer: 7-8 sections

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Figure 7. The H_4R antagonist JNJ7777120 (JNJ) induces less formation of α-synuclein-positive inclusions in the SNpc. Representative image of α-synuclein inclusions in rotenone (Rot) (12 µg, ICV) unlesioned side (A) and lesioned side (B). The side with JNJ administration (ICV, 5 µg/day for three weeks) (C) showed

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significantly less α-synuclein inclusions. α-synuclein levels were assessed by analysis of relative optical densitometry (ROD) from α-synuclein antibody stained sections (7-8 per animals, quantified by Image J). The ratio of α-synuclein inclusion between the lesioned side and unlesioned side was significantly decreased by JNJ (D). Bar plots show the mean, S.E.M of the lesioned side/unlesioned side ratio, and the individual data $^{**}P < 0.01$, Scale bar=200 μm for A,B and C. Rotenone group: n=6 animals; Rotenone + JNJ group: n=7 animals.

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From Qing:ok

Figure 8. The H_4R antagonist JNJ7777120 (JNJ) did not protect the damage of SH-SY5Y cells induced by rotenone (Rot). H_4R-mRNA is expressed in SH-SY5Y cells (184 bp PCR products)(A). The rotenone-induced SH-SY5Y cells were treated with 50nM 100nM 250nM 500nM 1μM concentrations of JNJ for 48h or 72h, respectively. Cell viability was determined using the Cell Counting Kit assay (B). The effect of JNJ was examined in cells treated with 100 nM rotenone for 72 h. The concentration range from 10Nm to 10μM of H_4R antagonist JNJ was added to the cultures 2 h prior to 100 nM rotenone**.** The cell viability test of different concentrations of JNJ on SH-SY5Y cells damaged by rotenone was tested by Cell Counting Kit assay (D). Representative image of the α-synuclein staining on SH-SY5Y cells with 100nM rotenone alone or rotenone + 100/500nM concentrations of JNJ (Ca-d). Similar α-synuclein protein expressions in 100nM rotenone alone or rotenone + 500nM JNJ treatedSH-SY5Y cells were tested by Western blots. The intensity of the bands of α-synuclein proteins (14 kDa) and its internal reference

β-actin (42 kDa) was quantified by densitometry. The fold protein expression was calculated relative to a normalized value of control cells. (E). Results are presented as mean ± SEM. Each independent experiments were repeated three times. $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bar, 200 μm. These results mean that….(give brief conclusion)

Figure 9. Schematic model of the H_4R antagonist JNJ7777120 prevents dopaminergic neuron degeneration and dopamine level diminishment in rotenone-lesioned rat PD model by reducing the M1 subtype pro-inflammatory microglia (marked by CD86 and IL-1β) but not M2 subtype neuroprotective microglia (marked by CD206 and Arg1).

Supplementary Figure 1. Transcript levels of interleukine-1 beta (IL-1β) (A), and arginase-1 (Arg1) (B), as determined by qPCR in the SNpc in vehicle + saline, rotenone (Rot)-lesioned PD rats and JNJ7777120+Rot treated PD rats, respectively. Bar plots show the mean, S.E.M. $^{**}P < 0.01$ and * P <0.05 (n=3 animals/group). Note that….(give brief conclusion).

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