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

Pei Zhou<sup>a,d</sup>, Judith R. Homberg<sup>c</sup>, Qiuyuan Fang<sup>a</sup>, Jiaqi Wang<sup>a</sup>, Weizhuo Li<sup>a</sup>, Xianzong Meng<sup>a</sup>, Junqing Shen<sup>a</sup>, Yi Luan<sup>a,e</sup>, Peng Liao<sup>a</sup>, Dick F. Swaab<sup>b</sup>, Ling Shan<sup>b,1,\*</sup>, Chunqing Liu<sup>a,1,\*</sup>

<sup>a</sup> College of Medical Laboratory, Dalian Medical University, Dalian, Liaoning 116044, China

<sup>b</sup> Netherlands Institute for Neuroscience, an Institute of the Royal Netherlands Academy of Arts and Sciences, Amsterdam, the Netherlands

<sup>c</sup> Department of Cognitive Neuroscience, Donders Institute for Brain, Cognition, and Behaviour, Radboud University Medical Center, Nijmegen, the Netherlands

<sup>d</sup> Department of Clinical Laboratory, The First College of Clinical Medical Science, China Three Gorges University, Yichang, Hubei 443003, China

<sup>e</sup> Department of Clinical Laboratory, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou 510120, China

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### ABSTRACT

The 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<sub>4</sub>R), thus providing a novel target that may prevent the progression of PD. However, this putative mechanism has so far not been validated. In our previous study, we found that mRNA expression of H<sub>4</sub>R was upregulated in PD patients. In the present study, we validated this possible mechanism using the rotenone-induced PD rat model, in which mRNA expression levels of H<sub>4</sub>R-, and microglial markers were significantly increased in the ventral midbrain. Inhibition of H<sub>4</sub>R in rotenone-induced PD rat model by infusion of the specific H<sub>4</sub>R antagonist JNJ7777120 into the lateral ventricle resulted in blockade of microglial activation. In addition, pharmacological targeting of H<sub>4</sub>R in rotenone-lesioned rats resulted in reduced apomorphine-induced rotational behaviour, prevention of 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<sub>4</sub>R antagonist in a commonly used PD rat model, and proposes the H<sub>4</sub>R as a promising target to clinically tackle microglial activation and thereby the progression of PD.

# 1. Introduction

Parkinson's disease (PD) goes together with a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Hirsch et al., 1988; Damier et al., 1999). A neuropathological hallmark of the disease, in multiple brain areas, is the presence of Lewy bodies and Lewy neurites, whose main component is the  $\alpha$ -synuclein (Braak et al., 2003; Shan et al., 2012c). There is accumulating evidence that inflammation plays an important role 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). The primary inflammatory cells in the brain, microglia,

are activated and play a pivotal role in dopaminergic neurotoxicity (Gao et al., 2002b; Imamura et al., 2003; Shao et al., 2013; Doorn et al., 2014; Liddelow et al., 2017).

Microglia can take on a range of subtypes *in vivo* and can be proinflammatory (M1) or neuroprotective (M2) (Lan et al., 2017). M1 subtype, a classical activation type, is associated with a typical M1 marker cluster of differentiation-86 (CD86) and massive inflammatory response releasing interleukin-1beta (IL-1b), tumour necrosis factor alpha (TNF-a) and interleukin-6 (IL-6) (Benson et al., 2015; Popiolek-Barczyk et al., 2015; Su et al., 2015). In contrast, the M2 subtype represents the states of both an alternative activation and an acquired deactivation with a typical M2 marker cluster of differentiation-206 (CD206) and anti-inflammatory response releasing arginase-1 (Arg1), Insulin-like growth factor-1 (IGF-1) (Benson et al., 2015; Popiolek-

E-mail addresses: l.shan@nin.knaw.nl (L. Shan), liuchunqing@dlmedu.edu.cn (C. Liu).

<sup>1</sup> Shared last and corresponding authorship.

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<sup>\*</sup> Corresponding authors at: College of Medical Laboratory, Dalian Medical University, No.9 West section, Lvshun South Road, Dalian, Liaoning 116044, China (C. Liu). Netherlands Institute for Neuroscience, KNAW, Meibergdreef 47, 1105 BA Amsterdam, the Netherlands (L. Shan).

### Barczyk et al., 2015; Su et al., 2015).

Brain histamine acts as a neurotransmitter (Haas and Panula, 2003) and an immune-response mediator (Shan et al., 2015b), which, in PD, also modulates microglial activity that interferes with dopamine neuron survival in the SNpc (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, 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<sub>4</sub>R) was robustly (4.2–6.3 fold) increased in the basal ganglia of PD patients (Shan et al., 2012a).

The H<sub>4</sub> receptor mediates its effects by coupling to Gi/o G-proteins and has low homology with other (H1R H2R and H3R) histamine receptors (Coge et al., 2001; Liu et al., 2001). This receptor has a distinct expression profile on immune cells, including mast cells, eosinophils, dendritic cells, T cells and microglia, and has modulatory functions, such as activation, migration, and production of cytokines and chemokines (O'Reilly et al., 2002, Hofstra et al., 2003). Brain H<sub>4</sub>R is mainly expressed by microglia (Schneider and Seifert, 2016). The effects of H<sub>4</sub>R on microglia in vitro studies were, however, divergent. A H<sub>4</sub>R agonist inhibited lipopolysaccharide (LPS)-induced pro-inflammatory cytokine IL-1b release in both the N9 microglia cell line and hippocampal organotypic slice cultures (Ferreira et al., 2012). Conversely, other reports showed that H<sub>4</sub>R activation has a pro-inflammatory effect. Dong et al. have shown that the activation of microglia was mediated by both the H1 and the H4 receptors and led to the production of the inflammatory cytokines IL-6 and TNF-a (Dong et al., 2014). This observation is in agreement with in vivo data showing that intracerebroventricular (ICV) infusion of an H<sub>4</sub>R agonist increased the total microglia cell number as indicated by the marker ionized calcium binding adaptor molecule 1 (Iba-1) in C57BL/6 wild-type mouse brains (Frick et al., 2016). The same study also showed that a H<sub>4</sub>R antagonist blocked the effects of histamine on microglial cells (Frick et al., 2016).

This shows the necessity of evaluating the efficacy of  $H_4R$  antagonists in PD. In the present study, we set out to define the microgliamediated inflammation responses to the  $H_4R$  antagonist JNJ7777120 in a rat model of PD, and effects on  $H_4R$ -mRNA levels, Lewy body formation, number of dopaminergic neurons, dopamine levels and PDrelated behaviour. Additionally, an *in vitro* PD experimental model, the rotenone lesioned SH-SY5Y cell line was investigated to test if the  $H_4R$ antagonist JNJ7777120 had a direct protective effect on dopaminergic neurons.

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 support  $H_4R$  as a promising novel therapeutic target for PD.

# 2. Materials and methods

### 2.1. Animals

We used male Sprague-Dawley (SD) rats (n = 69) from Dalian Medical University Laboratory Animal Centre, at 4 month age when rats were weighing 280–320 g, due to 1.5 times more prevalent in men than in women in PD (Mayeux et al., 1992; Hirsch et al., 2016). 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 the 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 and off at 08:00 p.m. The experiments were designed to include control groups for all experiments as well as randomized procedures and to apply blinded analysis whenever possible. The quantitative PCR (qPCR) experiments were replicated in a separate batch.

# 2.2. Experimental protocol

We used animal surgery procedures as described in detail in our previous studies, 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). Like in 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) = 1.6 mm; antero-posterior (AP) = -4.8 mm; and dorso-ventral (DV) = 8.2 mm) at a flow rate of 1 µl/min by a micro-infusion apparatus (Harvard, Holliston, MA, USA). Sham-operated rats received an identical volume of the 50% DMSO + 50% PEG400 vehicle only. A fixed cannula was implanted in the left lateral ventricle (LV) (L = 1.5 mm; AP = -1.0 mm; DV = 3.8 mm) for intracerebroventricular administration of  $H_4R/H_3R$ antagonists and M1 subtype microglia inhibitor. To avoid post-surgery complications and further brain damage, the cannula was implanted on the left side of brain at some distance from the rotenone lesion site that was on right side of brain.

The H<sub>4</sub>R 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<sub>4</sub>R-mRNA levels in the ventral midbrain of PD rats was performed first. After unilateral lesion of substantia nigra (SN) by rotenone, 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 $\mu$ g/day) was infused into the ventricles via a permanent cannula during the animal's recovery period. 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 or protein extractions were performed.

Three animals from each group were selected randomly, the ventral midbrain was homogenized by hand and the tissues were divided into two parts. One part was used for qPCR, the other part for Western blotting. The expression of  $H_4R$ , M1 subtype microglia marker CD86 and two of its released factors, IL-1b, and TNF-a, were measured in the ventral midbrain by quantitative PCR (qPCR) in triplicate fashion. The same went for an M2 subtype microglia marker CD206 and two of its released factors: Arg1, and IGF-1.

A second batch of twenty-six rats was randomly divided, after surgery, into five groups. These groups were: vehicle + JNJ7777120 (n = 6), rotenone + saline (n = 6), rotenone + H<sub>4</sub>R antagonist JNJ7777120 (ICV,  $5 \mu g/day$ ) (n = 7), rotenone + Donepezil hydrochloride monohydrate (Don, D6821, Sigma-Aldrich St. Louis, MO, USA) (ICV,  $5 \mu g/day$ ) (n = 7) and rotenone + Carcinine dihydrochloride (Car, SML0329, Sigma-Aldrich St. Louis, MO, USA) (ICV, 5µg/day) (n = 6). To determine the effect of JNJ7777120 on microglia and the specificity of H<sub>4</sub>R antagonist on dopaminergic neurons in SNpc, the M1 subtype microglia inhibitor Don and the well characterized H<sub>3</sub>R antagonist Car, which we found to have no effect on dopaminergic neurons in SN in a pilot study (data not shown), 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, 0.1 M, pH 7.4). Brain tissue was then harvested, coded and stored in 4% PF. Before cutting the brains with a freezing microtome, the brain was immersed in phosphate-buffered saline (PBS, 0.01 M, 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<sub>4</sub>R antagonist JNJ7777120 group (n = 6), which were tested for apomorphine-induced rotation behaviour every week up to three weeks (National Institutes for Food and Drug Control, Beijing, China). After three weeks of treatment, the animals were decapitated and the striatum was freehand dissected. This tissue was snap frozen on top of dry ice and stored at -80 °C. The level of dopamine was measured by high performance liquid chromatography-mass spectrometry (HPLC-MS/MS).

Two groups of experiments were included to show that a lack of endogenous effects of JNJ7777120 in the absence of rotenone. Group 1: the control group that received a vehicle injection just once, similar to the one time injection of rotenone. Subsequently they were given JNJ7777120 for three weeks, to study the possible toxicity effect of JNJ7777120. The results were compared to the unlesioned side of the rotenone group for TH cell number and microglia marker Iba-1. Group 2: to follow the European efforts to reduce animal experimental usage, 3R principle (Reduction, Refinement, Replacement), we also designed the HPLC experiments in such a way that each animal served as its own control. The rotenone was infused into the right side of the SNpc, while JNJ7777120 was intracerebroventricularly administered. The left, unlesioned side (without rotenone) therefore served as control for any endogenous effects of JNJ7777120. Within the same animal, regarding our HPLC data, we did not observe any significant endogenous effect of JNJ7777120 in the absence of rotenone or vehicle + saline (data not shown). It should be noted that omission of a JNJ7777120 alone dataset from these measures is a limitation of the study.

# 2.3. RNA isolation, cDNA synthesis, and qPCR

RNA isolation and qPCR have been described in 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 were determined with NanoDrop 2000 spectrophotometer (Thermo Fisher, Carlsbad, CA, USA). The first step was to synthesize cDNA from 1  $\mu$ g RNA by the *TransScript*<sup>®</sup> One-Step gDNA (AQ131, Transgen Biotech, Beijing, China) and cDNA synthesis SuperMix (AT311, Transgen Biotech, Beijing, China).

Reactions were performed in 20 µl volume containing cDNA diluted 1:10, 10 µl × *TransStart*<sup>®</sup> 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 Supplementary 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 30 s, denaturizing at 95 °C for 5 s, annealing at 55 °C for 30 s, 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 βactin (ACTB), which was stable in all 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 50 µl volume containing cDNA diluted at 1:25,  $25 \mu l 2 \times$  The EasyTaq<sup>®</sup> PCR SuperMix (AS111, Transgen Biotech, Beijing, China), and a mixture of 0.2 µM forward and reverse H<sub>4</sub>R primers. GenBank accession number and sequence for each primer pair are provided in Supplementary 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 30 s (SD rats) / 52 °C (SH-SY5Y cells) for 30 s, 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. Sterile water (non-template control) and omission of reverse transcriptase (non-reverse transcriptase control) during cDNA synthesis served as negative controls.

# 2.5. Immunohistochemical staining and cell counting

The midbrain SN was cut coronally on a frozen sliding microtome (Leica, Wetzlar, Germany) at  $30 \,\mu$ m. 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 °C in PBS with 0.25% Triton X-100 (PBST). The next day, all the brain slices were brought to room temperature for 60 min. They were then incubated 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). Iba1 is a microglia/macrophage-specific calcium-binding protein (Ohsawa et al., 2004). The protein  $\alpha$ -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- $\alpha$ -synuclein antibody (10842-1-AP, Proteintech, Chicago, IL, USA) (1:2,000).

The degree of SN lesions was determined by counting the number of TH-immunoreactive (TH-ir) neuron profiles on the ipsilateral (lesioned) and the contralateral (unlesioned) side of each section, respectively. An Olympus IX-71 microscope with a three-axis motorized stage, video camera and Image J (National Institutes of Health, Bethesda, MD, USA) was used for image analysis. Seven-Eighth sections of each rat (one out of six slices distributed from rostral to caudal throughout the SN for staining TH, IBA-1 and  $\alpha$ -synuclein, respectively) were taken for counting, and matched for level as closely as possible from animal to animal. The mean for all the brain sections per rat is presented in Fig. 1. To get the same amount of light, the intensity of light was adjusted for unstained control areas in the same section. The collected Iba-1 and  $\alpha$ synuclein staining images were transformed into optical density (OD) images by use of a standard transformation curve, as described in a previous publication in detail (Shan et al., 2018). The ratio of positive cells between the ipsilateral side and 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 100 nM formic acid solution. The homogenates of striatal tissue were centrifuged at

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**Fig. 1.** The number of tyrosine hydroxylase (TH) positive cells and fibers was significantly reduced by rotenone injection but JNJ7777120 injection alone did not influence TH cell numbers. And H<sub>4</sub>R antagonist JNJ7777120 treatment kept microglia marker Iba-1 stable. Rats were treated with rotenone (Rot) (12 µg, intranigra injection). (A) Representative images of tyrosine hydroxylase (TH) positive cells and fibers were made after three weeks of treatment. There was a large number of dark brown TH positive cells and fibers in the substantia nigra pars compacta (SNpc) at the unlesioned side of the Rot-lesioned rats (Aa). A significant cell degeneration was observed at the lesioned side (Ab). (Ae) The average number of TH positive cells at the unlesioned side was significantly higher than that at the lesioned side. The number of TH positive cells at the lesioned and unlesioned sides of the SNpc in vehicle + JNJ7777120 (JNJ) (ICV, 5 µg), animals was stable compared with those in the unlesioned side of Rot-treated animals (Ac, non-vehicle injection side and Ad, vehicle injection side). The average number of TH positive cells numbers between the lesioned side and unlesioned side in the individual groups is shown in Ae. (B) Representative images of microglial activation in the SN, as stained for Ionized calcium binding adaptor molecule-1 (Iba-1). Rot lesion (Bb) greatly increased the number of Iba-1 positive cells compared with the unlesioned side. (Bc, non-vehicle injection side). The average number of Iba-1 positive cells numbers between the lesioned of Iba-1 positive cells numbers between the lesioned of Iba-1 positive cells numbers between the lesioned side. (Bc, non-vehicle injection side and Bd, vehicle injection side). The average number of Iba-1 positive cells numbers between the lesioned side. (Bc, non-vehicle injection side and Bd, vehicle injection side). The average number of Iba-1 positive cells numbers between the lesioned side and unlesioned side of each sample (n = 6 animals/group, each point is the m

14,000 rpm at 4 °C for 10 min. 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  $\mu$ l) into 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 °C; 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:  $2.1 \text{ mm} \times 150 \text{ mm}, 5 \mu\text{m};$  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  $(0 \text{ min}) \rightarrow 90\%$  A (linear increase in 5 min)  $\rightarrow 0\% \text{ A} (5 \text{ min}) \rightarrow 98\% \text{ A} (5 \text{ min})$ . The column temperature was maintained at 50 °C. 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 $154 \rightarrow 137$  (collision energy, 6 eV; declustering potential, 22 V). Data

acquisition and processing were performed using Analyst supplied by AB Sciex.

# 2.8. Western blot

Total protein of the midbrain in rats was extracted by a total protein extraction kit (KGP250, KeyGEN BioTECH, Nanjing, China). Due to limited amount of tissue, all the ventral midbrain tissues from individual animals in each treatment group were pooled together. After the protein concentrations were determined, protein samples were mixed with  $6 \times$  Protein Loading buffer (DL101, Transgen Biotech, Beijing, China). Subsequently, 20 µg protein was dissolved by 12% SDS-PAGE and subsequently electrotransferred onto a PVDF membrane by electrophoresis (Millipore, MA, USA). The non-specific protein binding-site on membranes was blocked by incubation for 2 h in 5% non-fat milk in TBST prior to incubation with primary antibodies (IL-1b: 1:1000, catalogue no. bs-6319R; TNF-a: 1:1000, catalogue no. bs-0078R; GAPDH:1:1000, catalogue no. bs-2188R Bioss, Beijing, China) overnight at 4 °C. After TBST washing, the membrane was incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Goat-anti-rabbit antibody: 1:5000, catalogue no. A0208; Goat-anti-mouse antibody: 1:1000, catalogue no. A0216, Beyotime, Shanghai, China) for 2 h at room temperature. Immunoreactive proteins

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were detected using ECL western blotting substrate (Abbkine, CA, USA). Blots were scanned and analyzed using the ImageQuant<sup>™</sup> LAS 500 system (GE Healthcare, Buckinghamshire, UK) and Odyssey 2.1 scanning software (Timezero, Barcelona, Spain). GAPDH signal was used to normalize the final protein quantifications.

# 2.9. Cell culture experiments

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 the 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<sub>2</sub> in an incubator (Thermo Fisher, Carlsbad, CA, USA). The culture medium was refreshed every 2-3 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<sub>4</sub>R antagonist JNJ7777120. We used a Cell Counting Kit (CCK) to test cell viability and immunocytochemistry and Western-blotting to detect the expression of *a*-synuclein.

# 2.10. Cell viability assay

Cells were seeded in 96-well plates  $(1\times10^4\,cells/well)$  and incubated for 24 h. Subsequently, cells were exposed to rotenone (50 nM, 100 nM, 250 nM, 500 nM, 1  $\mu$ M) for an additional period of either 48 h or 72 h. H<sub>4</sub>R antagonist JNJ7777120 (10 nM, 100 nM, 500 nM, 1  $\mu$ M, 10  $\mu$ M) was added to the cultures 2 h prior to rotenone, once a certain concentration and time point of rotenone had been selected to establish PD cell model.

Cell viability was determined using the *TransDetect*<sup>®</sup> 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)  $\times$  100%.

# 2.11. Immunocytochemical staining of SH-SY5Y cells

SH-SY5Y cells were cultured in 24-well plates and divided into four groups: 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 72 h administration, immunocytochemical staining was performed to detect the expression of  $\alpha$ -synuclein. First, the cells were washed two times with PBS, and then the cells were fixed with 4% PF for 15 min. Then we applied the standard immunohistochemical staining. Cells immersed in PBS in a 24-well were analysed under an inverted microscope (IX-71, Olympus, Tokyo, Japan).

# 2.12. Western blot of SH-SY5Y cells

SH-SY5Y cells were cultured in 6-well plates (2.5  $\times$  10  $^5$  cells/well).

There were three groups: control group (only cell medium), rotenone model group, and the rotenone + 500 nM JNJ7777120 group. After 72 h of treatment, total protein 40  $\mu$ g/sample was extracted from cells for Western-blotting.

The procedure of protein extraction, the protein concentration determination, SDS-PAGE and Blots in SH-SY5Y cells have been described in the Western blot section. The primary antibodies ( $\alpha$ -synuclein: 1:500,  $\beta$ -actin: 1:1000, TA-09) were purchased from ZSGB-Bio (Beijing, China).

# 2.13. Statistical analysis

Statistical analyses were carried out using SPSS Statistics version 21.0 (SPSS Inc, Chicago, IL). The data between control and PD model group were analysed using independent Student t-test. Multiple groups were analyzed by one-way ANOVA followed by a Fisher's least significant difference (LSD) post-hoc correction. To analyse JNJ7777120 treatment effects across time points a time x treatment two-way repeated measures ANOVA was used. Significant interactions were followed by LSD post-hoc testing. The figures were made by GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). P values lower than 0.05 (\*) and lower than 0.01 (\*\*) were considered as statistically significant. Values are presented as mean  $\pm$  standard error of the mean (S.E.M.). Percentage-changes were calculated using the mean values.

# 3. Results

# 3.1. Decrease in dopaminergic neurons and increased in microglia marker Iba-1 in the SNpc in rotenone-lesioned rats

The rotenone-induced PD rat model showed a decrease in dopaminergic neurons and an increase in microglia marker Iba-1 in the SNpc. There were no endogenous effects of JNJ7777120 (JNJ) on dopaminergic neuron marker TH and microglia marker Iba-1. When we compared the contralateral (unlesioned) side to those on the ipsilateral (lesioned) side (Fig. 1Aa and Ab) (180  $\pm$  16 vs 77  $\pm$  8, T<sub>(10)</sub>=5.688, P < 0.01<sup>\*\*</sup>, Fig. 1Ae) after three weeks of unilateral rotenone administration, the number of TH positive neurons was reduced by 43%. The number of Iba-1 positive cells in the rotenone lesioned side compared to the unlesioned side in the SNpc was greatly increased (788  $\pm$  125 vs 373  $\pm$  56, T<sub>(10)</sub> = -3.026, P < 0.05<sup>\*</sup>, Fig. 1Be). There were no endogenous effects of JNJ7777120 (JNJ) on number of TH neurons (Fig. 1Ac, Ad and Ae) or microglia marker Iba-1 (Fig. 1Bc, Bd and Be) when rotenone was absent.

# 3.2. Validation of $H_4R$ changes and the effect of JNJ7777120 on the changes in unilateral rotenone-lesioned animal model

H<sub>4</sub>R-mRNA was expressed in the ventral midbrain of rats (Fig. 2A). One-way ANOVA revealed altered H<sub>4</sub>R-mRNA levels in the ventral midbrain of vehicle + saline, rotenone-lesioned PD rats and JNJ7777120 + rotenone treated PD rats (H<sub>4</sub>R,  $F_{(2,18)} = 3.89$ , P < 0.05<sup>\*</sup>). Post-hoc comparison showed that H<sub>4</sub>R-mRNA levels in the ventral midbrain of rotenone-lesioned PD rats were significantly increased compared with the vehicle + saline group (H<sub>4</sub>R, 4.2 ± 1.4 vs 1.0 ± 0.4, P < 0.05<sup>\*</sup>, Fig. 2B). Compared to the rotenone-lesioned rats treated with saline, the co-administration of rotenone and H<sub>4</sub>R antagonist JNJ7777120 significantly down-regulated H<sub>4</sub>R-expression (H<sub>4</sub>R, 1.5 ± 0.7 vs 4.2 ± 1.4, P < 0.01<sup>\*\*</sup>, Fig. 2B). Compared with the control group, H<sub>4</sub>R-mRNA of the H<sub>4</sub>R antagonist JNJ7777120 + rotenone group almost returned to the level of the vehicle + saline group (H<sub>4</sub>R, 1.5 ± 0.7 v s1.0 ± 0.4, P = 0.70, Fig. 2B).



**Fig. 2.** The H<sub>4</sub>R antagonist JNJ7777120 inhibits the H<sub>4</sub>R-mRNA expression in the rotenone-lesioned rats. Agarose gel electrophoresis of RT-PCR amplification product shows that the H<sub>4</sub>R gene was expressed in the ventral mesencephalon of control rats (A). (B) shows that H<sub>4</sub>R-mRNA levels were significantly upregulated in response to Rotenone exposure, while the H<sub>4</sub>R antagonist JNJ7777120 (JNJ, ICV, 5 µg/day for 3 weeks) downregulated the expression of this gene. Data represent the mean  $\pm$  S.E.M. (n = 6–7 animals/group). <sup>\*\*</sup>P < 0.01.

# 3.3. $H_4$ R-, but not $H_3$ R-antagonist treatment protects TH positive neurons in the SNpc from loss in rotenone-lesioned PD rats

One-way ANOVA revealed that treatment with H<sub>3</sub>R- or H<sub>4</sub>R-antagonists ( $F_{(3,21)} = 107.49$ , P < 0.01<sup>\*\*</sup>) yielded significant changes in the number of TH positive neurons on the lesioned side relative to the number in the non-lesioned contralateral side. Subsequent post-hoc analysis of the treatment effects indicated that there was a degeneration of TH positive cells of about 43% at the lesioned side of rotenonetreated rats compared to the lesioned side of vehicle-treated animals (0.43 ± 0.01 vs 1.00 ± 0.03, P < 0.01<sup>\*\*</sup>, Fig. 3A and E). H<sub>4</sub>R-antagonist JNJ7777120 had a protective effect on rotenone-induced TH positive neuron degeneration (0.43 ± 0.01 vs 0.69 ± 0.03, P < 0.01<sup>\*\*</sup>, Fig. 3A, C and E). In contrast, H<sub>3</sub>R-antagonist Car administration did not influence the cell degeneration caused by rotenone treatment (0.43 ± 0.01 vs 0.48 ± 0.07, P = 0.42, Fig. 3A, B and E).

# 3.4. The $H_4R$ -antagonist JNJ7777120 increases dopamine content in the striatum of rotenone-lesioned PD rats

One-way ANOVA revealed unchanged dopamine levels under rotenone treatment 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 rotenone 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^{\*\*}, Fig. 4A). The amount of dopamine in the striatum was significantly increased (62.4%, 122.6 ± 12.9 vs 75.5 ± 8.6, P = 0.04<sup>\*</sup>) in the JNJ7777120 + rotenone group compared to the rotenone-lesioned along group (Fig. 4A). The level of dopamine in the JNJ7777120 + rotenone group was 57.5% of the vehicle + saline treatment control groups (122.6 ± 12.9 vs 213.4 ± 22.2, P < 0.01<sup>\*\*</sup>, Fig. 4A).

# 3.5. The $H_4R$ -antagonist JNJ7777120 ameliorates apomorphine-induced rotational behaviour

A significant treatment × weeks of treatment interaction was observed for apomorphine-induced rotation ( $F_{(3,30)} = 69.92$ ,  $P < 0.01^{**}$ , Fig. 4B), with a main treatment effect ( $F_{(3,30)} = 15.24$ ,  $P < 0.01^{**}$ , Fig. 4B). Subsequent analysis of the four time points × two treatments interaction indicated that rotenone + JNJ7777120 rats showed significantly less apomorphine-induced rotation behaviour during the 1st

week. i.e. (57. 4%, 63. 7  $\pm$  10.53 vs 148.1  $\pm$  13.0,  $T_{(10)}$  = 4.43, P < 0.01\*\*, Fig. 4B) and the 3rd week, i.e. (70.6%, 78.9  $\pm$  10.8 vs 268.4  $\pm$  19.9,  $T_{(10)}$  = 5.98, P < 0.01\*\*, Fig. 4B) of treatment, and a trend during the 2nd week of (41.16%, 133.2  $\pm$  14.8 vs 226.5  $\pm$  31.7,  $T_{(10)}$  = 2.11, P = 0.06) compared to their rotenone counterparts (Fig. 4B).

3.6. The expression of M1 subtype microglial makers is inhibited by the  $H_4R$  antagonist JNJ7777120 in the ventral midbrain of rotenone-lesioned PD rats

One-way ANOVA revealed altered CD86-, IL-1b-, TNF-a-, CD206-, Arg1- and IGF-1-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-1b,  $F_{(2,18)} = 4.835$ , P <  $0.05^{*}$ ; TNF-a,  $F_{(2,17)} = 3.707$ , P <  $0.049^{*}$ ; CD206,  $F_{(2,18)} = 3.67$ , P <  $0.05^{*}$ ; Arg1,  $F_{(2,18)} = 1.613$ , P = 0.230; IGF-1,  $F_{(2,17)} = 0.691$ , P = 0.516).

CD86-, IL-1b- and TNF-a-mRNA levels were significantly upregulated in the ventral midbrain of rotenone-lesioned PD rats compared to controls (CD86,  $2.9 \pm 0.5 \text{ vs} 1.0 \pm 0.2$ , P  $< 0.01^{**}$ ; IL-1b,  $2.3 \pm 0.2 \text{ vs} 1.0 \pm 0.2$ , P  $< 0.01^{**}$ ; TNF-a,  $1.9 \pm 0.5 \text{ vs} 1.0 \pm 0.1$ , P  $< 0.05^*$ , Fig. 5ABC). However, when compared to the rotenone lesion, the H<sub>4</sub>R antagonist JNJ7777120 + rotenone treatment significantly down-regulated CD86-, IL-1b- and TNF-a-mRNA levels (CD86,  $2.9 \pm 0.5 \text{ vs} 1.8 \pm 0.4$ , P  $< 0.05^*$ ; IL-1b,  $2.3 \pm 0.2 \text{ vs} 1.4 \pm 0.4$ , P  $< 0.05^*$ ; TNF-a,  $1.9 \pm 0.5 \text{ vs} 1.1 \pm 0.2$ , P  $< 0.05^*$ , Fig. 5ABC). Furthermore, H<sub>4</sub>R antagonist JNJ7777120 + rotenone treatment did not influence CD86-, IL-1b- or TNF-a-mRNA expression compared to controls (CD86, P = 0.14; IL-1b, P = 0.377 and TNF-a P = 0.717, Fig. 5ABC).

The H<sub>4</sub>R antagonist treatment significantly up-regulated mRNA levels of CD206 (CD206,  $1.00 \pm 0.21$  vs  $2.47 \pm 0.53$ , P <  $0.05^{*}$ , Fig. 5D) in comparison with their control groups. No any significant difference was found in Arg-1 and IGF-1 among groups.

Due to the limited amount of tissue, we pooled all the ventral midbrain tissue from individual animals in treatment groups for Western-blotting. IL-1b and TNF-a protein levels (Supplementary Fig. 1ABC) were in good agreement with qPCR mRNA expression results (Fig. 5BC).

# 3.7. The H<sub>4</sub>R antagonist JNJ7777120 and 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 on the lesioned side compared to the unlesioned side (Fig. 1Ba and Bb). The ratio of Iba-1 positive cells between the lesioned and unlesioned side in the SNpc was significantly different among the rotenone, H<sub>4</sub>R antagonist JNJ7777120 + rotenone, and M1 subtype microglia inhibitor Don + rotenone groups ( $F_{(3,22)} = 31.45$ , P <  $0.01^{**}$ , Fig. 6E). Compared with the rotenone-only group (Fig. 6A), this ratio  $(1.3 \pm 0.1 \text{ vs } 2.1 \pm 0.1, \text{ P} < 0.01^{**}, \text{ Fig. 6E})$  was significantly lower in the H<sub>4</sub>R antagonist (JNJ7777120) + rotenone group (Fig. 6C), and M1 subtype microglia inhibitor Don + rotenone (Fig. 6B)  $(1.1 \pm 0.1 \text{ vs})$  $2.1 \pm 0.1$ , P <  $0.01^{**}$ , Fig. 6E) as well. The ratio was stable between the rotenone + JNJ7777120 and the rotenone + Don treatment groups  $(1.3 \pm 0.1 \text{ vs } 1.1 \pm 0.1, P = 0.156)$  or vehicle + JNJ7777120 and rotenone + Don treatment  $(0.9 \pm 0.1 \text{ vs } 1.3 \pm 0.1, P = 0.188,$ Fig. 6E). Additionally, the morphology of Iba-1 positive cells was associated with the more irregular processes in the rotenone-lesioned group compared to the rotenone + JNJ7777120 group (Fig. 6A and C).

# 3.8. The $H_4R$ antagonist JNJ7777120 reduces Lewy Body-like formation in the SN of rotenone-lesioned PD rats

The number of  $\alpha$ -synuclein positive bodies (Lewy Body-like structures) in the SNpc on the rotenone-lesioned side (Fig. 7B) was 219%



**Fig. 3.** The H<sub>4</sub>R antagonist JNJ7777120 prevent dopaminergic neuron degeneration. Representative image of TH positive neurons in the SNpc in the lesioned side of the Rot-lesioned rats (12 µg, intranigra injection) with a significant cell degeneration at the Rot-lesioned side (A). The H<sub>4</sub>R antagonist JNJ7777120 (JNJ, ICV, 5 µg/ day for 3 weeks) (C), but not the H<sub>3</sub>R antagonist carcinine dihydrochloride (Car, ICV, 5 µg/day for 3 weeks) (B), prevented the Rot induced-degeneration of TH positive cells in SNpc (E). Bar plots show the mean, S.E.M., and individual data points. <sup>\*\*</sup>P < 0.01, Scale bar = 1000 µm.

higher than that on the unlesioned side (Fig. 7A). A significant treatment effect of JNJ7777120 + rotenone (Fig. 7C) was observed in the number of  $\alpha$ -synuclein positive bodies in the SNpc (1.4 ± 0.1 vs 2.2 ± 0.1, T<sub>(9)</sub> = 3.456, P < 0.05<sup>\*</sup>, Fig. 7D).

# 3.9. No protective effect of histamine $H_4R$ antagonist JNJ7777120 on rotenone-lesioned SH-SY5Y cells

 $H_4R$ -mRNA is also expressed in SH-SY5Y cells (Fig. 8A). As shown in Fig. 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 vivo* experiment, we decided to use 72 h of 100 nM rotenone treatment for SH-SY5Y cells, based on the outcome of Fig. 8B.

As representative Fig. 8Ca shows, SH-SY5Y cells were epithelioid and polygonal, arranged in a neat, uniform way, with weak and homogeneous  $\alpha$ -synuclein staining. The 100 nM rotenone significantly reduced the number of cells after 72 h (0.70  $\pm$  0.02, P < 0.01<sup>\*\*</sup>)

compared to the control group, and some of the cells became irregular, with more dark brown stained  $\alpha$ -synuclein inclusions (Fig. 8Cb). 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, the JNJ7777120 concentration up to  $10\,\mu$ M (0.45  $\pm$  0.04, P  $< 0.01^{**}$ , Fig. 8D) increased cell toxicity. At other lower concentrations there was a lack of significant protective effects of JNJ7777120 on cell viability (10 nM (0.66  $\pm$  0.04, P = 0.44), 100 nM (0.69  $\pm$  0.03, P = 0.76) (Fig. 8Cc), 500 nM (0.65  $\pm$  0.05, P = 0.34, Fig. 8Cd), 1  $\mu$ M (0.61  $\pm$  0.04, P = 0.10)).

Following up with western-blotting, 500 nM of H<sub>4</sub>R antagonist JNJ7777120 had no significant effect on the expression of  $\alpha$ -synuclein compared with the rotenone group (F<sub>(2,8)</sub> = 1.44, P = 0.31, Fig. 8E). The SH-SY5Y cells of the control group expressed a small amount of  $\alpha$ -synuclein (Fig. 8E). After 72 h of 100 nM rotenone treatment the expression of  $\alpha$ -synuclein in SH-SY5Y cells was 169% higher than in the control group (Fig. 8E). Compared to the 100 nM rotenone treatment



**Fig. 4.** The H<sub>4</sub>R antagonist JNJ7777120 prevents apomorphine-induced rotational behavioural and dopamine deficits. (A) Striatal dopamine (DA) levels were significantly lower in the rotenone (Rot) treatment versus vehicle + saline treatment group. Compared with Rot treatment, the H<sub>4</sub>R antagonist JNJ7777120 (JNJ) treatment significantly increased DA levels. (B) Apomorphine (APO)-induced rotational behaviour was observed within 1 h after APO injection. JNJ (ICV, 5  $\mu$ g/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 < 0.05, \*\*P < 0.01 (n = 6 animals/group).

group, the 500 nM histamine H<sub>4</sub>R antagonist JNJ7777120 (added to the cultures 2 h prior to 100 nM rotenone) did not significantly change the expression of  $\alpha$ -synuclein (F<sub>(2,16)</sub> = 2.78, P = 0.10, Fig. 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). Here, we provide evidence for 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 JNJ777120 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 dopa-minergic neuron degeneration and diminishment of extracellular dopamine levels, improvement of motor impairment and reduction of Lewy body-like formation pathology (Fig. 9). To the best of our



**Fig. 5.** The H<sub>4</sub>R antagonist JNJ7777120 inhibits the expressions of the M1 subtype microglia marker cluster of differentiation-86 (CD86)-mRNA and its two released cytokines (interleukine-1 beta (IL-1b) and tumour necrosis factor alpha (TNF-a)), but not the expressions of the M2 subtype microglia marker cluster of differentiation-206 (CD 206) and its two released cytokines (arginase-1 (Arg1) and Insulin-like growth factor-1(IGF-1)). Transcript levels of M1 subtype microglia marker CD86 (A), M2 subtype microglia marker CD206 (D), IL-1b (B), TNF-a (C), Arg1 (E) and IGF-1(F) as determined by qPCR in the ventral mesencephalon in vehicle + saline, rotenone (Rot)-lesioned PD rats and Rot + JNJ7777120 (ICV, 5 µg/day for 3 weeks) treated PD rats, respectively. Bar plots show the mean, S.E.M. and the individual data points. <sup>\*\*</sup>P < 0.01 and <sup>\*</sup>P < 0.05 (n = 6–7 animals/group). H<sub>4</sub>R antagonist JNJ7777120 suppressed the over-activation of M1 subtype microglia marker (CD86, IL-1b and TNF-a) but not M2 subtype microglia marker (CD206, Arg1 and IGF-1) in the rotenone-induced PD rat model.

# Rot Rot + Don

Rot + JNJ



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Fig. 6. H<sub>4</sub>R antagonist JNJ7777120 treatment led to a significant lower number of Iba-1 positive cells at the lesioned sides of SNpc. Representative images of microglial activation in the SNpc, as stained for Ionized calcium binding adaptor molecule-1 (Iba-1). Rotenone (Rot) (12 µg, intranigra injection) lesion (A) greatly increased the number of Iba-1 positive cells (A). The H<sub>4</sub>R antagonist JNJ7777120 (JNJ) (ICV, 5 µg/day for three weeks) treatment led to a significantly lower number of Iba-1 positive cells at the lesioned sides (C). The M1 subtype microglia inhibitor Donepezil hydrochloride monohydrate (Don) (ICV, 5µg/day for three weeks) treatment in Rot rats was also associated with fewer Iba-1-positive cells at the rotenone lesioned side (B). The ratio of Iba-1 positive cell profiles between the lesioned side and unlesioned side was significantly decreased after JNJ and Don treatment (B, C and E). The ratio is similar in the JNJ and Don treatment groups (E, n = 6-7 animals/ group). Bar plots show the mean  $\pm$  S.E.M. of the lesioned side/unlesioned side ratio, and individual data points. \*\*P < 0.01, Scale bar =  $50 \,\mu m$ .



knowledge, our study is the first to indicate that pharmacological inhibition of H<sub>4</sub>R suppresses neuroinflammation and modulates the M1/ M2 ratio in the SNpc of the rotenone-induced PD rodent model. We thus provide a new strategy to target the microglia-mediated immune response in PD.

H<sub>4</sub>R 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 could be translated into clinical trials for pruritus and atopic dermatitis (Thurmond et al., 2008; Thurmond et al., 2017). However, no information is available on the ability of H<sub>4</sub>R antagonists to treat neurological disorders such as PD. In the present study, we tested the possible effect of H<sub>4</sub>R antagonists as a novel therapeutic strategy for PD by acting on microglial activation in a PD animal model that closely resembles PD patients (Shan et al., 2015a).

### 4.1. The rotenone-lesion rat model closely resembles PD

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<sub>4</sub>R-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<sub>4</sub>R antagonist. It should be noted that commercialized H<sub>4</sub>R antibodies lack specificity (Beermann et al., 2012), which currently hampers immunocytochemical localization experiments on brain tissue (Beermann et al., 2012).

### 4.2. Translational treatment

To the best of our knowledge, this is the first time that it has been

Rot(Lesioned)

Rot+JNJ

# **Rot(Unlesioned side) Rot(Lesioned side)** Rot + JNJ D LB-like lesioned/unlesioned) Ratio

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Fig. 7. The H<sub>4</sub>R antagonist JNJ7777120 (JNJ) induces decreased formation of a-synuclein-positive inclusions in the SNpc. asynuclein levels were assessed by analysis of relative optical densitometry (ROD) from asynuclein antibody stained sections (7-8 per animal, quantified by Image J). There were few a-synuclein-positive inclusions in the SNpc unlesioned side in Rot-treated rats (A), while a-synuclein-positive inclusions significantly increased in the lesioned side (B). However, JNJ significantly decreased the  $\alpha$ synuclein-positive inclusions induced by Rot (C). The ratio of  $\alpha$ -synuclein inclusion between the lesioned and unlesioned side was significantly decreased by JNJ. Bar plots show the mean S.E.M of the lesioned side/ unlesioned side ratio, and the individual data <sup>\*\*</sup>P < 0.01, Scale bar =  $200 \,\mu m$  for A, B and C. Rotenone group: n = 6 animals; Rotenone + JNJ group: n = 7 animals.

demonstrated that the H<sub>4</sub>R antagonist JNJ7777120 significantly reduced PD-related alterations in a validated rodent PD model. Three weeks of continuous JNJ7777120 administration protected about 30% of the TH-positive neurons against rotenone degeneration. Accordingly, the JNJ7777120 treatment group had 62.4% more striatal dopamine compared to the rotenone-lesioned group. An apomorphine challenge significantly reduced ipsilateral rotation behaviour, by 57%, 41%, and 70% after the first, second and third week of treatment, respectively. Furthermore, JNJ7777120 treatment significantly reduced the main neuropathological hallmark of PD, i.e. the presence of Lewy body-like structures.

# 4.3. Mechanism of action

 $H_4 R$  antagonist JNJ7777120 treatment in the rotenone animal model directly suppressed H<sub>4</sub>R-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<sub>4</sub>R (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 and others showed that histamine induced an inflammatory response during dopaminergic neuronal survival through a mechanism involving oxidative signaling pathways and microglial phagocytosis (Vizuete et al., 2000; Liu et al., 2007; Rocha et al., 2016). We tested the hypothesis that the H<sub>4</sub>R-mRNA down-regulation and positive treatment effects of the H<sub>4</sub>R antagonist were mediated through inhibition of microglia activation.

Rotenone, a common pesticide and inhibitor of mitochondrial complex I, induces neuronal degeneration and microglial activation, but the neurotoxicity mechanisms are not entirely clear. In vitro experiments suggest that rotenone directly stimulates microglial phagocytic activity by increasing reactive oxygen species (ROS), and promotes neuronal death through phagocytosis of viable neurons (Gao et al., 2002a; Sherer et al., 2003; Emmrich et al., 2013). Interestingly,

H<sub>4</sub>R activation is at least partially involved in microglial phagocytosis and ROS production (Rocha et al., 2016). The H<sub>4</sub>R antagonist treatment in our current experiments, therefore, may act on this target to inhibit microglial ROS production.

A previous in vitro study demonstrated that JNJ7777120 suppressed microglia activation, and simultaneously inhibited pro-inflammatory cytokines IL-6 and TNF-a release (Dong et al., 2014). We showed that the H<sub>4</sub>R antagonist JNJ7777120 largely inhibited the mRNA levels of M1 subtype microglia associated genes CD68 IL-1b and TNF-a, but not of the anti-inflammatory CD206, Arg1 and IGF-1. Also, the IL-1b and TNF-a western-blot data were in good agreement with the mRNA expression results. This is also in line with the result that seven days of JNJ7777120 decreased the plasma levels of the IL-1b and TNF-a in a cerebral ischemia rat model (Dettori, 2017). In addition, the effect of H<sub>4</sub>R 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.

Furthermore, we tested whether the H<sub>4</sub>R antagonist JNJ7777120 had a direct effect on dopaminergic neurons. The H<sub>4</sub>R gene was expressed in SH-SY5Y cells, and rotenone treatment indeed induced toxicity. a-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 in this cellular PD model, not even when applied at high concentration. Moreover, the JNJ7777120 treatment did not modulate  $\alpha$ -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<sub>4</sub>R 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<sub>4</sub>R antagonist JNJ7777120 was found to reduce the pro-inflammatory microglia response, prevent dopaminergic neuron degeneration and



Fig. 8. The H<sub>4</sub>R antagonist JNJ7777120 (JNJ) did not alter the degeneration of SH-SY5Y cells induced by rotenone (Rot). H<sub>4</sub>R-mRNA is expressed in SH-SY5Y cells (184 bp PCR products) (A). The rotenone-induced SH-SY5Y cells were treated with 50 nM 100 nM 250 nM 500 nM 1  $\mu$ M concentrations of JNJ for 48 h or 72 h, 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 10 nm to 10  $\mu$ M of H<sub>4</sub>R 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 a Cell Counting Kit assay (D). Representative image of the  $\alpha$ -synuclein staining on SH-SY5Y cells with 100 nM rotenone alone or rotenone + 100/500 nM concentrations of JNJ (Ca-d). Similar  $\alpha$ -synuclein expressions in 100 nM rotenone alone or rotenone + 500 nM JNJ treated SH-SY5Y cells were tested by Western blots. The intensity of the bands of  $\alpha$ -synuclein (14 kDa) and its internal reference  $\beta$ -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  $\pm$  SEM. Each individual experiment was repeated three times. \*P < 0.05, \*\*P < 0.01. Scale bar, 200  $\mu$ m. JNJ7777120 did not directly prevent rotenone-induced damage in SH-SY5Y cell line (a dopaminergic cell line).



**Fig. 9.** Schematic model of the H<sub>4</sub>R antagonist JNJ7777120. JNJ prevents dopaminergic neuron degeneration and dopamine level diminishment in the rotenonelesioned rat PD model by reducing the M1 subtype pro-inflammatory microglia (marked by CD86, IL-1b and TNF-a) but not M2 subtype neuroprotective microglia (marked by CD206, Arg1 and IGF-1).

dopamine level diminishment, improve motor impairment, and reduce Lewy body-like formation pathology (Fig. 9). We thus provide 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 of clinical trials to test  $H_4R$  antagonists for PD as a next step.

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### Contributors

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

### **Competing interests**

All authors report no potential conflicts of interest.

# Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2018.11.006.

### References

- Anichtchik, O.V., Rinne, J.O., Kalimo, H., Panula, P., 2000. An altered histaminergic innervation of the substantia nigra in Parkinson's disease. Exp. Neurol. 163, 20–30.
- Beermann, S., Seifert, R., Neumann, D., 2012. Commercially available antibodies against human and murine histamine H(4)-receptor lack specificity. Naunyn Schmiedebergs Arch. Pharmacol. 385, 125–135.
- Benson, M.J., Manzanero, S., Borges, K., 2015. Complex alterations in microglial M1/M2 markers during the development of epilepsy in two mouse models. Epilepsia 56, 895–905.
- Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., Greenamyre, J.T., 2000. Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat. Neurosci. 3, 1301–1306.
- Braak, H., Del Tredici, K., Rub, U., de Vos, R.A., Jansen Steur, E.N., Braak, E., 2003. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging 24, 197–211.
- Chen, Z., Sakurai, E., Hu, W., Jin, C., Kiso, Y., Kato, M., Watanabe, T., Wei, E., Yanai, K., 2004. Pharmacological effects of carcinine on histaminergic neurons in the brain. Br. J. Pharmacol. 143, 573–580.
- Chiu, C.C., Yeh, T.H., Lai, S.C., Wu-Chou, Y.H., Chen, C.H., Mochly-Rosen, D., Huang,

# P. Zhou et al.

Y.C., Chen, Y.J., Chen, C.L., Chang, Y.M., Wang, H.L., Lu, C.S., 2015. Neuroprotective effects of aldehyde dehydrogenase 2 activation in rotenone-induced cellular and animal models of parkinsonism. Exp. Neurol. 263, 244–253.

- Cicchetti, F., Drouin-Ouellet, J., Gross, R.E., 2009. Environmental toxins and Parkinson's disease: what have we learned from pesticide-induced animal models? Trends Pharmacol. Sci. 30, 475–483.
- Coge, F., Guenin, S.P., Rique, H., Boutin, J.A., Galizzi, J.P., 2001. Structure and expression of the human histamine H4-receptor gene. Biochem. Biophys. Res. Commun. 284, 301–309.
- Damier, P., Hirsch, E.C., Agid, Y., Graybiel, A.M., 1999. The substantia nigra of the human brain. II. patterns of loss of dopamine-containing neurons in Parkinson's disease. Brain 122, 1437–1448.
- Deleidi, M., Gasser, T., 2013. The role of inflammation in sporadic and familial Parkinson's disease. Cell. Mol. Life Sci.: CMLS 70, 4259–4273.
- Dettori, I.G., Melani, L., Lucarini, L., Pepeu, G., Masini, E., Pedata, F., 2017. Effects of chronic administration of the selective histamine H4 receptor antagonist JNJ7777120 on cerebral injury in a model of transient brain focal ischemia in the rat. Annual Neuroscience meeting. Society For Neuroscience, Washington, DC.
- Dong, H., Zhang, W., Zeng, X., Hu, G., Zhang, H., He, S., Zhang, S., 2014. Histamine induces upregulated expression of histamine receptors and increases release of inflammatory mediators from microglia. Mol. Neurobiol. 49, 1487–1500.
- Doorn, K.J., Moors, T., Drukarch, B., van de Berg, W., Lucassen, P.J., van Dam, A.M., 2014. Microglial phenotypes and toll-like receptor 2 in the substantia nigra and hippocampus of incidental Lewy body disease cases and Parkinson's disease patients. Acta Neuropathol. Commun. 2, 90.
- Dzamko, N., Geczy, C.L., Halliday, G.M., 2015. Inflammation is genetically implicated in Parkinson's disease. Neuroscience 302, 89–102.
- Emmrich, J.V., Hornik, T.C., Neher, J.J., Brown, G.C., 2013. Rotenone induces neuronal death by microglial phagocytosis of neurons. FEBS J. 280, 5030–5038.
- Ferreira, R., Santos, T., Goncalves, J., Baltazar, G., Ferreira, L., Agasse, F., Bernardino, L., 2012. Histamine modulates microglia function. J. Neuroinflamm. 9, 90.
- Frick, L., Rapanelli, M., Abbasi, E., Ohtsu, H., Pittenger, C., 2016. Histamine regulation of microglia: Gene-environment interaction in the regulation of central nervous system inflammation. Brain Behav. Immun. 57, 326–337.
- Gao, H.M., Hong, J.S., Zhang, W., Liu, B., 2002a. Distinct role for microglia in rotenoneinduced degeneration of dopaminergic neurons. J. Neurosci. 22, 782–790.
- Gao, H.M., Jiang, J., Wilson, B., Zhang, W., Hong, J.S., Liu, B., 2002b. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J. Neurochem. 81, 1285–1297.
- Haas, H., Panula, P., 2003. The role of histamine and the tuberomamillary nucleus in the nervous system. Nat. Rev. 4, 121–130.
- Hirsch, E., Graybiel, A.M., Agid, Y.A., 1988. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. Nature 334, 345–348.
- Hirsch, L., Jette, N., Frolkis, A., Steeves, T., Pringsheim, T., 2016. The incidence of parkinson's disease: a systematic review and meta-analysis. Neuroepidemiology 46, 292–300.
- Hofstra, C.L., Desai, P.J., Thurmond, R.L., Fung-Leung, W.P., 2003. Histamine H4 receptor mediates chemotaxis and calcium mobilization of mast cells. J. Pharmacol. Exp. Ther. 305, 1212–1221.
- Hsieh, G.C., Chandran, P., Salyers, A.K., Pai, M., Zhu, C.Z., Wensink, E.J., Witte, D.G., Miller, T.R., Mikusa, J.P., Baker, S.J., Wetter, J.M., Marsh, K.C., Hancock, A.A., Cowart, M.D., Esbenshade, T.A., Brioni, J.D., Honore, P., 2010. H4 receptor antagonism exhibits anti-nociceptive effects in inflammatory and neuropathic pain models in rats. Pharmacol. Biochem. Behav. 95, 41–50.
- Imamura, K., Hishikawa, N., Sawada, M., Nagatsu, T., Yoshida, M., Hashizume, Y., 2003. Distribution of major histocompatibility complex class II-positive microglia and cytokine profile of Parkinson's disease brains. Acta Neuropathol 106, 518–526.
- Korecka, J.A., van Kesteren, R.E., Blaas, E., Spitzer, S.O., Kamstra, J.H., Smit, A.B., Swaab, D.F., Verhaagen, J., Bossers, K., 2013. Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. PLoS One 8 e63862.
- Lan, X., Han, X., Li, Q., Yang, Q.W., Wang, J., 2017. Modulators of microglial activation and polarization after intracerebral haemorrhage. Nat Rev. Neurol. 13, 420–433.
- Liddelow, S.A., Guttenplan, K.A., Clarke, L.E., Bennett, F.C., Bohlen, C.J., Schirmer, L., Bennett, M.L., Munch, A.E., Chung, W.S., Peterson, T.C., Wilton, D.K., Frouin, A., Napier, B.A., Panicker, N., Kumar, M., Buckwalter, M.S., Rowitch, D.H., Dawson, V.L., Dawson, T.M., Stevens, B., Barres, B.A., 2017. Neurotoxic reactive astrocytes are induced by activated microglia. Nature 541, 481–487.
- Liu, C.Q., Chen, Z., Liu, F.X., Hu, D.N., Luo, J.H., 2007. Involvement of brain endogenous histamine in the degeneration of dopaminergic neurons in 6-hydroxydopamine-lesioned rats. Neuropharmacology 53, 832–841.
- Liu, C.Q., Hu, D.N., Liu, F.X., Chen, Z., Luo, J.H., 2008. Apomorphine-induced turning behavior in 6-hydroxydopamine lesioned rats is increased by histidine and decreased by histidine decarboxylase, histamine H1 and H2 receptor antagonists, and an H3 receptor agonist. Pharmacol. Biochem. Behav. 90, 325–330.
- Liu, C., Ma, X., Jiang, X., Wilson, S.J., Hofstra, C.L., Blevitt, J., Pyati, J., Li, X., Chai, W., Carruthers, N., Lovenberg, T.W., 2001. Cloning and pharmacological characterization of a fourth histamine receptor (H(4)) expressed in bone marrow. Mol. Pharmacol. 59, 420–426.
- Liu, C.Q., Shan, L., Balesar, R., Luchetti, S., Van Heerikhuize, J.J., Luo, J.H., Swaab, D.F., Bao, A.M., 2010. A quantitative in situ hybridization protocol for formalin-fixed paraffin-embedded archival post-mortem human brain tissue. Methods 52, 359–366.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using realtime quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

### Brain, Behavior, and Immunity xxx (xxxx) xxx-xxx

Lucin, K.M., Wyss-Coray, T., 2009. Immune activation in brain aging and neurodegeneration: too much or too little? Neuron 64, 110–122.

- Mayeux, R., Denaro, J., Hemenegildo, N., Marder, K., Tang, M.X., Cote, L.J., Stern, Y., 1992. A population-based investigation of Parkinson's disease with and without dementia. relationship to age and gender. Arch. Neurol. 49, 492–497.
- McGregor, R., Shan, L., Wu, M.F., Siegel, J.M., 2017. Diurnal fluctuation in the number of hypocretin/orexin and histamine producing: implication for understanding and treating neuronal loss. PLoS One 12 e0178573.
- Ohsawa, K., Imai, Y., Sasaki, Y., Kohsaka, S., 2004. Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity. J. Neurochem. 88, 844–856.
- O'Reilly, M., Alpert, R., Jenkinson, S., Gladue, R.P., Foo, S., Trim, S., Peter, B., Trevethick, M., Fidock, M., 2002. Identification of a histamine H4 receptor on human eosinophils-role in eosinophil chemotaxis. J. Recept. Signal Transduction Res. 22, 431–448.
- Popiolek-Barczyk, K., Kolosowska, N., Piotrowska, A., Makuch, W., Rojewska, E., Jurga, A.M., Pilat, D., Mika, J., 2015. Parthenolide relieves pain and promotes M2 microglia/macrophage polarization in rat model of neuropathy. Neural Plast. 2015 676473.
- Rinne, J.O., Anichtchik, O.V., Eriksson, K.S., Kaslin, J., Tuomisto, L., Kalimo, H., Roytta, M., Panula, P., 2002. Increased brain histamine levels in Parkinson's disease but not in multiple system atrophy. J. Neurochem. 81, 954–960.
- Rocha, S.M., Pires, J., Esteves, M., Graca, B., Bernardino, L., 2014. Histamine: a new immunomodulatory player in the neuron-glia crosstalk. Front. Cell. Neurosci. 8, 120.
- Rocha, S.M., Saraiva, T., Cristovao, A.C., Ferreira, R., Santos, T., Esteves, M., Saraiva, C., Je, G., Cortes, L., Valero, J., Alves, G., Klibanov, A., Kim, Y.S., Bernardino, L., 2016. Histamine induces microglia activation and dopaminergic neuronal toxicity via H1 receptor activation. J. Neuroinflamm. 13, 137.
- Saravanan, K.S., Sindhu, K.M., Mohanakumar, K.P., 2005. Acute intranigral infusion of rotenone in rats causes progressive biochemical lesions in the striatum similar to Parkinson's disease. Brain Res. 1049, 147–155.
- Schneider, E.H., Neumann, D., Seifert, R., 2015. Histamine H4-receptor expression in the brain? Naunyn Schmiedebergs Arch. Pharmacol. 388, 5–9.
- Schneider, E.H., Seifert, R., 2016. The histamine H4-receptor and the central and peripheral nervous system: a critical analysis of the literature. Neuropharmacology 106, 116–128.
- Shan, L., Bossers, K., Unmehopa, U., Bao, A.M., Swaab, D.F., 2012b. Alterations in the histaminergic system in Alzheimer's disease: a postmortem study. Neurobiol. Aging 33, 2585–2598.
- Shan, L., Bao, A.M., Swaab, D.F., 2015a. The human histaminergic system in neuropsychiatric disorders. Trends Neurosci. 38, 167–177.
- Shan, L., Dauvilliers, Y., Siegel, J.M., 2015b. Interactions of the histamine and hypocretin systems in CNS disorders. Nat. Rev. Neurol. 11, 401–413.
- Shan, L., Bossers, K., Luchetti, S., Balesar, R., Lethbridge, N., Chazot, P.L., Bao, A.M., Swaab, D.F., 2012a. Alterations in the histaminergic system in the substantia nigra and striatum of Parkinson's patients: a postmortem study. Neurobiol. Aging 33 1488 e1481-e1413.
- Shan, L., Liu, C.Q., Balesar, R., Hofman, M.A., Bao, A.M., Swaab, D.F., 2012c. Neuronal histamine production remains unaltered in Parkinson's disease despite the accumulation of Lewy bodies and Lewy neurites in the tuberomamillary nucleus. Neurobiol. Aging 33, 1343–1344.
- Shan, L., Guo, H.Y., van den Heuvel, C., van Heerikhuize, J., Homberg, J.R., 2018. Impaired fear extinction in serotonin transporter knockout rats is associated with increased 5-hydroxymethylcytosine in the amygdala. CNS Neurosci. Ther.
- Shao, W., Zhang, S.Z., Tang, M., Zhang, X.H., Zhou, Z., Yin, Y.Q., Zhou, Q.B., Huang, Y.Y., Liu, Y.J., Wawrousek, E., Chen, T., Li, S.B., Xu, M., Zhou, J.N., Hu, G., Zhou, J.W., 2013. Suppression of neuroinflammation by astrocytic dopamine D2 receptors via alphaB-crystallin. Nature 494, 90–94.
- Sherer, T.B., Betarbet, R., Testa, C.M., Seo, B.B., Richardson, J.R., Kim, J.H., Miller, G.W., Yagi, T., Matsuno-Yagi, A., Greenamyre, J.T., 2003. Mechanism of toxicity in rotenone models of Parkinson's disease. J. Neurosci. 23, 10756–10764.
- Sindhu, K.M., Saravanan, K.S., Mohanakumar, K.P., 2005. Behavioral differences in a rotenone-induced hemiparkinsonian rat model developed following intranigral or median forebrain bundle infusion. Brain Res. 1051, 25–34.
- Su, F., Yi, H., Xu, L., Zhang, Z., 2015. Fluoxetine and S-citalopram inhibit M1 activation and promote M2 activation of microglia in vitro. Neuroscience 294, 60–68.
- Thurmond, R.L., Desai, P.J., Dunford, P.J., Fung-Leung, W.P., Hofstra, C.L., Jiang, W., Nguyen, S., Riley, J.P., Sun, S., Williams, K.N., Edwards, J.P., Karlsson, L., 2004. A potent and selective histamine H<sub>4</sub> receptor antagonist with anti-inflammatory properties. J. Pharmacol. Exp. Ther. 309, 404–413.
- Thurmond, R.L., Gelfand, E.W., Dunford, P.J., 2008. The role of histamine  $H_1$  and  $H_4$  receptors in allergic inflammation: the search for new antihistamines. Nat. Rev. Drug Discovery 7, 41–53.
- Thurmond, R.L., Venable, J., Savall, B., La, D., Snook, S., Dunford, P.J., Edwards, J.P., 2017. Clinical development of histamine H<sub>4</sub> receptor antagonists. Handb. Exp. Pharmacol. 241, 301–320.
- Vizuete, M.L., Merino, M., Venero, J.L., Santiago, M., Cano, J., Machado, A., 2000. Histamine infusion induces a selective dopaminergic neuronal death along with an inflammatory reaction in rat substantia nigra. J. Neurochem. 75, 540–552.
- Zhang, Q., Chen, S., Yu, S., Qin, J., Zhang, J., Cheng, Q., Ke, K., Ding, F., 2016. Neuroprotective effects of pyrroloquinoline quinone against rotenone injury in primary cultured midbrain neurons and in a rat model of Parkinson's disease. Neuropharmacology 108, 238–251.