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Simvastatin reverses the downregulation of M1/4 receptor binding in 6-hydroxydopamine-induced parkinsonian rats: the association with improvements in long-term memory

Qing Wang
University of Wollongong, dwang@uow.edu.au

Xiaobo Wei
Third Affiliated Hospital of Sun Yat-sen University

H Gao
Third Affiliated Hospital of Sun Yat-sen University

Jin Li
Third Affiliated Hospital of Sun Yat-sen University

Jinchi Liao
Third Affiliated Hospital of Sun Yat-sen University

See next page for additional authors

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Abstract
Background It is believed that muscarinic M1/4 receptors are closely correlated to the dopaminergic system and are strongly involved in the pathogenesis of Parkinson's disease (PD). In addition to regulating lipid metabolism and protection from stroke, statins have been used to regulate the declined cognition. We aimed to explore the regional changes in M1/4 receptors in the 6-hydroxydopamine (6-OHDA)-lesioned rat brain. Methods PD rat model was set up by injecting 6-OHDA into the unilateral medial forebrain bundle; while simvastatin (10 mg/kg/day) or saline was orally administrated for 3 weeks, respectively. Long-term memory was measured using the Morris water maze. [3H]pirenzepine binding autoradiography was applied to investigate the alterations of M1/4 receptors in the PD rat brains. Results 6-OHDA induced long-term memory deficits along with downregulation of M1/4 receptors in the hippocampus, the medial amygdala, the posteromedial cortical and the piriform cortex; simvastatin administration significantly ameliorated the impaired memory and reversed the downregulation of M1/4 receptors in the examined brain regions. Profound positive correlations were verified between the decline in long-term memory activity and the restoration of M1/4 receptors in different brain regions after simvastatin treatment. Conclusions/significance Our results provide strong evidence that M1/4 receptor modulation after simvastatin administration did, at least partially, contribute to the improvement in the long-term memory in 6-OHDA-induced PD rats. These results provide a possible mechanism for simvastatin treatment in psycho-neurological diseases such as PD via M1/4 receptors.

Keywords
Parkinson's disease, simvastatin, M1/4 receptors, memory

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Authors
Qing Wang, Xiaobo Wei, H Gao, Jin Li, Jinchi Liao, X Liu, Bing Qin, Yinghua Yu, Chao Deng, B Tang, and Xu-Feng Huang

Simvastatin reverses the downregulation of M1/4 receptor binding in 6-hydroxydopamine-induced Parkinsonian rats: the association with improvements in long term memory

Qing Wang MD, Ph.D 1 2 †*, Xiaobo Wei MD 1†, Huimin Gao MD 1†, Jin Li MD1, Jinchi Liao MD 1, Xu Liu MD 1, Bing Qin MD 1, Yinghua Yu Ph.D 2, Chao Deng Ph.D 2, Beisha Tang MD, Ph.D.3, Xu-Feng Huang Ph.D MBBS 2

1 Department of Neurology, The Third Affiliated Hospital of Sun Yat-Sen University, Tianhe Road 600, Guangzhou, Guangdong 510630, P.R. China
2 Centre for Translational Neuroscience, School of Health Sciences, University of Wollongong, NSW 2522, Australia
3 Department of Neurology, Xiangya Hospital of Central South University, Changsha, Hunan 410008, P. R. China

Correspondence and reprint request to:
Prof. (Dr.) Qing Wang MD, Ph.D
Vice-Head, Department of Neurology,
Director of Neurological Research Lab,
The Third Affiliated Hospital of Sun Yat-Sen University,
600 Tianhe Road, Guangzhou,
Guangdong 510630,
P.R. China
Phone: +86-20-85252238, FAX: +86-20-85253117
Email: wqdennis@gmail.com or denniswq@yahoo.com
† The same contribution

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Abstract

BACKGROUND: It is believed that muscarinic M1/4 receptors is closely correlated to the dopaminergic system and are strongly involved in the pathogenesis of Parkinson's disease (PD). In addition to regulating lipid metabolism and protection from stroke, statins have been used to regulate the declined cognition. We aimed to explore the regional changes in M1/4 receptors in the 6-hydroxydopamine (6-OHDA) lesioned rat brain. **METHODS:** PD rat model was set up by injecting 6-OHDA into the unilateral medial forebrain bundle; while simvastatin (10mg/kg/day) or saline was orally administrated for three weeks, respectively. Long-term memory was measured using the Morris water maze. [3H]pirenzepine binding autoradiography was applied to investigate the alterations of M1/4 receptors in the PD rat brains. **RESULTS:** The 6-OHDA induced long-term memory deficits along with downregulation of M1/4 receptors in the hippocampus, the medial amygdala, the posteromedial cortical and the piriform cortex; simvastatin administration significantly ameliorated the impaired memory and reversed the downregulation of M1/4 receptors in the examined brain regions. Profound positive correlations were verified between the decline in long-term memory activity and the restoration of M1/4 receptors in different brain regions after simvastatin treatment. **CONCLUSIONS/SIGNIFICANCE:** Our results provide strong evidence that M1/4 receptor modulation after simvastatin administration did, at least partially, contribute to the improvement in the long-term memory in 6-OHDA induced PD rats. These results provide a possible mechanism for simvastatin treatment in psycho-neurological diseases such as PD via M1/4 receptors.

Keywords: Parkinson's disease, Simvastatin, M1/4 receptors, Memory

Highlights:

- 6-OHDA resulted in the impaired long-term memory in PD rats.
- Simvastatin ameliorated the impaired long-term memory in PD rats.
- 6-OHDA decreased M1/4 receptor binding in Hip, Me, PMCo, and Pir of PD rat brains.
- Simvastatin reversed M1/4 receptor binding in the examined brain regions.
- The altered M1/4 receptor binding was related to the long-term memory in PD rats.

Abbreviations:
ABC, avidin-biotin-peroxidase complex staining system;
Acb, accumbens nucleus;
AD, Alzheimer's disease;
AI, agranular insular cortex;
Amy, amygdala;
ANOVA, analysis of variance;
AP: anteroposterior;
Arc, arcuate hypothalamic nucleus;
CA1: Cornu Ammonis area 1
Cg, cingulate cortex;
CPu, caudate putamen;
DAB, diaminobenzidine;
DMH, dorsomedial hypothalamic nucleus;
DPX, Di-N-Butyle Phthalate in Xylene;
DV, dorsoventral;
Ent, entorhinal cortex;
GPCR, G-protein-coupled receptors;
HEPES, 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid;
Hip, hippocampus;
HMG-CoA, hydroxymethylglutaryl-coenzyme A;
LDL, low-density lipoprotein;
LTP, long-term potentiation;
M1, primary motor cortex;
M1/4 receptor, muscarinic1/4 receptor;
Me, medial amygdala;
MFB, medial forebrain bundle;
ML, mediolateral;
MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine;
MWM, morris water maze;
NMDA, N-methyl-D-aspartic acid;
PBS, phosphate buffered saline;
Pir, piriform cortex;
PD, Parkinson's disease;
PMCo, posteromedial cortical amygdala;
PRh, perirhinal cortex;
RSG, retrosplenial granular cortex;
SNpc, compact part of substantia nigra;
SNr, reticular part of substantia nigra;
TH, tyrosine hydroxylase;
Tha, thalamus;
Tu, olfactory tubercle ;
VDB, vertical limb of the diagonal band;
VMH, ventromedial hypothalamus;
VTA, ventral tegmental area;
6-OHDA, 6-hydroxydopamine.

Introduction

The cholinergic system and its neurodegeneration associated with Neurodegenerative diseases such as Parkinson's diseases (PD) and Alzheimer's disease (AD) have obtained considerable attention. Muscarinic receptors, one of the important elements in the cholinergic system, have been widely explored. Muscarinic receptors are G-protein-coupled receptors (GPCR) that include five subtypes M1–M5 (Bymaster *et al.*, 2003). Among these five subtypes, muscarinic M1 receptors that are localized to the postsynaptic membrane are the most studied and abundant in the cerebral cortex, the amygdala, the caudate putamen, and the hippocampus (Wang *et al.*, 2008). Increasing evidence indicates important roles for M1 receptors in learning and functional memory regulation among the various neuropsychological disorders (Auld *et al.*, 2002; Hu *et al.*, 2008; Digby *et al.*, 2012). In aged rats with a decline in memory and adult-onset cognitive dysfunction, the densities of M1 and M4 receptors were profoundly decreased in brain regions including the Cornu Ammonis area 1 (CA1) and the frontal and occipital cortices (Tayebati *et al.*, 2006). In Alzheimer's and Parkinson's disease patients with impaired cognitive function, the expression of muscarinic receptors was found to be decreased in the brain (Rodriguez-Puertas *et al.*, 1997; Araki *et al.*, 2000).

Statins, hydroxymethylglutaryl-coenzyme (HMG-CoA) reductase inhibitors, were normally recognized to lower plasma cholesterol. In addition to lowering cholesterol, statins have gradually been used to reduce the risk of heart attack, cerebral ischemia, and have been demonstrated to exert potential beneficial effects in different neurological diseases (Wang *et al.*, 2007; Keener & Sanossian, 2008; van der Most *et al.*, 2009; Sugawara *et al.*, 2011; Ayer *et al.*, 2013). Although the precise

mechanisms are unclear and contrasting results have been observed (Reiss & Wirkowski, 2007; Becker *et al.*, 2008), additional studies have found that statins display important effects on cognitive related neurological diseases such as PD (Wu *et al.*, 2008; van der Most *et al.*, 2009; Wang *et al.*, 2011; Lee *et al.*, 2013; Xu *et al.*, 2013). If statins would have a potentially useful impact on impaired memory regulation in the progression of PD, this presents an interesting challenge. Besides characterized by typical disturbances of dopaminergic system, PD patients also display widely unbalances in other systems including cholinergic system (Gubellini *et al.*, 2004; Lester *et al.*, 2010; Xu *et al.*, 2012). It has been indicated that the interruption of dopaminergic system may influence the cholinergic system in PD (Andersson *et al.*, 2010; Lester *et al.*, 2010). Our studies and Selley (Selley, 2005; Wang *et al.*, 2005) have shown that simvastatin regulated dopaminergic systems including receptors and transmission in the brain. Also, our study found the alterations of NMDA and M1/4 receptors in the brains following simvastatin treatment (Wang *et al.*, 2008; Wang *et al.*, 2009b; Yan *et al.*, 2011). Whether the application of simvastatin in Parkinsonian rats also influences the expression of M1 receptors raises an interesting question.

Based on the information above, we propose that simvastatin may reverse the decline in M1/4 receptors in memory-related regions of the brain in 6-OHDA-induced PD rats. To test this hypothesis, [3H]pirenzepine binding autoradiography was applied to verify the reactions of muscarinic receptors M1/4 to 3-week treatment of simvastatin in the 6-OHDA-induced PD rat model. Besides, we also investigated the impairment of memory in this PD rat model, as well as the influence of simvastatin on declined

memory in the PD rat model. The current study provides a possible association of simvastatin treatment with M1/4 receptors in the Parkinsonian rat brain.

Materials & Methods

Animals and drug treatments

Twenty-two Sprague-Dawley rats (male, 230-250 g/rat) were purchased from the Animal Resources Centre (Perth, Western Australia, Australia) and kept in specifically environmental conditions as previous description (Li *et al.*, 2010). Rats were housed for one week to be accustomed to the new environment before experiments start. The rats were randomly assigned to groups with a total of sixteen rats for the 6-OHDA lesioned group, among which eight of the rats were administered with simvastatin (10 mg/kg/day) orally and the rest rats were orally administrated with saline. One 6-OHDA lesioned rat receiving saline died after surgery. In the control group, six rats received 0.9% saline for three-week oral administration as the vehicle control. After three weeks of 6-OHDA lesion, all rats were sacrificed to examine M1/4 receptor binding. This study was approved by the local Animal Ethics Committee, and followed the instructions in compliance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23)* revised 1996 guidelines and National Health and Medical Research Council (NHMRC) *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2004).*

6-OHDA lesioned Parkinsonian rats

The 6-OHDA lesioned rat was created as described in Yan's work (Yan *et al.*, 2011). Briefly, the rats were anesthetized with 75 mg/kg ketamine and 10 mg/kg xylazine

(Troy laboratories Pty, Ltd., Australia). 4 µl 6-OHDA was diluted in the normal saline (8 µg/µl in normal saline containing 0.2 mg/ml ascorbic acid, Sigma-Aldrich, St. Louis, MO) were unilaterally injected into the medial forebrain bundle (0.8 µl/min; at AP −4.4 mm, ML −1.4 mm and DV −7.8 mm, from Bregma (Paxinos & Watson, 1997). The control group were orally administrated with the 0.9% saline vehicle, and received the 0.9% saline injection into the MFB. These animal procedures were conducted according to the protocols set up by the University of Wollongong, adapted from Howard-Jones (Howard-Jones, 1985).

Morris water maze (MWM)

Three weeks after the 6-OHDA lesion was performed, rats were tested in the MWM to assess the level of long-term memory. The MWM was described in previous studies (Andringa *et al.*, 2000; Wisman *et al.*, 2008). Briefly, the pool dimensions were a diameter of 185 cm and a water depth of 30 cm, and the water temperature was 21° Celsius. A hard plastic circular cover that fits inside the wall of pool was used as a protective sheath so that the rats would not scratch the rubber pool material. Four starting positions were conventionally indicating east, south, west, and north, which divided the pool into 4 quadrants. A target platform (10 x 10 cm) was placed midway between the center and the western point. The platform consisted of transparent plexiglass and was submerged 1.5 cm beneath the surface of the water. Rats were tested 2 days in succession, with two training trials conducted in the first day for the learning phase. On the second day, the test trial was performed to test the long-term memory. The distance that the rat travelled and the time that the rat took to reach the

platform was recorded by a camera placed 2 meters above the water maze. Distance and duration was traced and calculated using Ethovision Color-Pro software (Nldus information Technology, Wageningen, The Netherlands).

Tyrosine hydroxylase immunohistochemistry staining

After the MWM behavioral test, the rats were used for tyrosine hydroxylase (TH) staining. TH staining was performed as described in Yuan's study (Yuan et al., 2005; Yan *et al.*, 2011). Briefly, distilled water was used to wash the brain sections for three times (5 min/per time), and the sections were immerged in citrate buffer for 20 minutes. 1.5% goat serum was used to block the non-specific binding (Vectastain rabbit IgG ABC kit). The sections were incubated with TH primary antibody (rabbit polyclonal anti-tyrosine hydroxylase, Millipore Corporation, AB152) overnight at 4 °C. On the following day, the sections were incubated with the secondary antibody for 60 min followed by PBS wash. After ABC (Vectastain rabbit IgG ABC kit) and DAB kits were applied (DAB, Vector SK-4100), the sections were determined by light microscopy. The TH staining density was detected by the NIH ImageJ Analysis software.

Histology and [3H]pirenzepine binding autoradiography

CO2 asphyxiation was used to kill the rats. Microscope slides (Polysine™, Menzel GmbH & Co KG) were mounted with the brain sections cut at 14 μm thickness (Li *et al.*, 2010; Yan *et al.*, 2011). Rat brain atlas was used to identify standard brain structures (Paxinos & Watson, 1997). [3H]Pirenzepine autoradiography was performed to detect the M1/4 receptor binding (Newell, 2007; Wang *et al.*, 2008). Briefly, brain sections were incubated in HEPES (pH 7.5) for 15 minutes, and then in the same buffer but adding 10 nM [3H]pirenzipine (specific activity 86 Ci/mmol; Perkin Elmer, Norwalk, CT) for 90 minutes. Nonspecific binding was identified with [3H]pirenzepine in the presence of 10 μM atropine.

Quantification and Statistical Analysis

Beta Imager (BioSpace, Paris, France) was applied to quantify the M1/4 receptor binding sites as described in our previous studies (Wang *et al.*, 2009b; Yan *et al.*, 2011). Briefly, brain sections were put in the chamber of Beta Imager followed by 3.5 h scanning. By quantifying the _-particles emitting from the brain sections, the levels of radioactivity in the tissue were directly determined; while the Beta Vision Plus program (BioSpace) was used to measure the activities in the interested regions. The radioligand binding signal was expressed in counts per minute per square millimeter (cpm/mm2). The radioligand binding signals was converted to nCi/mg tissue equivalents. Three to five adjacent brain sections were used to quantify the average density of M1/4 receptor by measuring [3H]pirenzepine binding in various brain regions. Data were expressed as the mean ± SEM. [3H]Pirenzepine binding densities for each brain region, and the behavioral data from the MWM were analysed using a one-way ANOVA followed by a post hoc Dunnett-T test with the SPSS 15.0 program (Chicago, IL). A Student's t-test was employed to analyze the

data from the TH immunohistochemistry staining in Cpu. P values of less than 0.05 were regarded as statistically significant.

Results

TH immunostaining in the striatum

In the Striatum: TH immunoreactivity showed that the lesion by 6-OHDA into the MFB induced an extent nigrostriatal denervation in the striatum (Fig. 1). Fig. 1B showed a large absence of TH immunoreactive fibers, and the average density of TH immunoreactivity was expressed as a mean percentage ± S.E.M. The density of TH-positive fibers in the lesioned side was reduced around 80% when compared to the controls (Student *t*-test: t=18.551, *** p< 0.001, Fig. 1C). In the 6-OHDA lesioned rat, the TH density in the intact side of striatum was the same as that of control group (Fig. 1A).

Effects of 6-OHDA lesion and simvastatin treatment on long-term memory

One-way ANOVA demonstrated that there was a significant effect of drug treatment on the total distance traveled in the MWM ($F[2,18] = 4.340$, p=0.029, Fig. 2A). Compared to the controls, 6-OHDA caused a significant increase in the total distance (235% of controls, p<0.05) (Fig. 2A). When compared to the 6-OHDA treated group, simvastatin significantly prevented this increase in the total distance (41.4% of 6 OHDA group, p<0.05). In addition, one-way ANOVA demonstrated that there was a significant effect of drug treatment on the ratio of the duration in the west arena to the total duration ($F[2,18] = 4.353$, p=0.029). A post hoc test indicated that 6-OHDA-lesion PD rats showed a significant decrease (27%, p<0.05) in the ratio of the duration in the west arena to the total duration compared to controls (Fig. 2B). In

comparison to the 6-OHDA-lesion PD rats, simvastatin oral administration significantly prevented this behavioral deficit in which simvastatin treated PD-rats had a significantly longer duration in the west arena (36%, $p<0.05$, Fig. 2B).

Effects of 6-OHDA lesion and simvastatin treatment on [3H]pirenzepine bindingin the brain regions

[3H]pirenzepine binding sites can be found in most examined areas (Fig. 3). One way ANOVA revealed significant changes in [3H]pirenzepine binding sites in some examined brain regions such as hippocampus, the medial amygdala, the posteromedial cortical amygdale, and the piriform cortex among the 6-OHDA induced PD rats. Specifically, a Tukey post-hoc analysis showed that [3H]pirenzepine binding in 6-OHDA-lesioned rats was profoundly reduced in the hippocampus (9.34%, $p<0.01$), the medial amygdala (11.91%, $p<0.01$), the posteromedial cortical amygdala (12%, $p<0.05$) and the piriform cortex (13.60%, $p<0.01$) compared to the controls (Figs 4 and 5). However, after three-weeks of administration of simvastatin, the down-regulation of [3H]pirenzepine binding sites in these examined regions were reversed. Specifically, simvastatin significantly increased the [3H]pirenzepine binding density in the hippocampus (11.4%, $p<0.01$), the medial amygdala (11.38%, $p<0.05$), the posteromedial cortical amygdala (12.45%, $p=0.064$) and the piriform cortex (15.23%, $p<0.001$) in comparison to the 6-OHDA MFB lesioned PD rats (Figs 4 and 5). However, compared to the controls, no significant changes in [3H]pirenzepine binding were observed in the substantia nigra of the 6-OHDA and simvastatin treated group. In the caudate putamen (11.07%, $p=0.001$), [3H]pirenzepine binding was significantly increased in the simvastatin treated group in comparison to the 6-OHDA and control groups.

Correlations between duration in west arena of MWM and [3H]pirenzepine binding density

A **moderate** positive correlation was observed between the [3H]pirenzepine binding density in the hippocampus and the duration in the west arena of the water maze (r=0.491 Pearson's correlation, p=0.024) in the MWM test (Fig 6A). There were also significant correlations between the [3H]pirenzepine binding density in the medial amygdala (r=0.636, p=0.002) with the duration in the west arena of the water maze (Fig 6B) in the MWM test. However, no significant correlation was observed between [3H]pirenzepine binding density in the posteromedial cortical amygdala (r=0.148, p=0.520) or the piriform cortex (r=0.315, p=0.164) and the duration in the west arena of the MWM.

Discussion

The current study was to explore the changes of [3H]pirenzepine binding density in 6-OHDA lesioned rat brain regions and its association with memory activity after chronic treatment of simvastatin. Although [3H]pirenzepine possess the higher concentration, [3H]pirenzepine binds largely to the M1 receptor subtype (Dean *et al.*, 1999). The present study used 10 nM [3H]pirenzepine, a concentration that was reported to label approximately 65% of M1 receptors and 18.5% of M4 receptors (Flynn & Mash, 1993). The current study showed that compared with control group, the 6-OHDA lesion significantly decreased M1/4 receptor binding in some brain areas such as the hippocampus, the medial amygdala, the posteromedial cortical amygdala and the piriform cortex.

In this study, the profoundly reduced TH immunostaining in the striatum of the 6-OHDA-lesioned side indicated a complete nerve terminal denervation and successful 6-OHDA induced PD rat model establishment. In the MWM, 6-OHDA lesioned rats travelled a longer distance and used more time to reach to the target platform compared to the controls (Fig 2), reflecting the effects of the 6-OHDA lesions, which induce a decline in long-term memory. Our result is similar to other studies, which indicated that 6-OHDA or MPTP lesioned PD rodents showed severely impaired long-term memory activity (Da Cunha *et al.*, 2002; Yang *et al.*, 2004). Clinical studies showed that before the motor feature deficits showed up, PD patients usually present nonmotor dysfunctions, especially, the neuropsychiatric dysfunctions, among which memory impairment is a typically characteristic; in this case, the prevalence rates were up to 67% in the PD population (Emre *et al.*, 2007). For example, in some cross-sectional clinical cohort studies, it has been shown that PD patients exhibited memory deterioration in the early stage and gradually developed progressive memory decline; while this memory impairment was improved by different medical interventions (Uc *et al.*, 2009; Wang *et al.*, 2009a; Caviness *et al.*, 2011), the precise mechanisms are not yet clear. Therefore, it is urgently to investigate the possible mechanisms on the memory impairment in PD; this study directly implies this cognitive profile in clinical PD patients.

Our study showed that a 6-OHDA lesion in the MFB induced a reduction of M1/4 receptor binding in the observed brain regions (Figs 4 and 5). This finding is consistent with other studies demonstrating that M1/4 receptors were decreased in the brain following 6-OHDA induced dopaminergic depletion (Joyce, 1991a; b). The precise reasons for the reduced M1/4 receptor following a 6-OHDA lesion are not yet

known. However, it has been documented that dopaminergic denervation in the striatal pathway resulted in hyperactivity of the cholinergic systems (Muma *et al.*, 2001; Zhang *et al.*, 2008). Therefore, we hypothesize that the possible mechanisms for the reduced M1/4 receptors involved in our study is due to enhanced striatal cholinergic neuron firing following dopamine denervation in the nigrostriatal pathway; however, we cannot exclude the possibility that the reduced M1/4 receptors in the observed brain areas may reflect the receptor hypo-innervations. However, the precisemechanisms behind it remain to be determined.

It has been well documented that the changes of M1/4 receptors in the some brain regions are closely correlated with the memory activity. A recent study by Anagnostaras et al. indicated that through Olton win-shift radial arm maze and morris water maze studies, M1 receptor knockout mice showed dramatic forgetfulness and impaired working and contextual memory, implying that the function of the M1 receptor directly contributes to memory processing (Anagnostaras *et al.*, 2003). By inhibiting M1 receptors using its antagonist pirenzipine, the long-term potentiation (LTP) induction that serves as a cellular mechanism for memory was abolished, strongly suggesting that M1 receptors mediate memory and learning function (Doralp & Leung, 2008). Although some early studies showed no significant decrease in M1 receptor expression in the brains of declined memory related disorders (Young & Penney, 1994), more recent studies showed that M1 receptor levels were significantly declined in some important brain regions, such as the cortex and the hippocampus, among the many memory impairment related disorders (Parasi *et al.*, 2007). Further, the declined memory in aged rats can be improved by elevating the expression of M1 receptors in memory related brain

regions, such as the hippocampus and the cortex (Hu *et al.*, 2005; Hu *et al.*, 2008). This study showed that a profound decrease in the M1/4 receptor binding was observed in the hippocampus, the medial amygdala, the posteromedial cortical, and the piriform cortex of the 6-OHDA lesioned side. This robust decrease of M1/4 receptor binding in the hippocampus and medial amygdala, of 6-OHDA lesioned rats was found to have significant positive correlations with the duration in the west arena of the water maze in the MWM test (Figs 6A and 6B). These findings strongly suggest that the M1/4 receptor hypofunction in the hippocampus and medial amygdale may explain the declined memory in the PD rats. This hypothesis is supported by the fact that the changes in the M1/4 receptors in the examined regions directly regulate the memory behaviors.

Similar to previous reports (Rezvani *et al.*, 2008), 6-OHDA lesioned rats in the current study showed deficits in long-term memory as indicated by the longer total distance travelled to reach the platform and the shorter duration in the west arena of the water maze. It is important that simvastatin treatment significantly increased the reduced total distance travelled in the MWM and the duration of the 6-OHDA-induced PD rats in the west arena of the water maze (Fig 2), demonstrating the ability of simvastatin to produce a profound improvement in impaired memory effects in the 6-OHDA lesioned PD rats. Consistent with our results, through behavioral tests, Chopp's team and others also found that simvastatin substantially improved deficit spatial learning and memory in an established mouse model of AD and rats subjected to traumatic brain injury (Li *et al.*, 2006; Lu *et al.*, 2007; Wu *et al.*, 2008). An increasing number of clinical studies have indicated close association between statins and improvement in declined memory in the elderly population. In a

follow-up cohort investigation, Solomon et al. demonstrated that subjects with pronounced impairment in episodic memory benefited from statins usage, implying that statins exert useful impacts on memory deficits in the elderly (Solomon *et al.*, 2009). Several prospective studies showed that poorer memory performance was significantly ameliorated in Parkinson's disease following statin therapy (Wahner *et al.*, 2008; Mutez *et al.*, 2009). However, the means by which statins affect memory and several possible mechanisms underlying memory regulation are under exploration. The current study showed that the reduced [3H]pirenzepine binding sites in the observed brain regions was reversed after chronic treatment of simvastatin, which is consistent with our previous study showing an increase of M1/4 receptors in the rat brain following simvastatin treatment (Wang *et al.*, 2008). This result indicated that the increase of M1/4 receptor binding in the hippocampus and the medial amygdala of 6-OHDA lesioned rats after chronic treatment of simvastatin was identified to have significant positive correlations with the duration in the west arena of the MWM (Fig 6). This finding implies that simvastatin ameliorated the declined memory in 6-OHDA lesioned rats via M1/4 receptor modulation.

In addition to the in vivo and in vitro studies, recent clinical studies indicated that some types of statins may be associated with a reduction in PD risk. A population-based study by Lee et al., indicated that the continuation of lipophilic statin, such as atorvastatin and simvastatin, therapy was associated with a decreased incidence of PD (Lee *et al.*, 2013). During 12 years of follow-up (1994-2006) study, Gao et al., found that the regular statin medication was correlated to a reduction in PD risk (Gao *et al.*, 2012). Similar clinical meta analysis and review on the association of statin therapy with the decreased risk of PD was also obtained in other studies (van der

Most *et al.*, 2009; Dolga *et al.*, 2011; Noyce *et al.*, 2012). Taken together, clinical and epidemiological investigations provided strong evidence that statin therapy may be used to slow down the progression of PD patients. In conclusion, the current study provides the solid evidence indicating the influence of simvastatin on M1/4 receptor binding in the PD rat brain. It also reveals possible M1/4 receptor modulation effects, which indicates an interesting and potential paradigm to ameliorate memory deficits. According to our results, we reasonably speculate that the improvement in long-term memory performance due to simvastatin administration in 6-OHDA-induced rats is, at least partially, associated with a reversal of the declined M1/4 receptor density. This work presents a possible mechanism for the amelioration of memory deficits in PD by simvastatin, suggesting that it is mediated by M1/4 receptor modulation. Although the rat brain resembles the human brain to a certain degree, it does not mean we can directly translate the results from animal data to humans. However, this current animal study could provide pre-clinical evidence for clinical studies on PD. A better understanding of the roles and correlations between statins and dopaminergic and muscarinic systems may provide new perspectives on the role of statins in regulating neurodegenerative diseases such as PD and AD.

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Potential Conflicts of Interest Nothing to report

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Figure legends

Fig 1. Effects of 6-OHDA lesion on TH immunohistochemistry staining in the caudate putamen. Photo (A) represents the striatum in the control group in which the TH immunoreactive fibers are the same as in the intact side. The right side of the photo (B) shows the dopamine-depleted striatum side, which is characterized by a significant lack of TH immunoreactive fibers, while the left side is the intact side. (C) represents the average density of TH-positive fibers for the lesioned side and the contralateral (intact) side (Student's t-test: t= 18.551, ***P < 0.001).

Fig 2. Effects of simvastatin on the long-term memory activity of control, 6-OHDA-lesioned, and 6-OHDA-lesioned with simvastatin administration rats in the MWM test. The graph shows the total distance travelled and the ratio of the duration in the west arena to the total duration in the MWM. The parameters are expressed as the total distance (A) and the percentage of time spent in the west zone to the total time (B) in the MWM. (A) represents the mean ±SEM, n=6-8. *p<0.05, 6-OHDA group versus control group; **p < 0.01, 6-OHDA + saline group versus 6-OHDA + simvastatin group. (B) represents the mean ±SEM, n=6-8. *p<0.05, 6-OHDA group versus control group; # p < 0.05, 6-OHDA + saline group versus 6-OHDA + simvastatin group.

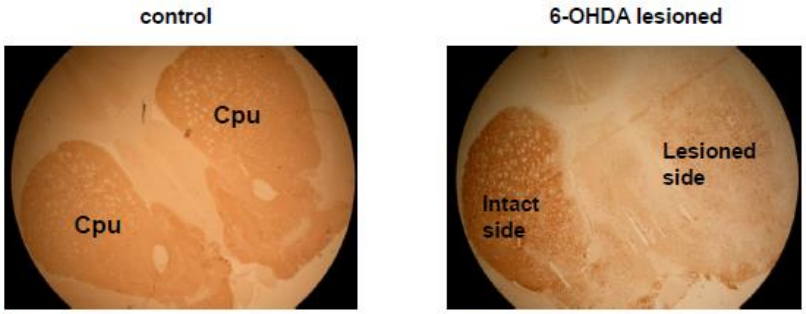
Fig 3 The maps of A, B and C are adapted from a rat brain atlas (Paxinos & Watson, 1997) indicating the levels where the [3H]pirenzepine binding density was measured. Autoradiographs (D, E, F) and (D', E', F') depict the expression of [3H]pirenzepine

binding and non-specific [3H]pirenzepine binding, respectively, at different rostro-caudal coronal levels of the rat brain.

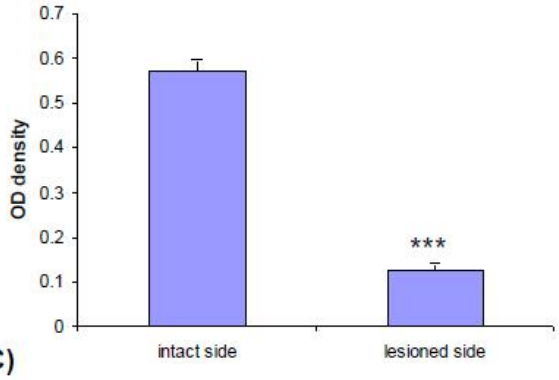
Fig 4 Typical autoradiographs depict the expression of M1 receptors in the hippocampus (Hippo) and amygdale (Amy) among the control rats, the 6-OHDA lesioned rats and the 6-OHDA lesioned rats with simvastatin treatment.

Fig 5 The effects of chronic simvastatin treatment on [3H]pirenzepine binding in the different groups of rat brain regions. *Note*: Units of measurement are in nCi/mg tissue. Data are represented as the mean ± SEM. Asterisks indicate significant differences from the control group (saline), and the cross indicates significant differences between the 6-OHDA rats and the 6-OHDA with simvastatin treated rats (*$p<0.05$; †$p<0.05$; ††$p<0.01$; one-way ANOVA followed by Tukey's test).

Fig 6 Correlations between the duration in the west arena of the MWM and [3H]pirenzipine binding density in brain regions. A significant positive correlation was identified between the [3H]pirenzepine binding density in the hippocampus ($r=0.491$ Pearson's correlation, $p=0.024$, Fig A), amygdala ($r=0.636$, $p=0.002$, Fig B), and the duration in the west arena of the MWM test. However, no significant correlation was observed between [3H]pirenzepine binding density in the posteromedial cortical amygdala ($r=0.148$, $p=0.520$), the piriform cortex ($r=0.315$, $p=0.164$), and the duration in the west arena of the MWM.


control
6-OHDA lesioned
Cpu
Cpu
Lesioned side
Intact side

(A) (B)


0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
OD density
***
intact side
lesioned side

(C)

Fig 1

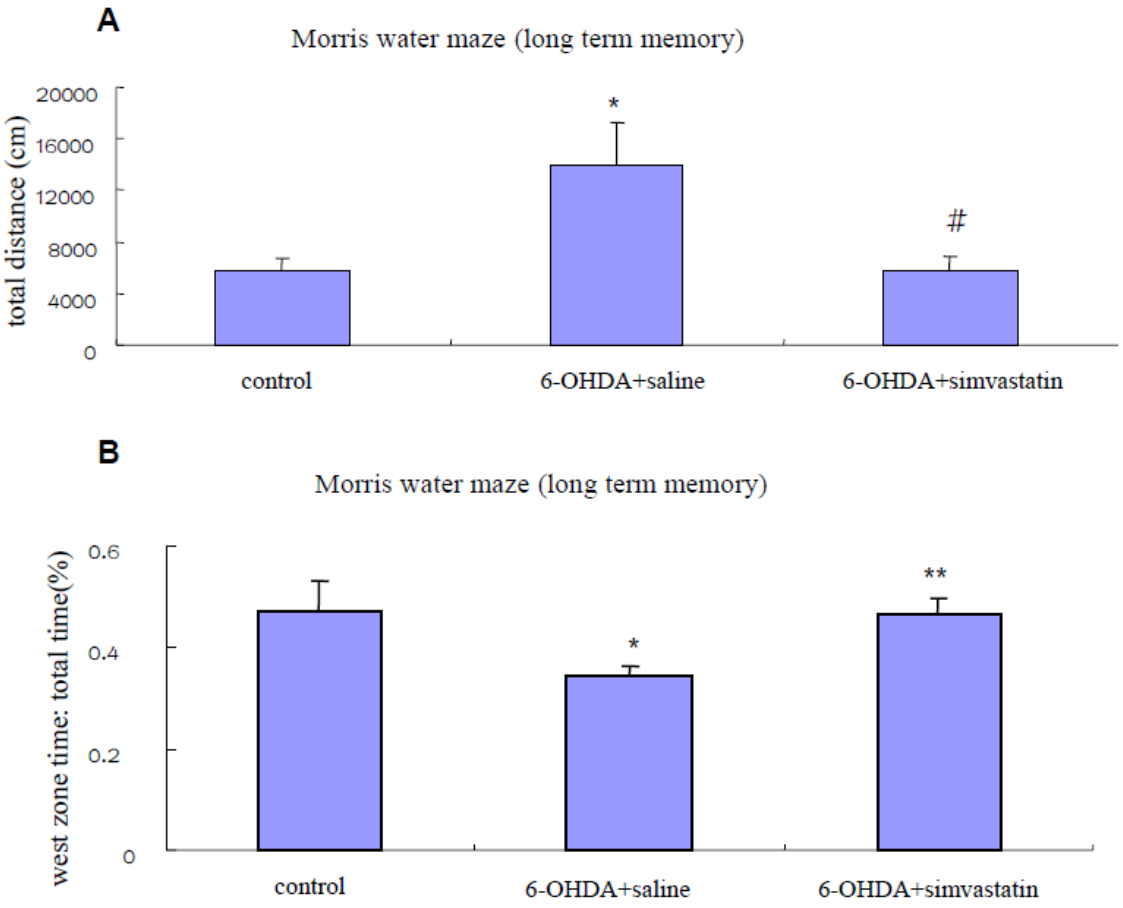

A
Morris water maze (long term memory)
total distance (cm)
20000
16000
12000
8000
4000
0
*
#
control
6-OHDA+saline
6-OHDA+simvastatin
B
Morris water maze (long term memory)
west zone time: total time(%)
0.6
0.4
0.2
0
*
**
control
6-OHDA+saline
6-OHDA+simvastatin

Fig 2

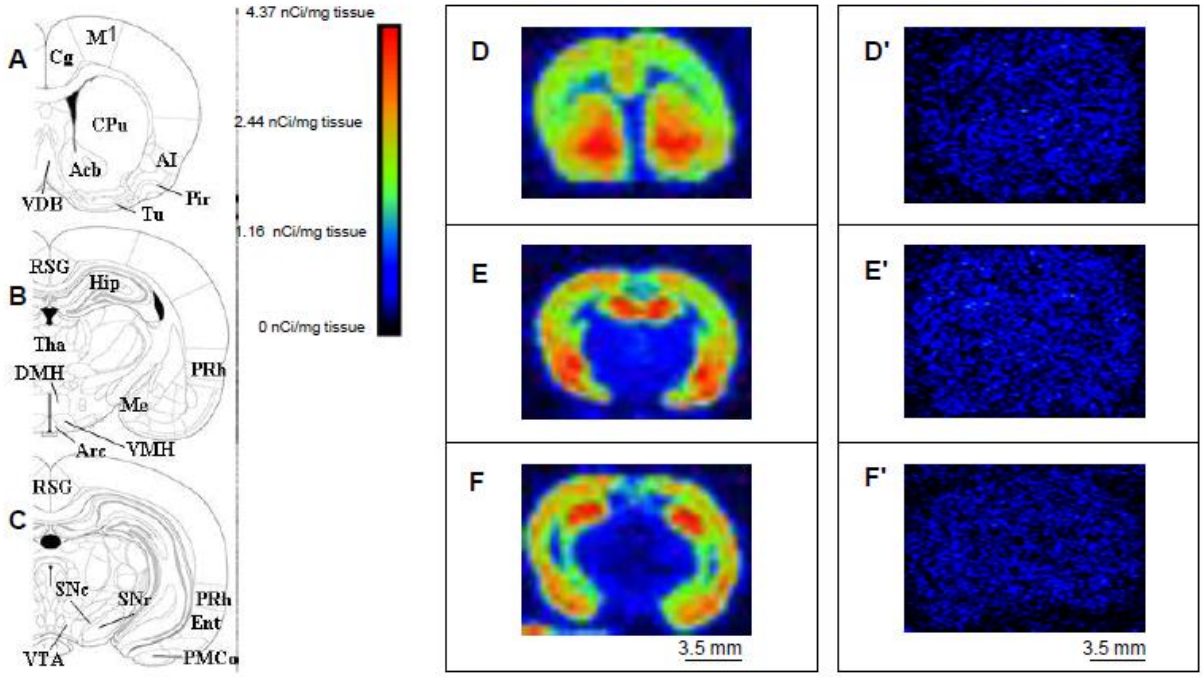

A
Cg
M1
CPu
Acb
AI
VDB
Tu
Pir
B
RSG
Hip
Tha
DMH
PRh
Me
Arc
VMH
C
RSG
SNc
SNr
PRh
Ent
VTA
PMCo
4.37 nCi/mg tissue
2.44 nCi/mg tissue
1.16 nCi/mg tissue
0 nCi/mg tissue
D
D'
E
E'
F
F'
3.5 mm
3.5 mm

Fig 3

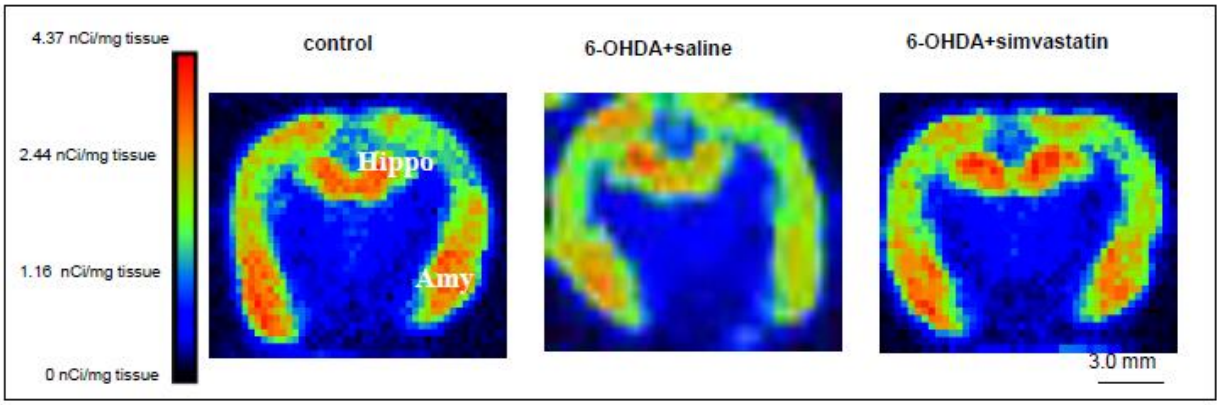

4.37 nCi/mg tissue
2.44 nCi/mg tissue
1.16 nCi/mg tissue
0 nCi/mg tissue
control
6-OHDA+saline
6-OHDA+simvastatin
Hippo
Amy
3.0 mm

Fig 4

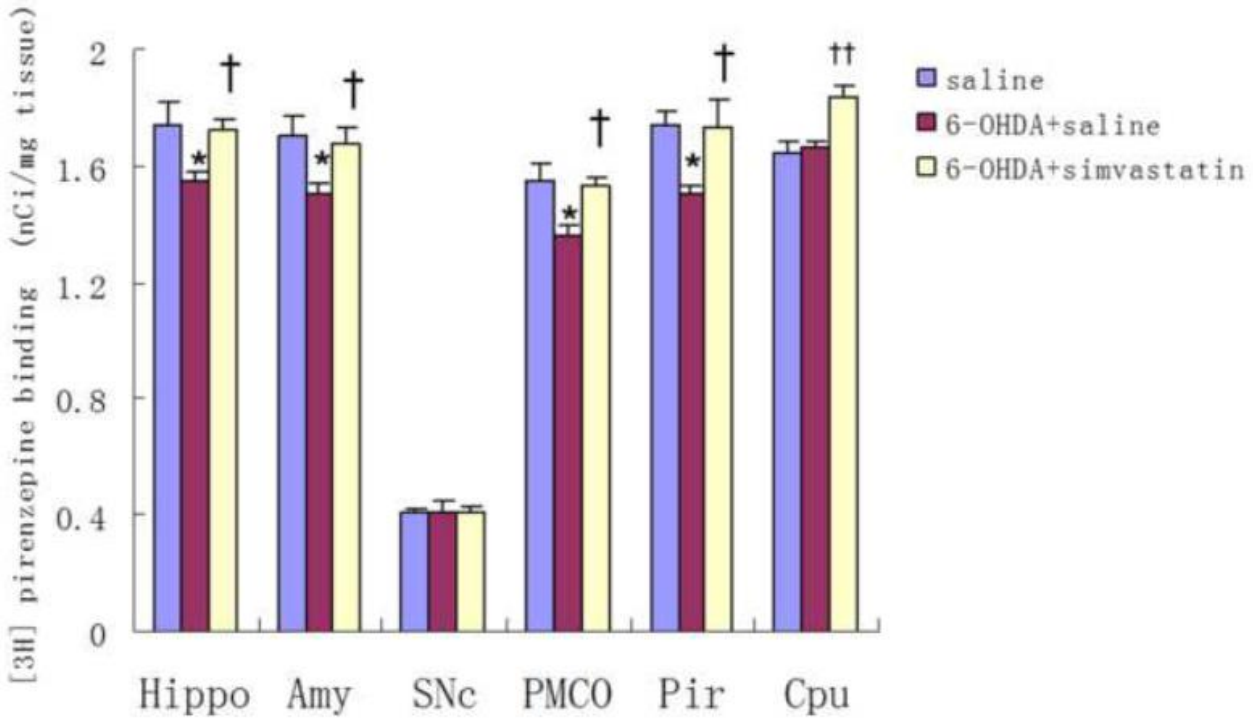

[3H] pirenzepine binding (nCi/mg tissue)
2
1.6
1.2
0.8
0.4
0
Hippo
Amy
SNc
PMCO
Pir
Cpu
saline
6-OHDA+saline
6-OHDA+simvastatin

Fig. 5

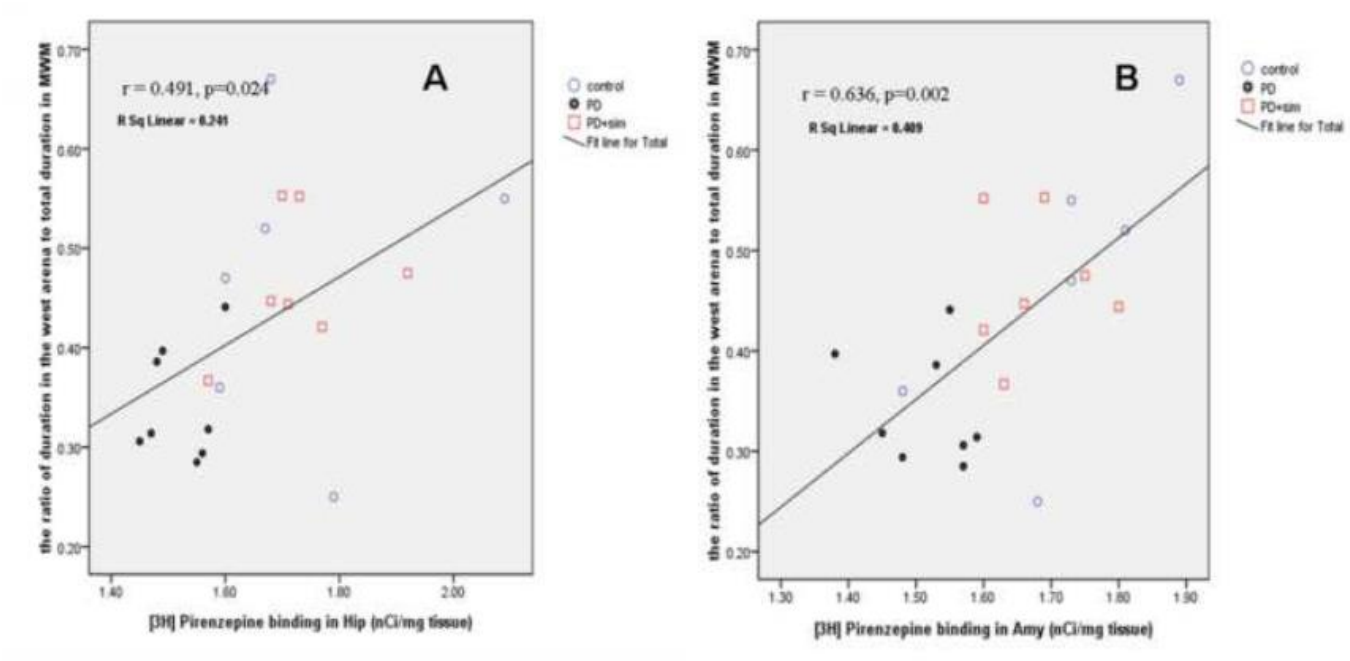

A
r = 0.491, p=0.024
R Sq Linear = 0.241
the ratio of duration in the west arena to total duration in MWM
[3H] Pirenzepine binding in Hip (nCi/mg tissue)
control
PD
PD+sim
Fit line for Total
B
r = 0.636, p=0.002
R Sq Linear = 0.409
the ratio of duration in the west arena to total duration in MWM
[3H] Pirenzepine binding in Amy (nCi/mg tissue)
control
PD
PD+sim
Fit line for Total

Fig.6