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RESEARCH ARTICLE

No synergism between bis(propyl)-cognitin and rasagiline on protecting dopaminergic neurons in Parkinson's disease mice

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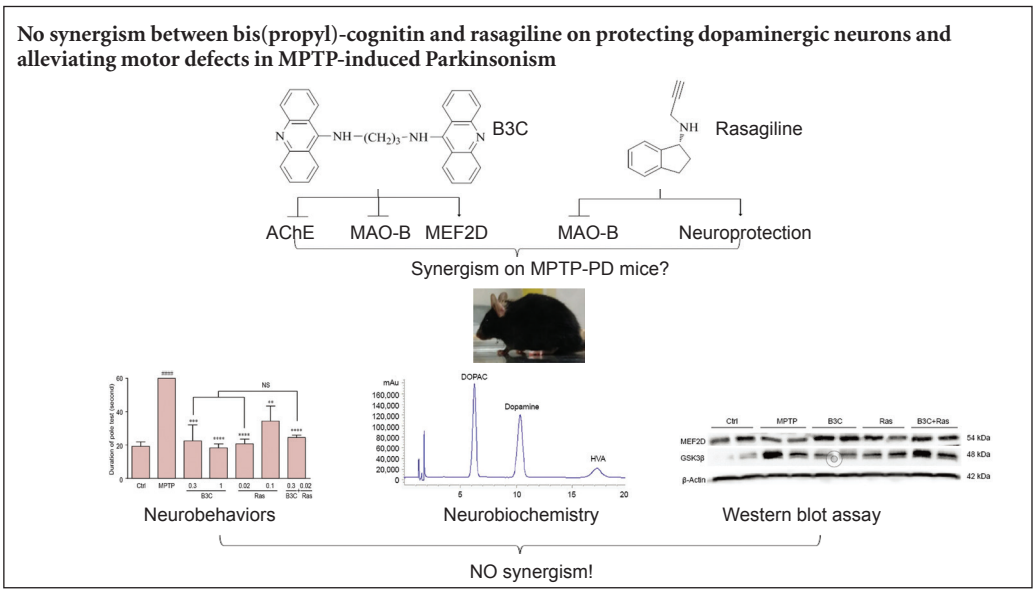
Graphical Abstract



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Abstract

Rasagiline, a monoamine oxidase-B inhibitor, and bis(propyl)-cognitin (B3C), a novel dimer are reported to be neuroprotective. Herein, the synergistical neuroprotection produced by rasagiline and B3C was investigated in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mice of Parkinsonism. By using neurobehavioural tests, high-performance liquid chromatography and western blot assay, we showed that B3C at 0.3 mg/kg, rasagiline at 0.02 mg/kg, as well as co-treatment with B3C and rasagiline prevented MPTP-induced behavioural abnormities, increased the concentrations of dopamine and its metabolites in the striatum, and up-regulated the expression of tyrosine hydroxylase in the substantia nigra. However, the neuroprotective effects of co-treatment were not significantly improved when compared with those of B3C or rasagiline alone. Collectively, we have demonstrated that B3C at 0.3 mg/kg and rasagline at 0.02 mg/kg could not produce synergistic neuroprotective effects.

Key Words: *nerve regeneration; Parkinson's disease; bis(propyl)-cognitin; rasagiline; monoamine oxidase B; dopamine; multitarget; synergism; neuroprotection; neural regeneration*

Introduction

Parkinson's disease (PD), the second most common neurodegenerative disorder, has emerged as one of the major public health problems worldwide (Jeanjean and Aubert, 2011). Unfortunately, the exact molecular pathology of PD remains to be elucidated. Currently used medications such as dopamine precursor (levodopa), dopamine agonists and monoamine oxidase-B (MAO-B) inhibitors (selegiline and rasagiline), are reported to have modest symptomatic benefits without obvious disease-modifying potential, because their primary target is not dopaminergic neuronal loss (Smith, 2010; Meissner et al., 2011). The ideal PD therapy aims to produce neuroprotective effects, concurrently relieve PD-associated symptoms and delay the loss of dopaminergic neurons in the substantia nigra (Schapira, 2004).

Rasagiline (**Figure 1**), a second-generation MAO-B inhibitor, has been approved by US Food and Drug Administration (FDA) for the treatment of PD (Degli Esposti et al., 2015). Rasagiline is reported to produce neuroprotective effects in various experimental models both *in vitro* and *in vivo* (Naoi et al., 2013). Interestingly, the neuroprotective effects of rasagiline might be associated with its anti-apoptotic activities rather than its MAO-B inhibition property (Youdim et al., 2001). However, the request for an on-label indication of rasagiline for neuroprotection in PD has been repeatedly denied by FDA, because there is limited evidence to prove its neuroprotective effects in clinical trials (Ahlskog and Uitti, 2010).

Bis(propyl)-cognitin (B3C; **Figure 1**), in which two tacrine moieties were linked by three methylene (-CH_2-) group, was originally synthesized as a novel acetylcholinesterase (AChE) inhibitor (Carlier et al., 1999). We have previously demonstrated the neuroprotective effects of B3C in various *in vivo* models of neurodegenerative disorders (Luo et al., 2010; Han et al., 2012). B3C was also reported to protect against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neuronal loss and Parkinsonian motor defects in mice (Yao et al., 2012). The neuroprotective effects of B3C are reported to be associated with its AChE inhibition (Carlier et al., 1999), myocyte enhancer factor-2D (MEF2D) enhancement (Yao et al., 2012), as well as uncompetitive N-methyl-D-aspartate receptor antagonism (Luo et al., 2010).

Considering the multi-factorial etiological nature of PD (Calabresi and Di Filippo, 2015), multiple drug therapy might offer a new hope by addressing complex pathological aspects. The combination of drug molecules with different modes of action could concurrently act on multiple targets and/or biological processes which cause the chronic and progressive neurodegeneration in PD (Reznichenko et al., 2010; Zhang et al., 2015). In the light of this rationale, we investigated whether the post-MPTP loss of dopaminergic neurons could be additively/synergistically restored by a combination of rasagiline and B3C. We also examined whether MPTP-induced behavioral abnormities and biochemcial changes could be reversed by these compounds.

Materials and Methods

Ethics statement

Animal treatment and maintenance were carried out in accordance with the guidelines established by the National Institutes of Health for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Jinan University in China (Ethical approval No. EAE-JNU-2013-0117). Precautions were taken to minimize suffering and the number of animals used in the study.

Treatments

Fifty-six specific-pathogen-free male C57BL/6 mice (25 ± 2 g and 7–8 weeks old) were purchased from the Animal Center of Guangdong Province in China (Certification No. SCXK (Yue)-2013-0002). Mice were housed under a 12-hour light/dark cycle, and allowed to acclimate for 7 days before treatment. Mice were randomly divided into seven groups (control, MPTP, MPTP + 0.3 mg/kg B3C, MPTP + 1 mg/kg B3C, MPTP + 0.02 mg/kg rasagiline, MPTP + 0.1 mg/kg rasagiline, and MPTP + 0.3 mg/kg B3C + 0.02 mg/kg rasagiline groups). Mice in the control group received saline (0.1 mL/10 mg). The remaining mice were given MPTP (30 mg/kg/day, Sigma-Aldrich, St. Louis., MO, USA) intraperitoneally once daily for 5 consecutive days to induce Parkinsonism. Resting period (3 days) was allowed for the conversion of MPTP to MPP^+ (Tatton and Greenwood, 1991). On day 8, B3C (0.3, 1 mg/kg), rasagiline (0.02, 0.1 mg/kg) (Sigma-Aldrich), or B3C (0.3 mg/kg) + rasagiline (0.02 mg/kg) were administered intragastrally once daily for 7 consecutive days according to the grouping. Mice in the control group or the MPTP group received equal volume of saline (0.1 mL/10 mg).

Behavioral analysis

On day 15, after final drug treatment, a serial of tests including catalepsy, pole, rotarod and foot-printing tests was used to analyze different aspects of Parkinsonism, such as hypokinetic disorder, rigidity and problem with gait (difficulty in walking). These tests were performed between 9 a.m. and 2 p.m. under normal room lighting. Behavioral experiments were randomized and blinded by an independent researcher.

The catalepsy test was performed according to a previous publication (Sedelis et al., 2001), by placing the forepaws of mice on a horizontal metal bar (2 mm in diameter), 15 cm above the tabletop. The duration until one of the hind paws caught the metal bar was recorded. The average of duration of three successive trials was measured. Between each trial, animals were allowed to rest for 1 minute.

The pole test was adapted from Ogawa et al. (1985). The pole test consisted of a 50 cm high steel pole, 0.5 cm in diameter, and wrapped with gauze to prevent slipping and the base position in the home cage. A rubber ball was glued on the top of the pole to prevent animals from sitting on the top and to help position the animals on the pole (by sliding the forepaws over the ball and holding the animal by the tail). The time that animals required to climb down the pole was measured. During pre-training as well as post-MPTP sessions, each animal was subjected to three successive trials,

with a 10-minute interval. The average time of three trials was used for statistical analyses.

Rotarod test was used to measure motor balance and coordination (Bao et al., 2012). Mice were placed on rotating rod with 3 cm diameter (Rotarod for mice, ZH-YLS-4C, Zhenghua, Anhui Province, China). Tested animals were separated by large disks. After the mice were placed on the rod at constant rotational speed of 5 r/min, the trial was started and rotational speed was automatically increased from 5 to 30 r/min within 5 minutes. The trial stopped when the mouse fell down, activating a switch that automatically stopped a timer, or when 5 minutes were completed. Mice were pre-trained on the rotarod for 3 consecutive days before MPTP treatment in order to reach a stable performance. The final test was performed in three sessions with an interval of 30 minutes. Rotarod performances in three sessions were recorded, and the average time on the rotarod was compared among groups.

For the footprint test, we followed the procedure described by Richter et al. (2007). Mice were first trained to pass straight forward through the wood corridor (5 cm wide, 85 cm long). Then mice with their forepaws with black ink colored and hindpaws with red ink colored were placed into the corridor. Their footsteps were recorded on a white absorbing paper. The duration of mice crossing the corridor was recorded, stride length and step width were also measured. Three trials were carried out and the results were averaged.

Tissue processing

After behavioral testing, animals were sacrificed by a 0.5 mL/10 g intraperitoneal injection of 10% chloral hydrate. The tissues of striatum and substantia nigra (Hayley et al., 2004; Jackson-Lewis and Przedborski, 2007) were dissected rapidly on ice and frozen in liquid nitrogen. Tissues were stored at –80°C until processed for high-pressure liquid chromatography (HPLC) or western blot assay.

Determination of dopamine and its metabolites, homovanilic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) levels by electrochemical HPLC

Striatal tissues were used for neurobiochemical analysis by electrochemical HPLC. Briefly, the striatum was weighed, and homogenized in 0.1 M perchloric acid ($HClO_4$) containing 0.01% ethylenediamine tetraacetic acid. The homogenate was centrifuged at the speed of 10,000 × *g* for 10 minutes at 4°C. The supernatant was filtered through 0.22 μm filter membrane and 20 μL samples were injected into the column. Dopamine and its metabolites (DOPAC and HVA) were analyzed using a HPLC system (Agilent-1200, Wakefield, MA, USA) coupled to a 2465 electrochemical detector (Waters, Milford, MA, USA) as described previously (Zhang et al., 2014). Concentrations of dopamine and its metabolites were expressed as ng/mg tissue.

Western blot assay

Tissues from the substantia nigra were homogenized with radioimmunoprecipitation assay lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktail (Pierce, Rockford, IL, USA) on ice. Lysis was centrifuged at 12,500 × *g* for 20 minutes at 4°C. The supernatant was separated and the amount of protein was determined using the bicinchoninic acid protein assay kit (Pierce). Protein sample (30 μg) was resolved using sodium dodecyl sulfate-polyacrylamide gel electropheresis and transferred to polyvinylidene fluoride membranes. The immunoblot was analyzed with the appropriate primary antibodies (rabbit anti-mouse antibodies against tyrosine hydroxylase (TH), MEF2D, glycogen synthase kinase-3β (GSK3β), β-actin; 1:1,000) at 4°C overnight. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:2,500) at room temperature for 2 hours were used to detect the proteins of interest through enhanced chemiluminescence. All primary and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Quantitative assessment of protein bands by densitometry was done with Gel Doc™ XRS equipped with Quantity One software (Bio-Rad, Hercules, CA, USA).

Activity of MAO-B inhibition

MAO-B activity was determined by MAO-GloTM Assay kit (Promega, Sunnyvale, CA, USA). The recombinant human MAO-B enzyme was purchased from Sigma-Aldrich. Briefly, B3C (1 μM–10 mM) and rasagiline (10 pM–10 μM) were incubated in 96-well opaque white plates with MAO substrate and recombinant human MAO-B (0.25 mg protein/mL). Reaction was started by the addition of recombinant human MAO-B. Reaction mixture was incubated for 1 hour at room temperature. Reaction was terminated by the addition of luciferin detection reagent, and sample was incubated for an additional 20 minutes to allow the development of luciferase-dependent luminescence. Relative luminescence was determined by a plate luminometer (BioTek, Winooski, VT, USA). Results were presented as the percent of vehicle (total MAO-B activity).

Statistical analysis

All data are expressed as the mean ± SEM and analyzed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). One-way analysis of variance and Dunnett's test were used to evaluate the statistical differences. A value of $P < 0.05$ was considered statistically significant.

Results

The effects of B3C and/or rasagiline on behavioral abnormities induced by MPTP in mice

MPTP injection significantly induced motor abnormalities, including postural rigidity, impaired balance and coordination and gait disorder in mice. At 15th day after MPTP injection, the latency in the catalepsy test was increased by 4.8-fold (**Figure 2A**), the time staying on the rotarod was decreased by 57.7% (**Figure 2B**), and the duration of pole test was increased by 3.1 times in the MPTP-treated mice, compared to the control mice (**Figure 2C**). In footprint test, the time taken to cross the corridor was significantly

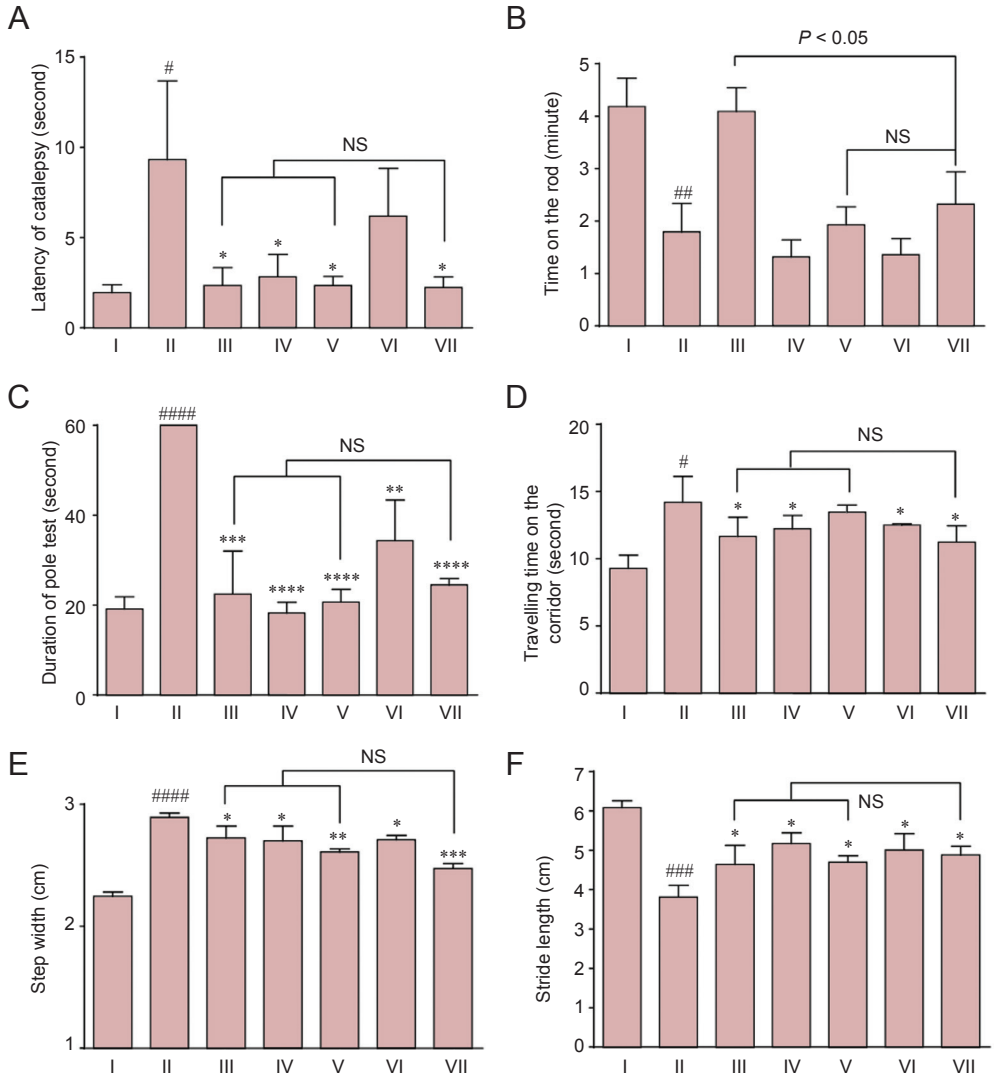

Figure 2 B3C, rasagiline and their combination alleviated MPTP-induced behaviour abnormities in mice.
At 15th day after MPTP injection, motor functions of mice were analyzed using (A) catalepsy test, (B) rotarod test, (C) pole test, and (D–F) footprinting test. In the footprinting test, (D) the travelling time on the corridor, (E) step width, and (F) stride length were recorded. All data are expressed as the mean ± SEM; $n = 8$ mice/group. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, ####$P < 0.0001$, *vs.* control group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, *vs.* MPTP group (one-way analysis of variance and Dunnett's test). Ras: Rasagilinie; S: second; NS: not significant; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. I: Control; II: MPTP; III: 0.3 mg/kg B3C; IV: 1 mg/kg B3C; V: 0.02 mg/kg Ras; VI: 0.1 mg/kg Ras; VII: 0.3 mg/kg B3C + 0.02 mg/kg Ras.

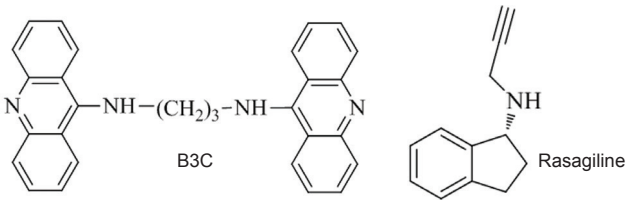

Figure 1 Chemical structures of bis(propyl)-cognitin (B3C) and rasagiline.

extended, accompanying increased step width and decreased stride length in the MPTP-treated mice, compared to the control mice (**Figure 2D–F**). B3C and rasagiline alone or their combination alleviated motor abnormities induced by MPTP ($P < 0.05$; **Figure 2**). Besides, neither rasagiline alone nor B3C+rasagiline co-treatment significantly reversed MPTP-induced behavioral abnormity in the rotarod test ($P > 0.05$; **Figure 2B**). In addition, there was no significant difference among B3C, rasagiline, and their combination on the reversion of motor abnormities in catalepsy, pole and footprint tests ($P > 0.05$).

The effects of B3C and/or rasagiline on contents of dopamine and its metabolites in the striatum of MPTP-injected mice

Representative HPLC chromatographic peaks of dopamine and its metabolites were shown in **Figure 3A**. Striatal dopamine, DOPAC and HVA were significantly reduced in

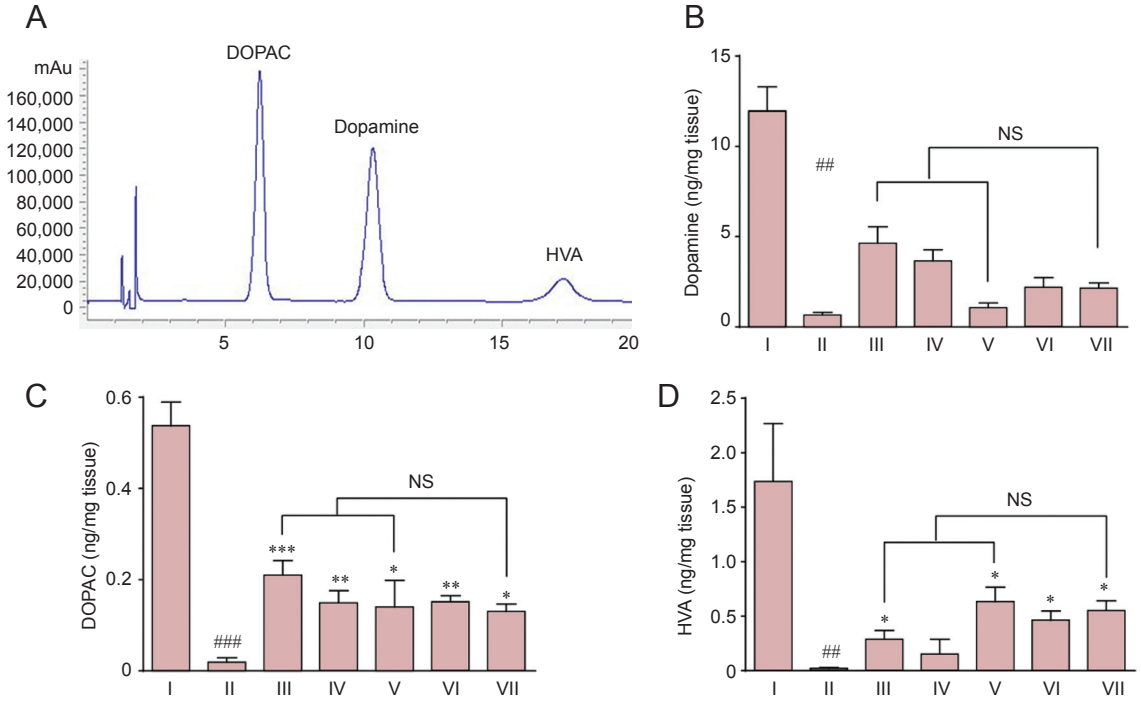

Figure 3 B3C, rasagiline and their combination reduced MPTP-induced decrease of dopamine and its metabolites in mice.
(A) Representative chromatographic profiles of dopamine, DOPAC and HVA detected by ECD-HPLC. At 15th day after MPTP injection, (B) the content of striatal dopamine, (C) the content of striatal DOPAC, and (D) the content of striatal HVA were analyzed by ECD-HPLC. All data are expressed as the mean ± SEM; $n = 8$ mice/group. ##$P < 0.01$, ###$P < 0.001$, *vs.* I; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, *vs.* II (one-way analysis of variance and Dunnett's test). Ras: Rasagilinie; NS: not significant; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; ECD-HPLC: high-pressure liquid chromatography equipped with an electrochemical detector; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. I: Control; II: MPTP; III: 0.3 mg/kg B3C; IV: 1 mg/kg B3C; V: 0.02 mg/kg Ras; VI: 0.1 mg/kg Ras; VII: 0.3 mg/kg B3C + 0.02 mg/kg Ras.

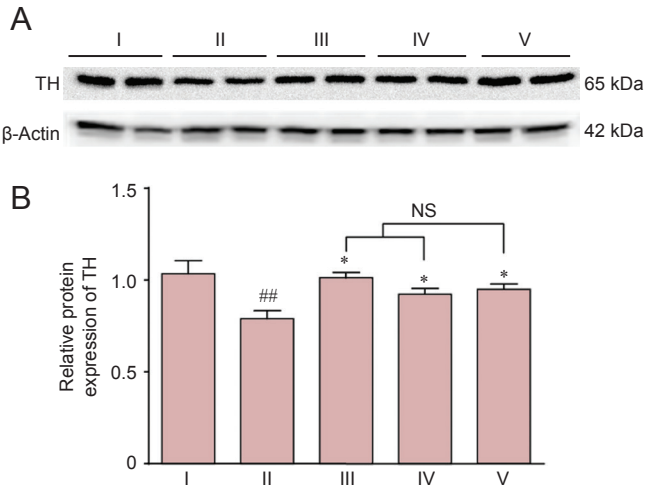

Figure 4 B3C, rasagiline and their combination reversed MPTP-induced decrease of TH expression in the substantia nigra (western blot assay).
At 15th day after MPTP injection, the expression of TH in the substantia nigra was evaluated. (A) Treatment with B3C, Ras and their combination up-regulated TH expression compared to MPTP group. (B) Densitometric analysis of optical density and relative protein expression normalized using β-actin as an internal standard. All data are expressed as the mean ± SEM; $n = 8$ mice/group. ##$P < 0.01$, *vs.* I; *$P < 0.05$, *vs.* II (one-way analysis of variance and Dunnett's test). NS: Not significant; TH: tyrosine hydroxylase; Ras: rasagiline; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. I: Control; II: MPTP; III: 0.3 mg/kg B3C; IV: 0.02 mg/kg Ras; V: 0.3 mg/kg B3C + 0.02 mg/kg Ras.

MPTP-treated mice when compared to control ($P < 0.05$; **Figure 3B–D**). B3C, rasagiline and their combination increased the levels of dopamine and its metabolites when compared to the MPTP group ($P < 0.05$). However, there was no significant difference among B3C, rasagiline and their combination ($P > 0.05$).

The effects of B3C and/or rasagiline on the TH expression in the substantia nigra of MPTP-injected mice

TH was widely accepted as a marker for dopaminergic neurons (Haavik and Toska, 1998). We used western blot assay to measure the expression of TH in the substantia nigra. MPTP significantly reduced the expression of TH when compared to the control group ($P < 0.05$). B3C, rasagiline and their combination increased TH expression when compared to the MPTP group ($P < 0.05$). However, there was no significant difference among B3C, rasagiline and their combination ($P > 0.05$; **Figure 4**).

Activities of B3C and rasagiline on MAO-B inhibition *in vitro*

B3C concentration-dependently inhibited MAO-B activity with an IC_{50} of 115.8 μM (**Figure 5**). Rasagiline, a well-known selective MAO-B inhibitor, inhibited MAO-B activity with an IC_{50} of 6.43 nM (**Figure 5**), which is similar to a previous report (Zheng et al., 2005).

Regulating the expressions of GSK3β and MEF2D by B3C and/or rasagiline in the substantia nigra of MPTP-injected mice

It was reported that MPP^+ could damage dopamine neurons *via* the inhibition of MEF2D transcriptional activity (Yao et al., 2012). MEF2D could be regulated by several signalling pathways. For example, GSK3β, an downstream molecule of PI3-K/Akt signaling pathway, could directly phosphorylate MEF2D and decrease the activity of MEF2D (Weinreb et al., 2005b; Wang et al., 2009; Yao et al., 2012). MEF2D could further protect dopamine neurons in the substantia nigra against neurotoxicity in PD animal models (Smith et al., 2006; She et al., 2011). In this study, MPTP signficanlty down-regulated the expression of MEF2D, while up-regulated the expression of GSK3β in the substantia nigra ($P < 0.05$; **Figure 6**). B3C, but not rasagiline or their combination, significantly reversed MPTP-induced alteration of GSK3β and MEF2D ($P < 0.05$; **Figure 6**).

Discussion

In the past decade, none of mono-drug therapies aimed to treat PD with disease-modifying potential was successful in clinical trials (Kalia et al., 2015). The multiple disease etiologies implicated in PD gave rise to a shift from a single-target to a multi-target therapy (NINDS NET-PD Investigators, 2006, 2007; Reznichenko et al., 2010).

It has been reported that continuous administration of rasagiline (0.05 mg/kg, oral administration) following MPTP lesion restored the loss of dopaminergic neurons, the decrease of striatal dopamine content, and the reduction of TH activity (Mandel et al., 2007). Therefore, rasagiline at 0.02 and 0.1 mg/kg was used in the present study. In our study, rasagiline at both dosages was effective to treat MPTP-induced Parkinsonism, while the higher dosage did not exert greater neuroprotection. Similar results were reported by Sagi et al. (2007), showing that doubling the dose of rasagiline to 0.1 mg/kg did not lead to the greater neuroprotection. It was demonstrated that rasagiline could not produce neuroprotection at very high doses (0.25–1 mg/kg) (Sagi et al., 2007). The molecular mechanism underlying the neuroprotection of rasagiline involved the increase of protein kinase Cε, the activation of mitogen-activated protein kinase pathway, and the induction of neurotrophic factors (Yogev-Falach et al., 2003; Bar-Am et al., 2005; Weinreb et al., 2005a).

B3C (1 mg/kg) reversed MPTP-induced loss of dopaminergic neurons and behavioral abnormitiies *via* effectively up-regulating MEF2D from the activation of Akt/GSK3β pathway (Yao et al., 2012). In the present study, 0.3 and 1 mg/kg B3C also alleviated behavioural abnormitiies, restored the contents of dopamine and its metabolites in the striatum, and up-regulated TH expression in the substantia nigra. To examine whether the effectiveness of B3C is associated with MAO inhibition, we have performed MAO-B activity assay. Our results showed that B3C could inhibit MAO-B with an IC_{50} of 115.8 μM. However, B3C could effectively prevent glutamate and K^+ deprivation-induced neurotoxicity with an IC_{50} at sub-nanomolar level (Luo et al., 2010; Hu et al., 2013). The concentration up to 100 μM is toxic to neurons and cannot be reached in the brain when 1 mg/kg of B3C was administrated to mice. Therefore, we deduced that the neuroprotective effects of B3C in MPTP-injected mice were independent of its MAO-B inhibition property. In consistent with the findings of a previous study (Yao et al., 2012), B3C up-regulated MEF2D and inhibited GSK3β in our study. However, rasagiline and the combination of B3C and rasagiline could not significantly alter the expressions of MEF2D and GSK3β. How could rasagiline counteract the effect of B3C on the expression of MEF2D and GSK3β? It needs to be investigated in our further study.

The reason why there is no synergism between B3C and rasagiline is possibly due to the concentrations of drugs used in the present study. In the study of Reznichenko et al. (2010), to test the additive/synergistic action of the combination of rasagiline and EGCG in MPTP mice, low/sub-effective dosages of drugs were chosen. And individual drug at used dosage did not exert positive effects. Such experimental design could circumvent potential "masking" of the contribution of rasagiline and EGCG to the neuroprotective effects. In our study, the dosages of B3C and rasagiline were close to their maximal effective dosages. Therefore, low/sub-effective dosages are required for further investigating the synergism between B3C and rasagiline on MPTP-induced model of Parkinsonism.

In the present study, we have investigated, for the first time, the synergistic effects between B3C and rasagiline in MPTP-induced mice model of Parkinsonism. In consistent with previous findings (Mandel et al., 2007; Sagi et al., 2007; Yao et al., 2012), both B3C and rasagiline significantly protected dopaminergic neurons against damage and reversed behavioral abnormities in MPTP-treated mice. However, the combination of B3C and rasagiline could not produce synergistic effects.

Author contributions: *ZJZ, YFH and SMYL designed the study. ZJZ and YQW wrote and revised the paper. CYZ and WC1 performed experiments and analyzed experimental data. BJG, WC2 and SHM participated in study design and performed experiments. All authors approved the final version of the paper.*

Conflicts of interest: *None declared.*

Plagiarism check: *This paper was screened twice using Cross-Check to verify originality before publication.*

Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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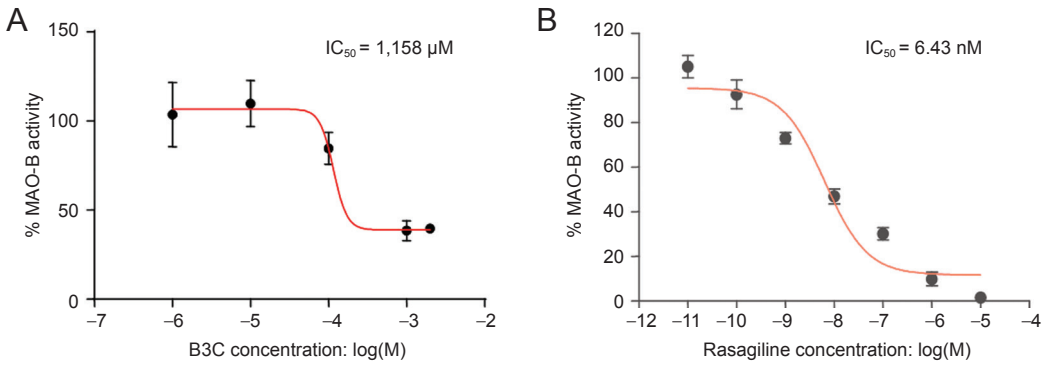

Figure 5 B3C and rasagiline inhibit MAO-B activity *in vitro*.
(A) B3C concentration-dependentaly inhibits MAO-B activity. (B) Rasagiline concentration-dependently inhibits MAO-B activity. MAO-B activity was determined by MAO-GloTM assay kit. All data are expressed as the mean ± SEM of three independent experiments. MAO-B: Monoamine oxidase-B; B3C: bis(propyl)-cognitin.

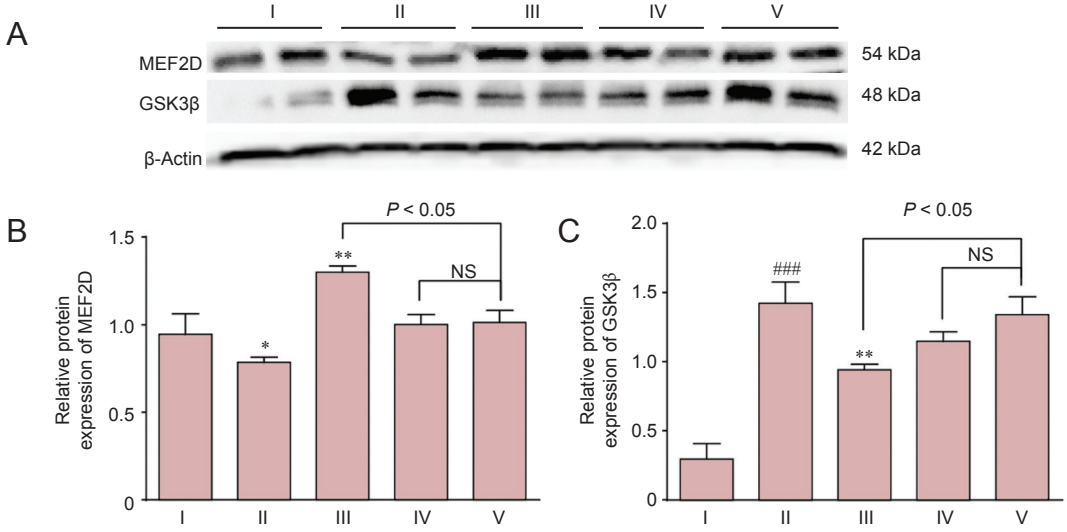

Figure 6 B3C, rasagiline and their combination reversed MPTP-induced alteration of MEF2D and GSK3β expressions in the substantia nigra (western blot assay).
At 15th day after MPTP injection, the expression levels of MEF2D and GSK3β in the substantia nigra were evaluated. (A) Treatment with B3C, but not Ras or their combination, significantly restored MPTP-induced alteration of MEF2D and GSK3β expression. (B) Densitometric analysis of optical density and relative protein expression normalized using β-actin as an internal standard. All data are expressed as the mean ± SEM; $n = 8$ mice/group. #$P < 0.05$, ###$P < 0.001$, *vs.* I; **$P < 0.01$, *vs.* II (one-way analysis of variance and Dunnett's test). NS: Not significant; Ras: rasagiline; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MEF2D: myocyte enhancer factor-2D; GSK3β: glycogen synthase kinase-3β. I: Control; II: MPTP; III: 0.3 mg/kg B3C; IV: 0.02 mg/kg Ras; V: 0.3 mg/kg B3C + 0.02 mg/kg Ras.

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