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NOBILETIN TREATMENT IMPROVES MOTOR AND COGNITIVE DEFICITS SEEN IN MPTP-INDUCED PARKINSON MODEL MICE

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Abstract—Nobiletin, a polymethoxylated flavonoid found in citrus fruit peel, reportedly improves memory impairment in rodent models. Here we report its effect on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced motor and cognitive deficits. Nobiletin administration (50 mg/kg i.p.) for 2 consecutive weeks improved motor deficits seen in MPTP-induced Parkinson model mice by 2 weeks, an effect that continued until 2 weeks after drug withdrawal. Drug treatment promoted similar rescue of MPTP-induced cognitive impairment at equivalent time points. Nonetheless, nobiletin treatment did not block loss of dopaminergic neurons seen in the MPTP-treated mouse midbrain, nor did it rescue decreased tyrosine hydroxylase (TH) protein levels seen in the striatum or hippocampal CA1 region of these mice. Interestingly, nobiletin administration (50 mg/kg i.p.) rescued reduced levels of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) autophosphorylation and phosphorylation at Thr-34 of dopamine- and cAMP-regulated phosphoprotein-32 (DARPP-32) in striatum and hippocampal CA1 to levels seen in sham-operated mice. Likewise, CaMKII- and cAMP kinase-dependent TH phosphorylation was significantly restored by nobiletin treatment. MPTP-induced reduction of dopamine contents in the striatum and hippocampal CA1 region was improved by nobiletin administration (50 mg/kg i.p.). Acute intraperitoneal administration of nobiletin also enhanced dopamine release in striatum and hippocampal CA1, an effect partially inhibited by treatment with nifedipine (a L-type Ca^{2+} channel inhibitor) or NNC 55-0396 (a T-type Ca^{2+} channel inhibitor) and completely abolished by combined treatment with both. Overall, our study describes a novel nobiletin activity in brain and suggests that nobiletin rescues motor and cognitive dysfunction in MPTP-induced Parkinson model mice, in part by enhancing dopamine release.

Key words: nobiletin, cognitive function, Ca^{2+}/calmodulin-dependent protein kinase II, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Voltage-dependent Ca^{2+} channel.

INTRODUCTION

Parkinson's disease (PD) is a progressive disorder marked by degeneration of dopaminergic neurons in the substantia nigra (SN) and causing extrapyramidal motor dysfunctions such as tremor, rigidity and akinesia (Olanow and Tatton, 1999). Notably, PD patients also exhibit psychological dysfunction including cognitive deficits, depression and anxiety (Pillon et al., 1989; Cummings 1992; Walsh and Bennett 2001). Although dopamine replacement therapies with levodopa or dopamine agonists in part improve motor dysfunction, effective therapeutics aimed at improving cognitive dysfunction in PD patients are not yet available.

Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively causes death of midbrain dopaminergic neurons. MPTP is metabolized to the toxic cation MPP^+ by monoamine oxidase-B enzymes, which in turn specifically penetrates dopaminergic neuron via dopamine transporters (Javitch et al., 1985). Since MPP^+ interferes with complex I of the electron transport chain in mitochondria, ATP levels decrease following MPTP administration, promoting loss of mitochondrial membrane potential and damage to dopaminergic neurons due to oxidative stress (Nicklas et al., 1987; Smeyne and Jackson-Lewis 2005). Recently, we observed markedly decreased Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) activity with concomitant impaired long-term potentiation (LTP) in the hippocampus of MPTP-treated mice (Moriguchi

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Abbreviations: ANOVA, analysis of variance; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CREB, cyclic-AMP-responsive-element-binding protein; DAB, diaminobenzidine; DARPP-32, dopamine- and cAMP-regulated phosphoprotein-32; ECD, electrochemical detector; ERK, extracellular signal-regulated kinase; HPLC, high-performance liquid chromatography; LTP, long-term potentiation; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate-buffered saline; PD, Parkinson's disease; PDE, phosphodiesterase; PET, positron emission tomography; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SN, substantia nigra; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VDCCs, voltage-dependent Ca^{2+} channels; VTA, ventral tegmental area.

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et al., 2012b). This activity likely accounts in part for cognitive dysfunction observed in these mice.

Nobiletin, a major polymethoxyflavone present in the peel of citrus fruit, has the ability to enhance cognitive function in mouse models of Alzheimer's disease (Onozuka et al., 2008) and brain ischemia (Yamamoto et al., 2009). Studies using these models show that peripheral administration of nobiletin enhances cyclic-AMP-responsive-element-binding protein (CREB) phosphorylation and CaMKII activity in hippocampus (Yamamoto et al., 2009). In addition, in rat hippocampal neurons, nobiletin treatment stimulates cAMP-dependent protein kinase (PKA) and extracellular signal-regulated kinase (ERK) pathways (Nagase et al., 2005a,b). However, mechanisms underlying these activities remain unclear. In *in vitro* studies using PC12 cells, nobiletin (100 μM) treatment potentiated forskolin-induced cAMP production via inhibition of phosphodiesterase (PDE) (Nagase et al., 2005b). In addition, nobiletin (1–100 μM) stimulates catecholamine secretion and $^{45}Ca^{2+}$ influx in a concentration-dependent manner in cultured bovine adrenal medullary cells by activating voltage-dependent Ca^{2+} channels (Zhang et al., 2010). However, pharmacological relevance of Ca^{2+} elevation by nobiletin in the brain has not been addressed.

Here, we asked whether nobiletin rescues motor and cognitive deficits in MPTP-treated mice and assessed potential mechanisms accounting for resultant improvements in cognition. We show that that nobiletin promotes dopamine release in the hippocampus and striatum in a voltage-gated calcium channel-dependent manner, an activity that likely underlies its improvement of MPTP-induced behavioral deficits and memory impairment.

EXPERIMENTAL PROCEDURES

Animals and MPTP treatment

Adult male C57/BL6N mice (8–9 weeks old) were obtained from Clea Japan Inc. (Tokyo, Japan). Animals were housed under conditions of constant temperature (23 ± 1 °C) and humidity (55 ± 5%) on a 12-h light–dark cycle (light; 9–21 h) and fed *ad libitum*. All animal procedures were approved by the Committee on Animal Experiments at the Tohoku University. Efforts were made to minimize suffering and reduce the number of animals used.

MPTP-treated model mice were prepared as described (Moriguchi et al., 2012b). Mice were treated with vehicle or MPTP (25 mg/kg, i.p.; Sigma, St Louis, MO, USA) once a day for consecutive 5 days.

Nobiletin administration

Nobiletin was isolated from *Citrus depressa* as described (Nagase et al., 2005a), and dissolved in saline containing 0.5% Tween 80. One day after final MPTP injection, MPTP-treated mice were administered 25 or 50 mg/kg of nobiletin intraperitoneally once a day for 14 consecutive days. Control mice were treated with the same volume of vehicle or 50 mg/kg of nobiletin.

Animals were subjected to behavioral tests including rotarod and beam walking to assess motor skills and novel object recognition and step-through passive avoidance tasks to evaluate cognition. To minimize stress effects, six sets of experiments were conducted using different animals. (1) Group I was subjected to immunohistochemical analysis 4 weeks after the final MPTP injection ($n = 4$ per groups). (2) Group II was subjected to behavioral analyses. Specifically, at 2 and 4 weeks after the final MPTP injection, animals underwent motor function tests and at 4 weeks after the final MPTP injection were tested for cognitive function [vehicle-treated controls: $n = 11$; nobiletin- (50 mg/kg, i.p.) treated controls: $n = 5$; vehicle-treated MPTP: $n = 10$; nobiletin- (25 mg/kg, i.p.) treated MPTP: $n = 5$; nobiletin-(50 mg/kg, i.p.) treated MPTP: $n = 11$]. (3) Mice from Group III were analyzed by western blotting analysis 4 weeks after the final MPTP injection ($n = 4$ per group). (4) Group IV was subjected to measurement of dopamine contents after evaluation of the sensitivities to electric foot [vehicle-treated controls: $n = 6$; nobiletin-(50 mg/kg, i.p.) treated controls: $n = 4$; vehicle-treated MPTP: $n = 5$; nobiletin- (50 mg/kg, i.p.) treated MPTP: $n = 6$]. (5) Group V consisted of naïve mice was subjected to measurement of nobiletin-induced dopamine levels in the striatum and hippocampal CA1 with or without treatment with voltage-dependent Ca^{2+} channel inhibitors ($n = 4$–5 per group). (6) Mice from Group VI were subjected to measurement of nobiletin-induced dopamine levels in the striatum and hippocampal CA1 4 weeks after the final MPTP injection ($n = 4$ per group). The protocol was shown in Fig. 1.

Rotarod task

The rotarod task was performed as described (Moriguchi et al., 2012b). The apparatus consisted of a base platform and a non-slippery iron rod of 3-cm diameter and 30-cm length. Before MPTP injection, mouse was placed on a drum rotating at 20 rpm for training. Mice ran on the drum repeatedly until the latency of falling from the beam exceeded 200 s. For the test, mice were placed on the rod rotating at 20 rpm and falling latency was recorded for up to 5 min.

Beam-walking task

The apparatus consisted of a rectangular beam (length: 870 mm × width: 5 mm, medium density fiberboard). Both ends of the beam were fixed by wooden pools at 500 and 315 mm from the floor and the 'goal box' (155 mm × 160 mm × 5 mm, Perspex) was placed on the higher end of beam. The beam-walking task was performed as described (Ferguson et al., 2010). Before MPTP injection mice were trained to traverse the beam by habituating them to the goal box for 3 min and then placing them on the beam at 10 cm from the goal. After traversing 10 cm and reaching the goal box mice were placed at increasing 30-, 50-, and 80-cm distances from the box and trained to traverse the beam for one trial at