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Full paper

Rolipram improves facilitation of contextual fear extinction in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mouse model of Parkinson's disease

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ABSTRACT

Cognitive impairment often occurs in Parkinson's disease (PD), but the mechanism of onset remains unknown. Recently, we reported that PD model mice produced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) show facilitation of hippocampal memory extinction, which may be the cause of cognitive impairment in PD. When we examined the cAMP/CREB signaling in the hippocampus, decreased levels of cAMP and phosphorylated CREB were observed in the dentate gyrus (DG) of MPTP-treated mice. Administration of rolipram improved the memory deficits with concomitant recovery of cAMP and phosphorylated CREB levels, suggesting that reduced cAMP/CREB signaling in the DG leads to cognitive impairment in MPTP-treated mice.

Parkinson's disease (PD) is a neurodegenerative disorder characterized by progressive loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) and reduction of DAergic fibers in the striatum, resulting in extrapyramidal motor dysfunctions such as tremor, rigidity, and bradykinesia.[1] Although non-motor symptoms in PD, especially cognitive impairment, appear in about 40% of PD patients,[2] the etiology of the cognitive impairment associated with PD is not well understood. Moreover, commonly used clinical medications such as L-dopa and dopamine agonists are effective for reversing the motor dysfunction in PD but are not fully effective for improving cognitive impairment.[3] Therefore, therapeutic methods that improve PD-induced cognitive impairment are needed.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes selective loss of DAergic neurons in the SNpc, leading to DA depletion in the striatum.[4,5] Furthermore, administration of MPTP in rodents causes similar pathological and neurochemical features as those seen in PD patients, including both motor and non-motor deficits.[4,5] Recently, we reported that MPTP-treated mice show facilitation of memory extinction and attenuation of memory retention, but consolidation and reconsolidation in the contextual fear conditioning test are not affected.[5] Extinction of contextual fear memory can be influenced by communication between the hippocampus and other brain regions.[6,7] Moreover, distinct hippocampal neurons have been implicated in new extinction learning.[6,7] However, how the hippocampus contributes to the facilitation of contextual fear extinction in PD mice is largely unknown.

The intracellular cascades involving cyclic adenosine monophosphate (cAMP) are implicated in long-term potentiation (LTP) and hippocampus-dependent learning, and also in the extinction of conditioned fear, through activation of protein kinase A (PKA) and phosphorylation of cAMP-dependent response element-binding protein (CREB).[7,8] Moreover, CREB signaling plays a critical role in memory extinction and in promoting memory strengthening upon retrieval.[9] Previous studies have reported that rolipram, a phosphodiesterase (PDE) IV inhibitor, stimulates the hippocampal cAMP-PKA-CREB pathway, leading to improvement in hippocampus-dependent memory.[10]

In the present study, to investigate how facilitation of memory extinction occurs in PD model mice produced by MPTP, we examined cAMP levels and expression of phosphorylated CREB (p-CREB) in the

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hippocampus. Moreover, we also examined whether rolipram can restore facilitation of the contextual fear extinction in MPTP-treated mice by stimulating the cAMP/CREB pathway in the hippocampus.

Maintenance of Male C57BL/6 mice (7–8 weeks old) and preparation of MPTP (Sigma-Aldrich, Tokyo, Japan)-treated PD model mice were conducted as described in our previous report.[5] All procedures for the care and use of experimental animals were approved by the Animal Research Committee at Obihiro University of Agriculture and Veterinary Medicine and were conducted in compliance with the Guiding Principles for the Use of Animals in Toxicology in 1989. Subsequent experiments were conducted 7 days after the last MPTP or saline injection. Rolipram (Tokyo Chemical Industry, Tokyo, Japan) was prepared by dissolving in saline containing 1% dimethylsulfoxide (DMSO). All procedures of the Contextual fear conditioning test were performed as described in our previous report.[5] Percentage of time spent freezing was measured during the test session (time spent freezing/total time × 100) as an index of memory.

Table 1
Analysis of cAMP levels in the hippocampus before and immediately after extinction training.

	Hippocampal cAMP levels (pmol/mg)	
	Before extinction	After extinction
Control-1%DMSO	39.95 ± 1.48	44.69 ± 3.23
MPTP-1%DMSO	32.13 ± 2.70*	32.10 ± 1.70†
Control-3-mg/kg-rolipram	63.99 ± 3.20$^{*,\#}$	57.59 ± 3.87†,§
MPTP-3-mg/kg-rolipram	52.21 ± 2.82$^{*,\#}$	48.76 ± 5.17§

Data are the mean ± SEM; $n = 6$–8. There were no significant differences between before and after extinction training in each group. $^{*}p < 0.05$ vs. control-1%DMSO before extinction, $^{\#}p < 0.05$ vs. MPTP-1%DMSO before extinction, $^{\dagger}p < 0.05$ vs. control-1%DMSO after extinction, $^{\S}p < 0.05$ vs. MPTP-1%DMSO after extinction.

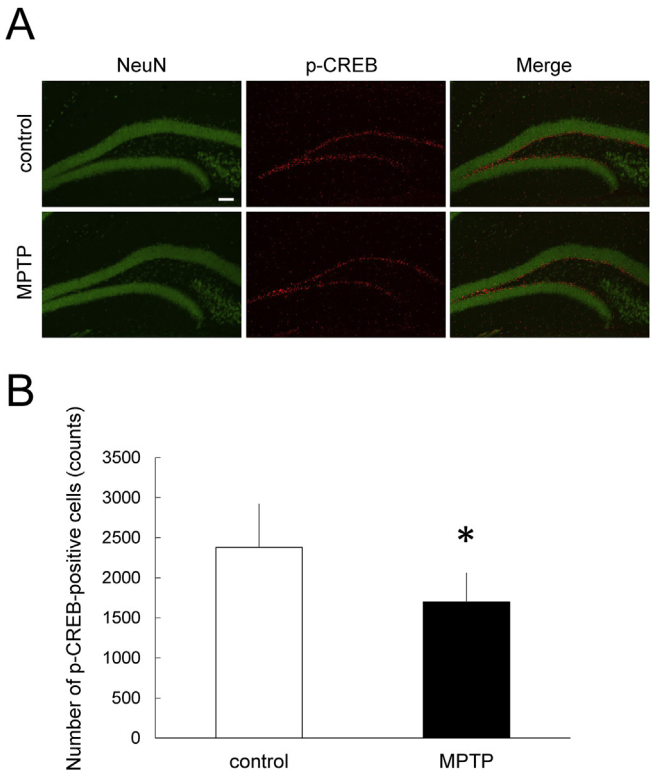

Fig. 1. Analysis of the number of p-CREB-positive cells in the DG of the hippocampus after extinction training. (A) Immunohistochemistry for p-CREB and NeuN in the DG after fear extinction. Scale bar = 100 μm. (B) The number of p-CREB-positive cells in the DG. Data are the mean ± SD; $n = 6$ per group. $^{*}p < 0.05$ with Student's t-test.

The cAMP levels were determined using a Cyclic AMP EIA kit (Cayman Chemical Company, Ann Arbor, MI). The hippocampus was collected before extinction training or immediately after the second extinction training. The samples were subjected to the enzyme-linked immunosorbent assay according to the manufacturer's protocol. The cAMP levels are shown in pmol per mg of hippocampal protein. Protein concentration was determined using the protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Fluorescent double immunohistochemistry was performed as described in our previous report.[11] The sections (consecutive 40-μm thick sections) were incubated for 48 h at 4 °C in rabbit anti-p-CREB or anti-p-ERK polyclonal antibody (1:500 in TPBS; Cell Signaling Technology Inc., Danvers, MA), followed by incubation with Alexa Fluor® 568 goat anti-rabbit IgG (1:5000 in T-PBS; Thermo Fisher Scientific, Waltham, MA) for 2 h at 4 °C. The sections were then incubated with mouse anti-NeuN monoclonal antibody (1:5000 in T-PBS; EMD Millipore, Billerica, MA) for 24 h at 4 °C, followed by incubation with Alexa Fluor® 488 goat anti-mouse IgG (1:5000 in T-PBS, Thermo Fisher Scientific) for 2 h at 4 °C. For each mouse, the total number of p-CREB-positive and p-ERK-positive cells in a section from the hippocampal DG (positioned 1.82–2.06 mm posterior to the bregma) was counted using confocal laser-scanning microscopy (C2+, Nikon, Tokyo, Japan).

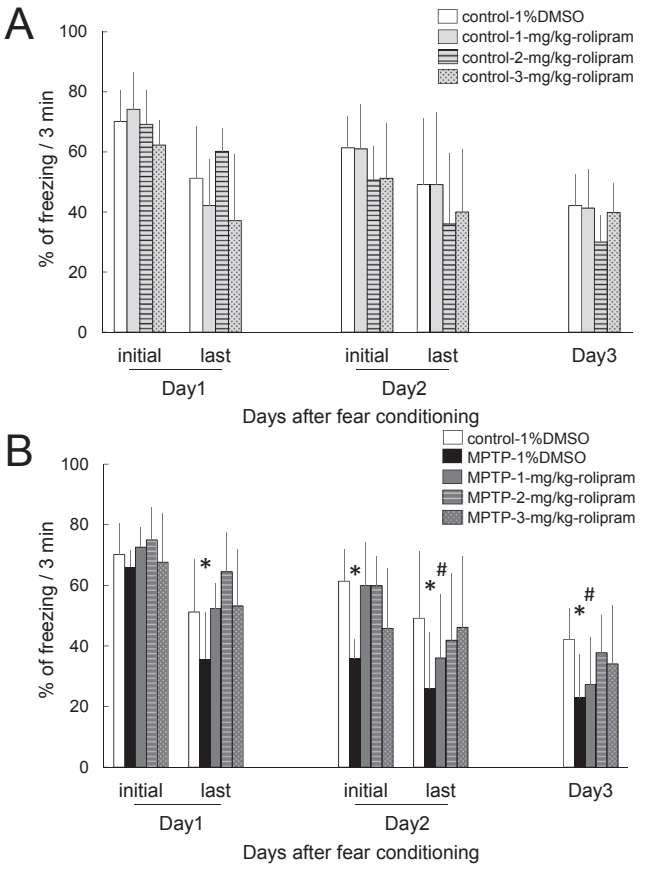

Fig. 2. Effect of rolipram on contextual fear extinction in mice. Mice were given intraperitoneal injection of rolipram at a dose of 1, 2, or 3 mg/kg in saline containing 1% DMSO or the vehicle only 2 h prior to both extinction training sessions. (A) Effect of rolipram on fear extinction in control mice. Data are the mean ± SD; $n = 6$–7. (B) Effect of rolipram on fear extinction in PD mice. Data are the mean ± SD; $n = 5$–7. $^{*}p < 0.05$ vs. MPTP-1%DMSO-initial-Day-1, $\#p < 0.05$ vs. MPTP-1-mg/kg rolipram-initial-Day-1 with two-way measures ANOVA followed by Tukey's post-hoc tests. There were no significant differences among all groups in Day 1 (both initial and last), Day 2 (both initial and last), and Day 3.

Comparisons between two-group data were analyzed with Student's *t*-test. Multiple group comparisons were assessed using one-way or two-way analysis of variance (ANOVA) followed by the post-hoc Tukey's test, or Kruskal–Wallis analysis followed by Mann–Whitney U-tests. Statistical differences were considered significant when $p < 0.05$. All statistical analyses were performed with SPSS 16.0 software (SPSS Japan Inc., Tokyo, Japan).

We first examined whether the hippocampal cAMP/CREB cascade is involved in facilitation of fear extinction in MPTP-treated mice. The enzyme-linked immunosorbent assay showed that the hippocampal cAMP levels in the MPTP-treated mice were significantly decreased in both before and after extinction training compared with control mice (Table 1). These results suggest that cAMP cascades in the hippocampus were attenuated throughout contextual fear extinction in MPTP-treated mice.

PKA is activated by cAMP and phosphorylates CREB, and this cascade in the hippocampus plays critical roles in memory retrieval, strengthening, and extinction.[8,9] We evaluated the number of p-CREB-positive cells after the second extinction training. The number of p-CREB-positive cells were significantly decreased in the hippocampal DG in MPTP-treated mice compared to control mice (Fig. 1A and B). On the other hand, the number of both p-CREB- and NeuN-positive cells was less than 30% of the total number of p-CREB-positive cells and was not significantly different between control and MPTP-treated mice (control: 28.01 ± 4.41%, MPTP: 26.88 ± 1.69%) (Fig. S1A). Moreover, there were no significant differences in the number of p-ERK-positive cells between control and MPTP-treated mice (Fig. S2). This result suggests that the lower number of p-CREB-positive cells in the DG may be critical for facilitation of hippocampal memory extinction in MPTP-treated mice.

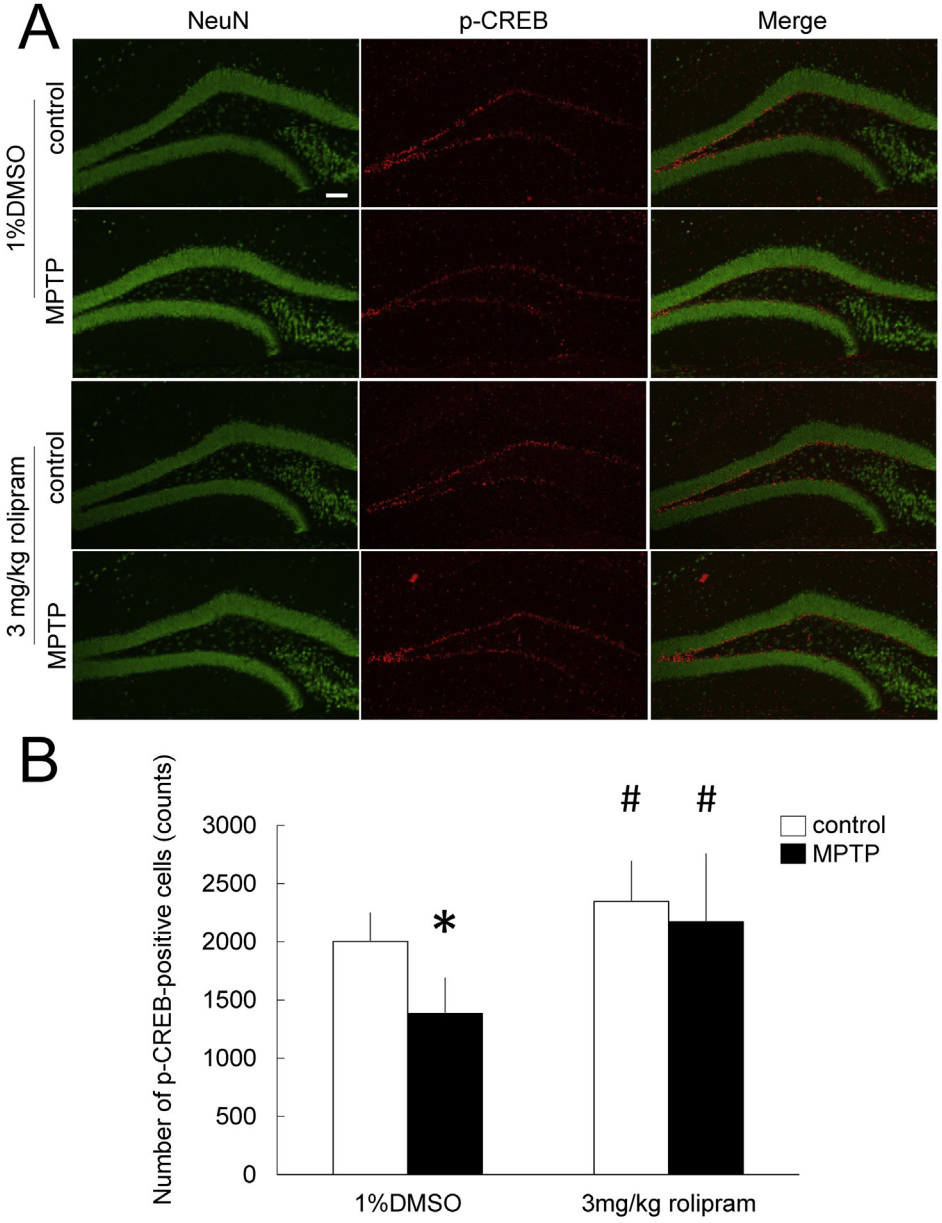

Fig. 3. Effect of rolipram on the number of p-CREB-positive cells in the DG of the hippocampus after fear extinction. (A) Immunohistochemistry for p-CREB and NeuN in the DG after fear extinction. Scale bar = 100 μm. (B) The number of p-CREB-positive cells in the DG. Data are the mean ± SD; $n = 4–9$. *$p < 0.05$ vs. control-1%DMSO, #$p < 0.05$ vs. MPTP-1%DMSO with two-way measures ANOVA followed by Tukey's post-hoc tests.

Next, we investigated whether rolipram, a PDE IV inhibitor, can improve facilitation of fear extinction in MPTP-treated mice. Systemic administration of rolipram (a single injection at 1, 2, or 3 mg/kg) 2 h before extinction training prevented facilitation of fear extinction in MPTP-treated mice in a dose-dependent manner (Fig. 2B), but did not show any significant effects on fear extinction in control mice (Fig. 2A). Moreover, higher dosages of rolipram (3 mg/kg) improved the facilitation of fear extinction in MPTP-treated mice over a longer time period (Fig. 2B) and restored cAMP levels in hippocampus to the level in control mice (Table 1). Similarly, administration of higher dosages of rolipram (3 mg/kg) to MPTP-treated mice significantly restored the number of p-CREB-positive cells in the hippocampal DG to the level in control mice (Fig. 3A and B). However, rolipram did not affect the number of double-positive cells that expressed both p-CREB and NeuN (control-1%DMSO: 29.40 ± 4.23%, MPTP-1%DMSO: 30.14 ± 2.80%, control-3-mg/kg-rolipram: 32.38 ± 4.41%, MPTP-3-mg/kg-rolipram: 28.58 ± 4.41%) (Fig. S1B).

Extinction occurs via original memory retrieval and new memory learning.[6,7] These processes require gene expression and protein synthesis in distinct brain regions.[7,9] In particular, the cAMP/CREB cascade plays critical roles in contextual fear memory extinction or strengthening via memory retrieval.[9,12] In this study, MPTP-treated mice showed a reduction in cAMP levels in the hippocampus (Table 1) and in the number of p-CREB-positive cells in the DG of the hippocampus (Fig. 1) after extinction training. Moreover, rolipram administration rescued the facilitation of contextual fear extinction in MPTP-treated mice (Fig. 2B). These results suggest that the cAMP/CREB pathway in the hippocampal DG is involved in facilitation of fear extinction in MPTP-treated mice and that loss of DAergic neurons in the SNpc affects the DG function via unknown neuronal pathways. On the other hand, rolipram did not affect fear extinction in control mice (Fig. 2A), suggesting that cAMP contents higher than a certain threshold level in hippocampal neurons are necessary for normal extinction.

DA plays critical roles in learning, working memory, and hippocampus-dependent memory.[13] Because DA modulates the intracellular cAMP cascade, a decline in cAMP signaling in MPTP-treated mice may be caused by dysfunction of the hippocampal DA system. However, L-dopa treatment has no effect on cognitive deficits of PD in either human patients or rodent models, suggesting that other neuronal pathways may be involved in cognitive deficits of PD.[3,14] SNpc neurons sparsely project to the dorsal and median raphe nuclei, which mostly contain serotonin neurons that project their axons to a variety of brain regions including hippocampus.[15] Therefore, functional impairment of these nuclei may secondarily affect hippocampal function.

In conclusion, we report that PD model mice induced by MPTP show a decrease in cAMP and p-CREB expression levels in the hippocampal DG. Furthermore, rolipram can rescue facilitation of contextual fear extinction in MPTP-treated mice via enhancement of the cAMP/CREB cascade in the DG. We therefore conclude that rolipram may be potentially useful as a therapeutic drug for treating the cognitive deficits of PD by improving the cAMP/CREB signaling pathway in the DG.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2017.04.002.

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