Brief Report
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Effects of Subdiaphragmatic Vagotomy in the MPTP-induced Neurotoxicity in the Striatum and Colon of Mice

Jiajing Shan, Youge Qu, Jiancheng Zhang, Li Ma, Kenji Hashimoto

Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, Chiba, Japan

Objective: Gut—microbiota—brain axis plays a role in the pathogenesis of Parkinson's disease (PD). The subdiaphragmatic vagus nerve serves as a major modulatory pathway between the gut microbiota and the brain. However, the role of subdiaphragmatic vagus nerve in PD pathogenesis are unknown. Here, we investigated the effects of subdiaphragmatic vagotomy (SDV) on the neurotoxicity in the mouse striatum and colon after administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).
Methods: Sham or SVD was performed. Subsequently, saline or MPTP (10 mg/kg × 3, 2-hour interval) was administered to mice. Western blot analysis of tyrosine hydroxylase (TH) and dopamine transporter (DAT) in the striatum and phosphorylated α-synuclein (p-α-Syn) in the colon was performed.
Results: Repeated administration of MPTP significantly caused reduction of TH and DAT in the striatum and increase of p-α-Syn in the colon of mice. However, SDV did not affect the reduction of TH and DAT in the striatum and increases in p-α-Syn in the colon after repeated MPTP administration.
Conclusion: These data suggest that subdiaphragmatic vagus nerve doses not play a role in the MPTP-induced neurotoxicity in the brain and colon.

KEY WORDS: Alpha-synuclein; Colon; Brain; MPTP; Vagus nerve.

INTRODUCTION

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects predominantly dopaminergic neurons in the striatum and substantia nigra. α-Synuclein is a key protein involved in the pathology of PD. Although the precise mechanisms underlying PD pathology remain unknown, increasing evidence suggests a crucial role of gut—microbiota—brain axis in the pathology of PD [1,2]

Using mice that overexpress α-synuclein, Sampson *et al.* [3] reported that gut—microbes are necessary for motor deficits and α-synuclein pathology. Microbiome depletion by antibiotic cocktail ameliorated these deficits in mice, while microbial re-colonization promoted these deficits. Interestingly, colonization of α-synuclein overexpressing mice with fecal microbiota transplantation (FMT) from PD patients enhanced physical impairments compared to FMT from healthy control subjects [3]. Furthermore, we reported that antibiotic-induced microbiome depletion protected against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity in the mouse brain [4]. Collectively, it is likely that the gut—microbiota—brain axis might play a key role in pathology of PD. The subdiaphragmatic vagus nerve serves as a major modulatory pathway between the gut microbiota and the brain [5-8]. However, the role of subdiaphragmatic vagus nerve on the MPTP-induced neurotoxicity in the brain and colon remains unknown.

This study was undertaken to investigate whether subdiaphragmatic vagotomy (SDV) affects MPTP-induced neurotoxicity in the mouse brain and colon.

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Address for correspondence: Kenji Hashimoto
Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, 1-8-1 Inohana, Chiba, Chuo-ku, Chiba 260-8670, Japan
E-mail: hashimoto@faculty.chiba-u.jp
ORCID: https://orcid.org/0000-0002-8892-0439

METHODS

Animals

Male adult C57BL/6 mice (13 weeks old) weighting 20−25 g bought from SLC, Inc. (Hamamatsu, Japan) were used. Animals were housed under controlled temperature and 12-hour light/dark cycles (lights on between 07:00−19:00) with libitum food (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water. All experiments were carried out according to the Guide for Animal Experimentation of Chiba University. The experimental protocol was approved by the Chiba University Institutional Animal Care and Use Committee (approval number: 2-446).

Vagotomy

Surgery of SDV and sham were performed, as previously reported [6-9]. Bilateral SDV was performed under anesthesia with 5% isoflurane on day 1 and day 2. Briefly, a 1 cm right transverse abdominal incision was made 0.5 cm below the xiphisternum, starting from the *linea alba*. The liver was carefully retracted with a small cotton pellet dampened with sterile normal saline and the costal arc was pulled using a vascular clamp, to expose the esophagus. The dorsal and ventral branches of the vagus nerve were exposed along the subdiaphragmatic esophagus under a surgical microscope (Leica, Heidelberg, Germany). Fourteen days after the operation, the observation of an increased stomach size indicated a successful SDV. For sham surgery, the trunk of the vagus nerve was gently exposed but not cut. In all mice that were subjected to SDV, particular care was taken to avoid any injuries to the subdiaphragmatic esophagus. The mice that underwent bilateral SDV were allowed to recover for more than 14 days (Fig. 1A).

Treatment of MPTP and Sample Collection

MPTP (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in saline. The procedure of MPTP-induced neurotoxicity was performed as previously reported [4,10,11]. Forty mice (13 weeks old) were divided into the following four groups: sham + saline group (n = 10); sham + MPTP group (n = 10); SDV + saline group (n = 10); SDV + MPTP group (n = 10). On day 18, MPTP (10 mg/kg × 3, 2-hour interval) or saline (5 ml/kg × 3, 2-hour interval) was injected intraperitoneally into mice (Fig. 1A). On day 25, the mice were anesthetized by 5% isoflurane and sodium pentobarbital (50 mg/kg) for collection of brain and colon (Fig. 1A). All tissues were stored at −80°C until use.

Western Blot Analysis

Western blot analysis was performed as previously reported [12]. The tissues were homogenized in freezing Laemmli lysis buffer, each specimen was performed separately, centrifuged at 3,000 × g at 4°C for 5 minutes to collect the supernatants. Use a DC protein assay kit (Bio-Rad, Hercules, CA, USA) to measure aliquots (60 μg) of proteins; and boiled at 95°C for 10 minutes with a quarter volume of 125 mM Tris-HCl, pH 6.8; 0.1% bromophenol blue; 4% sodium dodecyl sulfate; 10% β-mercaptoethanol and 20% glycerol. Proteins were separated by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (catalog #: 4568125, Mini-PROTEAN TGX™ Stain-Free Gels; Bio-Rad) and then were transferred onto

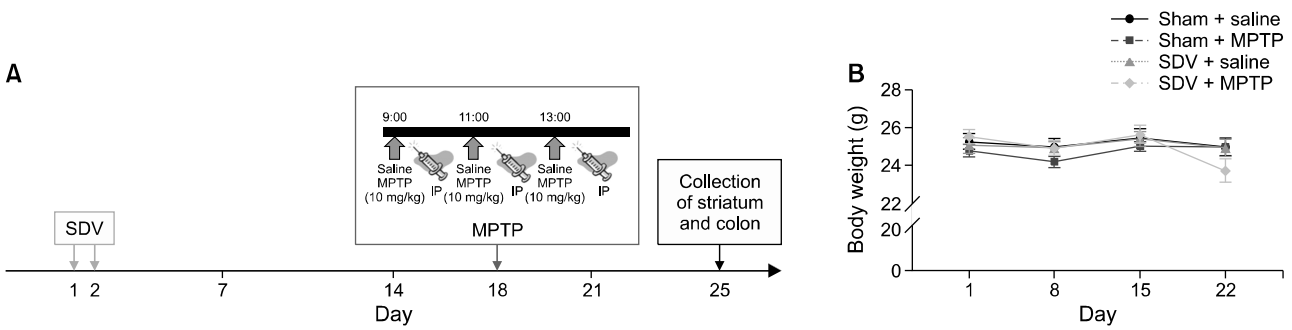

Fig. 1. Experimental schedule and body weight changes. (A) Surgery of SDV or sham was performed on day 1 or day 2, and then recovered until day 18. On day 18, MPTP (10 mg/kg × 3, 2-hour interval) or saline (10 ml/kg × 3, 2-hour interval) was administered into mice. On day 25, samples of striatum and colon were collected. (B) Body weight (repeated measure two-way ANOVA, time: $F_{3,108} = 9.770$, $p < 0.001$; group: $F_{3,36} = 0.253$, $p = 0.859$; interaction (time × group): $F_{9,108} = 3.733$, $p < 0.001$). Data represent the mean ± SEM (n = 10).
SDV, subdiaphragmatic vagotomy; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

polyvinylidene difluoride membranes using a Trans-Blot Mini Cell apparatus (Bio-Rad). For immunodetection, the polyvinylidene difluoride membranes were sealed with blocking solution (3% bovine serum albumin [BSA] in Toris buffer saline [TBS] + 0.1% Tween-20 [TBST]) at room temperature for 1 hour, the membranes for detecting dopamine transporter (DAT) were incubated with the appropriate dilution of the primary antibody against DAT (1:1,000, catalog number: NBP2-22164; NOVUS, Littleton, CO, USA), the membranes for detecting tyrosine hydroxylase (TH) were incubated with the appropriate dilution of the primary antibody against TH (1:1,000, catalog number: #AB152; Millipore, Temecula, CA, USA), while the membranes for detecting phosphorylated α-synuclein (p-α-Syn) were incubated with the appropriate dilution of the primary antibody against p-α-Syn (1:200, Catalog number: #ab51253; Abcam, Cambridge, UK), and β-actin (1:10,000, Catalog number: A5441; Sigma-Aldrich Co., Ltd., St Louis, MO, USA) at 4°C overnight. The next day, wash the polyvinylidene difluoride membranes in three washes of TBST, 10 minutes each. Then the polyvinylidene difluoride membranes were selectively incubated with a recommended dilution of labeled secondary antibody in 3% blocking buffer in TBST (anti-mouse antibody [1:5,000, catalog number: NA931; GE Healthcare, Tokyo, Japan] or a horseradish peroxidase-conjugated anti-rabbit antibody [1:5,000, catalog number: NA934; GE Healthcare]) at room temperature for 1 hour. After three final washes in TBST, 10 minutes each. The bands in the polyvinylidene difluoride membranes were detected by using enhanced chemiluminescence plus a Western Blotting Detection system (GE Healthcare).

Statistical Analysis

The data were presented as the mean ± standard error of the mean (SEM). The statistical analysis was performed using SPSS Statistics 20 (IBM Co., Armonk, NY, USA). Data of body weight were analyzed using repeated two-way analysis of variance (ANOVA), followed by *post-hoc* Fishers Least Significant Difference (LSD) test. Data of DAT, TH and p-α-Syn were analyzed by two-way ANOVA, followed by *post-hoc* Fishers LSD test. The p values of less than 0.05 were considered statistically significant.

RESULTS

Effects of SDV on Body Weight

Repeated measures two-way ANOVA showed no significant differences in the body weight changes among the four groups (Fig. 1B).

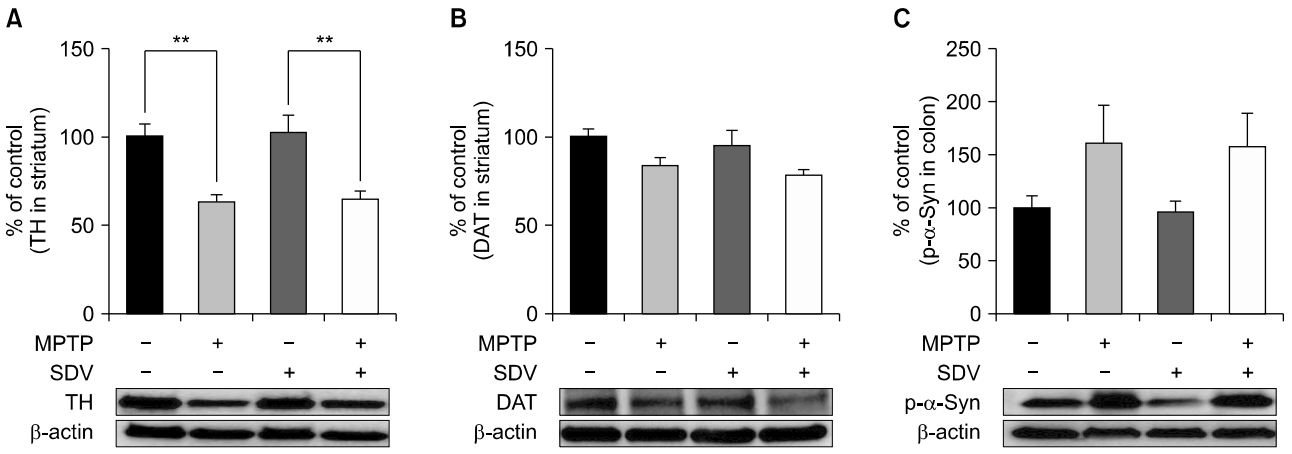

Fig. 2. Effects of SDV on neurotoxicity in the striatum and colon after MPTP administration. (A) Expression of TH in the striatum (two-way ANOVA, SDV: $F_{1,36} = 0.094$, $p = 0.761$; MPTP: $F_{1,36} = 26.85$, $p < 0.001$; interaction (SDV × MPTP): $F_{1,36} = 0$, $p = 0.992$). (B) Expression of DAT in the striatum (two-way ANOVA, SDV: $F_{1,36} = 0.729$, $p = 0.399$; MPTP: $F_{1,36} = 7.862$, $p = 0.008$; interaction (SDV × MPTP): $F_{1,36} = 0$, $p = 0.996$). (C) Expression of p-α-Syn in the colon (two-way ANOVA, SDV: $F_{1,36} = 0.016$, $p = 0.899$; MPTP: $F_{1,36} = 5.892$, $p = 0.02$, interaction (SDV × MPTP): $F_{1,36} = 0$, $p = 0.99$). Data represent the mean ± SEM (n = 10).
SDV, subdiaphragmatic vagotomy; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TH, tyrosine hydroxylase; DAT, dopamine transporter; p-α-Syn, phosphorylated α-synuclein.
**$p < 0.01$.

Effects of SDV on MPTP-induced Neurotoxicity in the Striatum and Colon

Two-way ANOVA of TH data in the striatum revealed statistical difference (SDV: $F_{1,36} = 0.094$, $p = 0.761$; MPTP: $F_{1,36} = 26.85$, $p < 0.001$; interaction (SDV × MPTP): $F_{1,36} = 0$, $p = 0.992$) among the four groups (Fig. 2A). Two-way ANOVA of DAT data in the striatum revealed statistical difference (SDV: $F_{1,36} = 0.729$, $p = 0.399$; MPTP: $F_{1,36} = 7.862$, $p = 0.008$; interaction (SDV × MPTP): $F_{1,36} = 0$, $p = 0.996$) among the four groups (Fig. 2B). These data suggest that SDV did not affect MPTP-induced reduction of TH and DAT proteins in the striatum of both groups (Fig. 2A, B).

Furthermore, two-way ANOVA of p-α-Syn data in the colon revealed statistical difference (SDV: $F_{1,36} = 0.016$, $p = 0.899$; MPTP: $F_{1,36} = 5.892$, $p = 0.02$; interaction (SDV × MPTP): $F_{1,36} = 0$, $p = 0.99$) among the four groups (Fig. 2C). Collectively, these data suggest that SDV did not affect the neurotoxicity in the striatum and colon after repeated MPTP treatment.

DISCUSSION

In this study, we found that SDV did not affect the reduction of TH and DAT in the striatum and increased expression of p-α-Syn in the colon after repeated administration of MPTP. The data suggest that subdiaphragmatic vagus nerve dose not play a role in the neurotoxicity in the striatum and colon after repeated MPTP administration.

It is suggested that pathologic α-Syn in the gastrointestinal tract might be transported into brain regions via the vagus nerve [13]. A recent study showed that truncal vagotomy prevented the gut—to—brain spread of α-Syn and its associated neurodegeneration and behavioral deficits [14]. Furthermore, truncal vagotomy or α-Syn deficiency could prevent behavioral abnormalities (i.e., cognitive deficits, depression-like phenotypes, olfactory dysfunctions) induced by α-Syn preformed fibrils (PFF) injection into the gut [14]. The data suggest that pathologic α-Syn is capable of spreading from the gastrointestinal tract via the truncal vagus nerve into the brain.

Previously, we reported that SDV significantly blocked the onset of depression-like phenotypes in antibiotic-treated mice after repeated oral administration of "depression-related microbes" [6,7,9], suggesting a key role of brain—gut—microbiota axis via subdiaphragmatic vagus nerve in depression-like phenotypes. Furthermore, we reported that SDV caused significant changes in relative abundance of several microbiome at genus and species levels although SDV did not alter alpha-diversity and beta-diversity of gut microbiota [8]. Moreover, microbiome depletion by antibiotic cocktail significantly attenuated MPTP-induced neurotoxicity in the brain [4], suggesting a role of gut microbiota in MPTP-induced neurotoxicity. In this study, MPTP was administered systemically to mice, indicating that MPTP may cause neurotoxicity in the striatum and colon directly by subdiaphragmatic vagus nerve-independent mechanisms. Thus, it is unlikely that subdiaphragmatic vagus nerve may play a role in MPTP-induced neurotoxicity in the brain and colon, although we did not perform the effect of truncal vagotomy on MPTP-induced neurotoxicity. Further detailed study is needed to confirm the relationship between MPTP-induced neurotoxicity in the brain and colon, and the gut—microbiome—brain axis. It is interesting to investigate the effects of SDV on α-Syn pathology and behavioral deficits induced by α-Syn PFF injection into the gut of mice.

It is reported that activation of N-methyl-D-aspartate receptor (NMDAR) glycine site can ameliorate neuropsychiatric symptoms of PD patients with dementia [15] and that gut microbiome with glutamate racemase can convert L-glutamate to D-glutamate which may influence the NMDAR and cognitive functions in PD patients [16]. Therefore, further study on the role of gut microbiota with glutamate racemase in PD is interesting.

In conclusion, the present study suggests that SDV did not affect neurotoxicity in the striatum and colon after repeated systemic administration of MPTP.

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■ **Conflicts of Interest**

No potential conflict of interest relevant to this article was reported.

■ Author Contributions

Design of the research and experiment: Kenji Hashimoto. Supervised the experimental analyses: Kenji Hashimoto. Performed the experiments: Jiajing Shan, Youge Qu, Jiancheng Zhang, Li Ma. Analyzed the data: Jiajing Shan. Wrote the paper: Jiajing Shan, Kenji Hashimoto. All authors read and approved this paper.

■ ORCID

Jiajing Shan	https://orcid.org/0000-0003-2875-1941
Youge Qu	https://orcid.org/0000-0002-3579-8628
Jiancheng Zhang	https://orcid.org/0000-0002-2820-0851
Li Ma	https://orcid.org/0000-0002-7157-7144
Kenji Hashimoto	https://orcid.org/0000-0002-8892-0439

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