



## Original

# Calcium carbonate supplementation causes motor dysfunction

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**Abstract:** We previously showed that a diet containing calcium carbonate causes impairments in spatial and recognition memory in mice. In this study, we investigated the effects of calcium carbonate supplementation on motor function. Motor function was determined using different tests that have been used to analyze different aspects of Parkinsonism. A catalepsy test for akinesia; a muscular strength assessment, pole test, beam-walking test, and gait analysis for motor coordination and balance assessment; and an open-field test for locomotor activity assessment were performed. The mice were fed diets containing 0.6% or 1.0% calcium carbonate for eight weeks, after which they were evaluated for motor functions. The diets containing calcium carbonate caused significant motor dysfunction, as revealed by the different tests, although the spontaneous locomotor activity did not change. Calcium carbonate supplementation decreased the dopamine content in the basal ganglia, including the striatum and substantia nigra, and the number of tyrosine hydroxylase-positive neurons in the substantia nigra. In addition, administration of L-dopa led to at least a partial recovery of motor dysfunction, suggesting that calcium carbonate supplementation causes motor dysfunction by decreasing the dopamine content in the basal ganglia. These results suggest that mice with calcium carbonate-induced motor dysfunction may be useful as a new animal model for Parkinson's disease and Huntington's disease.

**Key words:** calcium carbonate supplements, dopaminergic neurons, motor dysfunction, Parkinson's disease

## Introduction

Calcium ions play an important role in various cellular functions and are essential for learning and memory formation [1–5]. Calcium signaling regulates neuronal functions, such as synaptic plasticity, neuronal growth, and dysregulation of calcium homeostasis, including a marked increase in the intracellular calcium content, and causes neuronal cell death and synaptic dysfunction, leading to neurodegenerative disorders [6, 7]. Calcium dysregulation in the brain has also been reported to be associated with the development of Alzheimer's, Parkinson's (PD), and Huntington's diseases [8–13]. Previously, we reported the influence of calcium carbonate supplementation on memory in mice and found that a diet containing calcium carbonate decreased the

expression of CREB in the brain and caused memory impairment [14]. In addition, mice that were fed a diet containing calcium carbonate and nimodipine (an L-type calcium channel antagonist) recovered from calcium carbonate-induced memory impairments, suggesting that the excessive entry of calcium into cells may cause memory impairments. These results indicate that calcium carbonate supplementation directly or indirectly influences calcium signaling in cells.

PD is a neurodegenerative disorder, the cause of which remains unclear. Patients with PD show typical motor dysfunctions, such as catalepsy, rigidity, and slowing of movement. In addition, mild cognitive impairment has been observed in these patients [15]. The characteristic pathology of this disease is the abnormal accumulation of  $\alpha$ -synuclein, referred to as Lewy bodies, in dopami-

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nergic neurons and the selective degeneration of dopaminergic neurons in the substantia nigra [16, 17].

The involvement of calcium in the development of PD has been demonstrated in several studies. Calcium channel blockers reportedly suppress the degeneration of dopaminergic neurons in animal models of PD induced by 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [18, 19]. A recent study showed that toxic synuclein aggregation, which is thought to contribute to the development of PD, might be caused by a higher calcium concentration in the brain [20].

The results of several cohort studies also support the notion that calcium intake is a risk factor for PD. In one cohort study, it was shown that the intake of total calcium and dairy vitamin D, which promotes the absorption of calcium, was significantly associated with an increased risk of PD [21, 22]. Conversely, in another study, calcium from dairy and non-dairy foods was not associated with an increased risk of PD [23]. The correlation between calcium intake and the development of PD remains unclear.

There have been no studies focusing on the relationship between calcium carbonate supplementation and motor dysfunction in rodents. In this study, we investigated the influence of calcium carbonate supplementation on motor function in mice.

## Materials and Methods

### Experimental mice and diets

Male ICR mice (age: 4 weeks) were purchased from CLEA (Tokyo, Japan). Mice were housed in an air-conditioned room at  $23 \pm 1^\circ\text{C}$  with a relative humidity of 55–65% and a light-dark cycle of 12 h (lights on from 8 a.m. to 8 p.m.). After acclimatization for at least a week, the mice (5 to 7 weeks old) were randomly assigned to three groups of five to eight mice per group

provided a fixed amount of a modified AIN-76 diet [14, 24] with or without calcium carbonate (4.5 g/day) and free access to water: the control, 0.6% calcium carbonate, and 1% calcium carbonate diet groups. The diet compositions are shown in Table 1. The amounts of calcium administered to the mice were 480 mg/kg/day, 912 mg/kg/day, and 1,200 mg/kg/day in the control, 0.6% calcium carbonate, and 1.0% calcium carbonate diet groups, respectively. Each mouse was weighed weekly. All mice were monitored daily by performing health checks. After eight weeks, behavior tests were performed between 9 a.m. and 2 p.m. in a quiet room with lighting (150–200 lux). Serum calcium concentrations of five mice of each group were measured using a Calcium Assay kit (Cayman Chemical, Ann Arbor, MI, USA) after 8 weeks of feeding. At the end of the experiment, the mice were euthanized under isoflurane anesthesia. Brains were rapidly excised, and basal ganglia were isolated and stored at  $-80^\circ\text{C}$ . Animal experiments were conducted following the guidelines of the Muroran Institute of Technology (approval number H29KS03) and were approved by the Ethics Committee on the Care and Use of Experimental Animals of the Muroran Institute of Technology, Japan.

### Behavioral tests for motor function

Pole tests, which are used to evaluate mice for movement disorders, were performed as follows: Each mouse was placed on top of a vertical pole (diameter, 10 mm; height, 80 cm) with its head pointed upward, and the time it took the mouse to reorient itself with its head pointed downward was measured. If the mouse did not reorient itself with its head pointed downward within 60 s, the time was considered to be 60 s.

Catalepsy tests were performed as follows: Each mouse was placed in an unusual posture with its forepaws on a horizontal bar (height from floor, 5 cm; diameter, 6 mm), and the time it maintained the unusual

**Table 1.** Compositions of the diets offered to the ICR mice

Ingredient (%)	Control	0.6% calcium carbonate diet	1.0% calcium carbonate diet
Casein	26.2	26.2	26.2
Corn starch	32	32	32
Cellulose	6.6	6.6	6.6
Sucrose	25.4	25.4	25.4
AIN-76 mineral mixture	2.3	1.7	1.3
AIN76A vitamin mixture	1.3	1.3	1.3
L-cysteine	0.4	0.4	0.4
Choline	0.3	0.3	0.3
Tripotassium citrate	2.2	2.2	2.2
Corn oil	3.3	3.3	3.3
Calcium carbonate	0	0.6	1
Total (%)	100	100	100

posture was measured.

Open-field tests were performed as described previously [25]. Briefly, the floor in the open-field chamber was divided into eight equal areas by lines. Locomotor activity was measured as the number of times the mice crossed the lines. The movements of the mice were recorded for 5 min using a video camera.

Beam-walking tests were performed as follows: An elevated narrow beam (length, 50 cm; diameter, 10 mm) was fixed between two desks at a height of 50 cm from the floor, and a black box was placed on one side of the beam. The mice were placed on the beam and allowed to walk across to the black box on the desk. The number of hind paw slips was counted.

Gait analyses were performed as follows: The forepaws and hind paws of each mouse were dipped in black and red ink, respectively, and each mouse was then placed on a white sheet of paper and allowed to walk across it. Stride length, step width, and front and hind footprint overlap were measured. The distance between the forepaw and hind-paw footprints on each side was evaluated as the overlap asymmetry.

#### Buried pellet test

To measure the ability of mice to smell odors, we performed the buried pellet test. The mice were housed individually and restricted from food for 1 day prior to the experiment. A piece of an AIN-76 pellet was buried 1 cm below the bedding in the test cage (30 cm × 20 cm × 15 cm). Each mouse was placed in the center of the cage, and the time it took to detect the pellet was recorded. A second buried pellet test was also performed in which the pellet was buried in a different spot, and the average and standard deviation (SD) values were calculated.

#### Effect of L-dopa administration

Ten to sixteen mice per group were fed the control, 0.6% calcium carbonate, or 1.0% calcium carbonate diet for 2 months. The mice from each group were divided into subgroups, which were intraperitoneally injected with L-dopa (60 mg/kg) or a vehicle, respectively. The dosage was selected based on the report of Liu *et al.* [26]. After one hour, catalepsy and pole tests were performed.

#### Determination of monoamine content

The concentrations of L-dopa and the monoamines dopamine, noradrenaline, and serotonin were determined according to the method described by Benedetto *et al.* [27]. After the behavioral tests, the brain basal ganglia were excised and extracted in a solution containing 1.5

ng/ml isoproterenol and 0.1 M perchloric acid, and the extract was separated using a Shim-pack CLC-ODS column at a flow rate of 0.5 ml/min. The mobile phase consisted of 12 mM acetic acid, 190 mg/l octyl sulfonic acid, 0.26 mM EDTA, and 10% (v/v) methanol, adjusted to pH 3.5 with phosphoric acid. Detection was performed at an excitation wavelength of 280 nm and an emission wavelength of 315 nm.

#### Western blotting

Western blotting was performed as described previously [14]. Briefly, the basal ganglia were excised from four mice and homogenized in a solution containing 2% SDS and 50 mM Tris-HCl (pH 7.5). After centrifugation at  $14,000 \times g$  for 5 min, the extract was separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 5% (w/v) skim milk and incubated with  $\beta$ -actin or tyrosine hydroxylase (TH) primary antibody (Biorbyt, St. Louis, MO, USA). After treatment with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Biorbyt), the color was developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The band intensities were measured using the ImageJ software.

#### Real-time PCR

The basal ganglia of the mice fed with the control diet or a diet containing calcium carbonate were excised and stored at  $-80^{\circ}\text{C}$  until use. Total RNA was prepared from the basal ganglia using RNAiso Plus (Takara, Shiga, Japan), and first-strand complementary DNA was synthesized using the oligo (dT) primer. PCR was performed using specific primers, the sequences of which are listed in Table 2. Real-time PCR was performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA).

The reaction mixture (total volume 12.5  $\mu\text{l}$ ) consisted of 1  $\mu\text{l}$  of diluted template cDNA, 5  $\mu\text{l}$  SYBR Green Supermix, and 200 nM of each sense and antisense primer. The amplification program consisted of 40 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 seconds and annealing/extension at  $60^{\circ}\text{C}$  for 60 s. Each reaction was run in triplicate, and the average of the  $C_t$  values was calculated. The expression levels of target genes relative to  $\beta$ -actin mRNA were determined by the comparative  $C_t$  method.

#### MDA content

The malondialdehyde (MDA) content was estimated to determine lipid peroxidation as described previously [14]. The basal ganglia (100 mg/ml) were extracted us-

**Table 2.** PCR primer used in real-time PCR

Gene	Forward primer	Reverse primer
$\beta$ -actin	5'-agccatgtacgtagccatcc-3'	5'-ctctcagctgtggtggtgaa-3'
Dopamine transporter (DAT)	5'-tgtgaggcatctgtgtggat-3'	5'-acaacgaagccagaggagaa-3'
Vesicular monoamine transporter (VMAT)	5'-acttactgtgttccagggg-3'	5'-cagcccaactgcacattct-3'
Dopamine D1 receptor	5'-accctgtcgaatctctcaa-3'	5'-tggggttcaggaggaaatc-3'
Dopamine D2 receptor	5'-aaaccggacctcccttaag-3'	5'-agaggactggtgggatgtt-3'
Heme oxygenase 1 (HO1)	5'-cacgcatatacccgtacct-3'	5'-ccagagtgttcattcgagca-3'
catalase	5'-aggtgtgaacgaggaggag-3'	5'-tgcgttaggtgtgaattgc-3'
Mn-superoxide dismutase (Mn-SOD)	5'-ggccaaggagatgttaca-3'	5'-gcttgatagcctccagcaac-3'
CuZn-SOD	5'-cggatgaagagaggcatgtt-3'	5'-caccttgcccaagtcattct-3'
Calretinin	5'-acaagatggaagcggctat-3'	5'-gagcacaatccaggctct-3'
Calmodulin	5'-aagcctctccctcttcgac-3'	5'-ggaaggcctctctatctcc-3'
Calbindin	5'-ctctgcagaaatgaagagca-3'	5'-cgccattcttatccagctcc-3'

ing 20% sucrose solution and mixed with 0.5% thiobarbituric acid, including 5% trichloroacetic acid. The mixture was heated at 95°C for 0.5 h and then cooled in ice to room temperature. After centrifugation at 14,000  $\times g$  for 10 min, the absorbance of the supernatant was measured at 540 nm. The extinction coefficient of MDA at 540 nm was 155 mM<sup>-1</sup> cm<sup>-1</sup>.

### TH immunostaining

The three mice from each group were anesthetized with sevoflurane. After transcardiac perfusion fixation with 4% paraformaldehyde in PBS, brains were removed and placed in 4% paraformaldehyde solution for at least 3 days. The brains were then embedded in paraffin, and more than 20 slices per region, including the substantia nigra (-3.0 to -3.6 mm from the bregma), striatum (0.2 to 0.9 mm from the bregma), and hippocampus (-1.8 to -2.5 mm from the bregma), were cut into 5  $\mu$ m-thick slices. The regions of interest (ROIs) in the slices were selected and analyzed. Immunohistochemical staining of TH was performed as follows: The cut tissues were washed twice with PBS for 10 min and then incubated with PBS containing 0.6% H<sub>2</sub>O<sub>2</sub> for 5 min, followed by washing with PBS twice for 10 min. They were then incubated in PBS containing 2% horse serum for 30 min and were incubated with a polyclonal anti-mouse antibody against TH overnight at 4°C. Subsequently, they were stained with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, washed three times with PBS for 10 min, stained with 0.02% 3,3'-diaminobenzidine tetrahydrochloride solution, and observed under an optical microscope. Slices including the hippocampus were stained with hematoxylin and eosin.

### Statistical analysis

Each experiment was replicated several times. All statistical analyses were performed using the BellCurve for Excel statistics software (SSRI, Tokyo, Japan). Data

are expressed as the mean  $\pm$  SD. After the Shapiro-Wilk test for normality was performed, data were analyzed using one-way analysis of variance and the Tukey-Kramer multiple comparison test for groups with normal distributions and the Mann-Whitney *U*-test for comparisons of nonparametric data. Results were considered significantly different at  $P < 0.05$ .

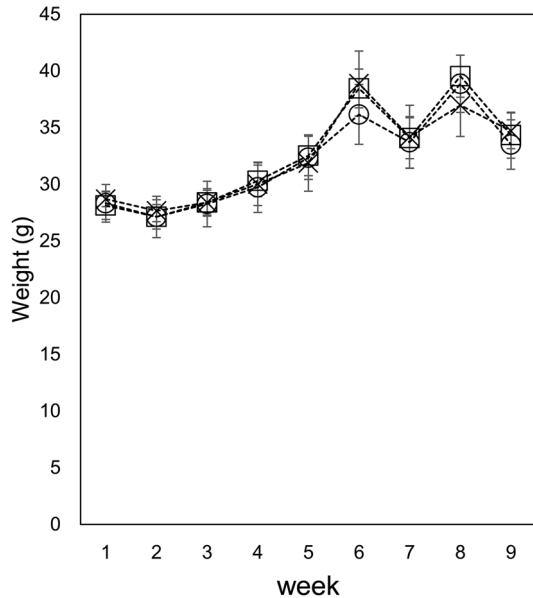
## Results

### Behavioral tests

We previously showed that a diet containing 1.0% calcium carbonate caused memory impairments in the object recognition, Y-maze, and Barnes maze tests in mice [14]. Papakonstantinou *et al.* showed that a high calcium diet reduces body weight [28]. Under the conditions in this study, calcium carbonate supplementation resulted in no changes in body weight during the eight weeks of feeding (Fig. 1). The serum calcium level also did not change. The steady-state serum calcium concentrations of the mice fed the control diet, 0.6% calcium carbonate diet, and 1.0% calcium carbonate diet were 12.0  $\pm$  0.76 mg/dl, 12.4  $\pm$  0.78 mg/dl, and 13.5  $\pm$  1.15 mg/dl, respectively, after eight weeks of feeding. Therefore, we used the diets containing 0.6% and 1.0% calcium carbonate to investigate the influence of calcium carbonate intake on motor function in this study. Behavior tests that have been used to analyze Parkinsonism with symptoms such as akinesia, bradykinesia, and rigidity were performed.

First, the catalepsy test was performed. The control mice maintained the unusual posture with their forepaws placed on the horizontal bar for approximately 0.4 s (Fig. 2a). The mice fed the diets containing calcium carbonate maintained the unusual posture for a prolonged period of time, with their times being approximately four times that of the control mice. Next, we performed the pole test to determine the degree of bradykinesia (slowness of movement), which is a typical symptom of PD (Fig.

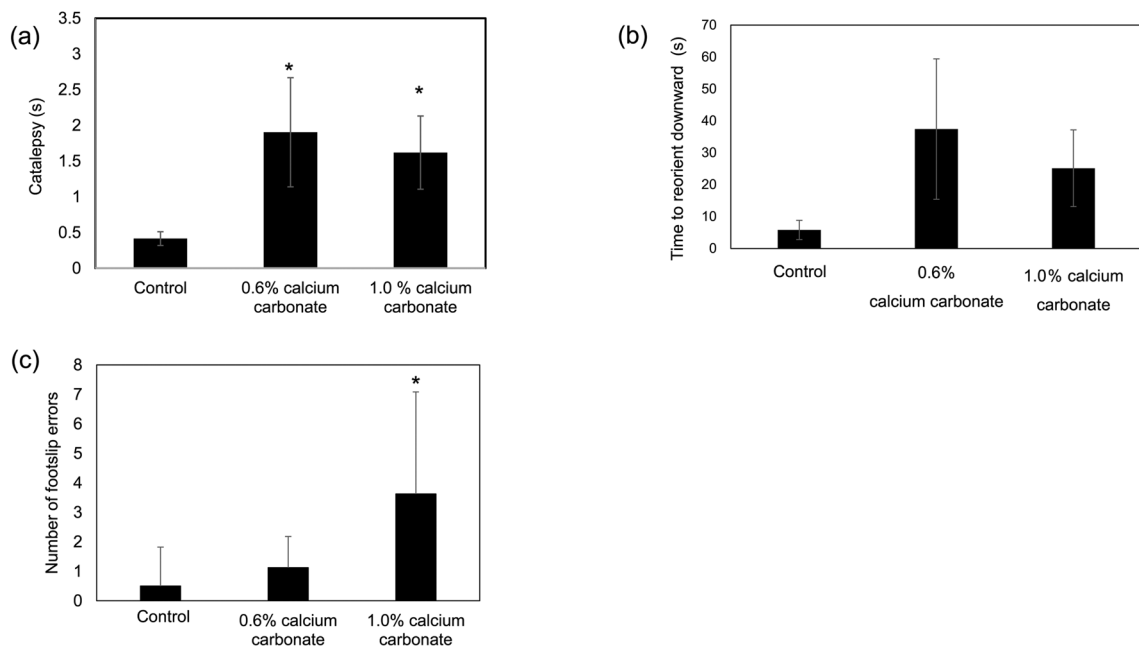
2b). Although no significant differences were observed, the time taken to reorient with the head pointed downward was longer in the mice fed the 1.0% calcium carbonate diet than in the control mice. Two of the eight



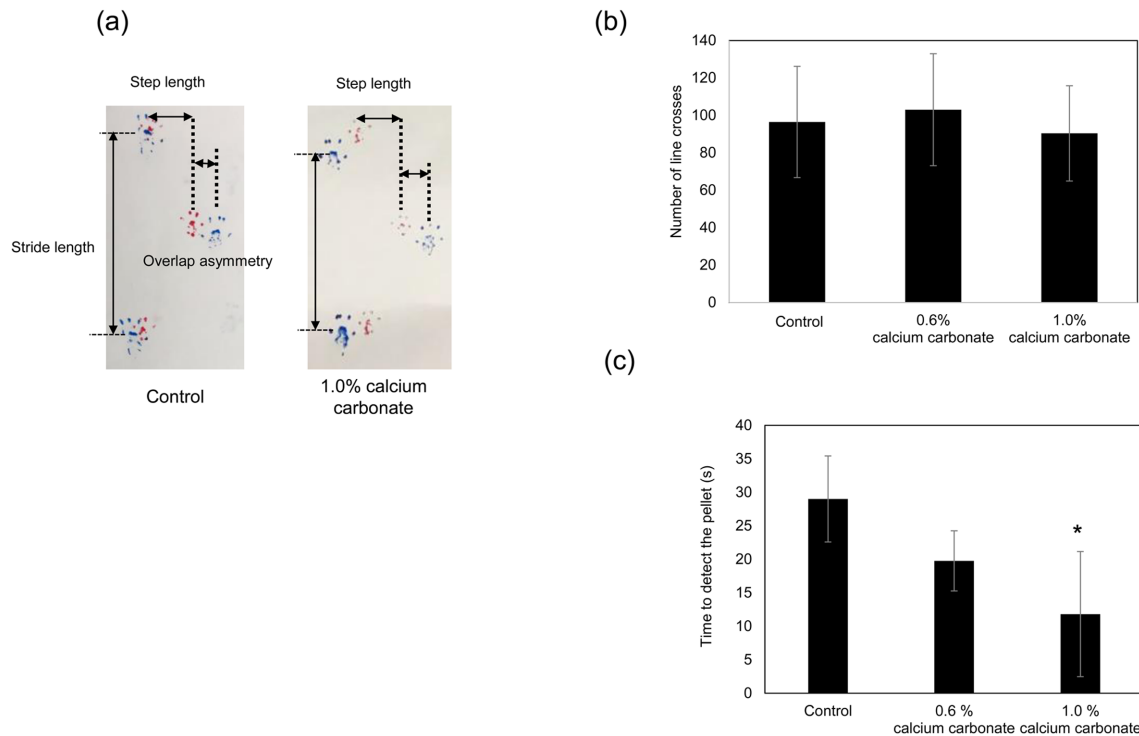
**Fig. 1.** Influence of calcium carbonate supplementation on body weight. Mice were provided the control diet (○), 0.6% calcium carbonate diet (×), or 1.0% calcium carbonate diet (□). The values of eight mice are presented as means ± SD (n=8).

animals did not reorient themselves with their heads pointed downward within 60 s among the mice fed the 1.0% calcium carbonate diet. These results suggest that the intake of calcium carbonate causes motor dysfunction in PD. To further confirm these results, we performed the beam-walking test and a gait analysis to evaluate the balance and motor coordination skills of the mice. In the beam-walking test, the mice fed the 1.0% calcium carbonate diet showed a significantly higher number of foot slips than the mice fed the control diet (Fig. 2c). Three of the eight mice fed the 1.0% calcium carbonate diet could not walk across the narrow beam. The time spent traversing the narrow beam could not be evaluated for these mice because they fell from the bar.

A gait analysis, which provides information regarding the balance of mice, was also performed (Fig. 3a). The representative footprint patterns of the control mice and mice fed the calcium carbonate diets were analyzed (Table 3). Although the stride length did not change, the step width of the forepaws was significantly shorter in the mice fed with the 1.0% calcium carbonate diet. The distance between the forepaw and hind paw placements on each side was measured to evaluate the accuracy of foot positioning and the uniformity of the steps. An increase in this distance was reported to be an early detectable behavioral abnormality. Our analysis showed a significant increase in the distance between the mice fed



**Fig. 2.** Effect of calcium carbonate supplementation on the catalepsy test (a), pole test (b), and beam-walking test findings (c). (a) The duration of catalepsy was measured when the forepaws of mice were placed on a horizontal bar. (b) The time reorient with the head pointed downward in the pole test was measured after the mice were placed with their head oriented upward on the pole. (c) The number of times the mice slipped off the beam was measured when they were made to walk across a narrow beam. The values of eight mice are presented as means ± SD (n=8). Asterisks indicate statistically significant differences relative to the control ( $P < 0.05$ ).



**Fig. 3.** Effect of calcium carbonate supplementation on the gait analysis (a), locomotor activity (b), and buried pellet test (c) findings. (a) In the gait analysis, paint was applied to the forepaws (red) and hind paws (blue) of the mice, and the mice were then allowed to walk on a piece of white paper to record the placement pattern of their footprints. The step length, step width of the hind paw and forepaw, and overlap asymmetry were evaluated (Table 3). (b) The number of line crosses of the mice within 5 min was measured. (c) After the mice were placed in the test box where the pellet was buried, the latency to pellet detection was measured. The values of eight mice are presented as means  $\pm$  SD ( $n=8$ ). Asterisks indicate statistically significant differences relative to the control ( $P<0.05$ ).

**Table 3.** Step length, stride length, and overlap asymmetry measurements

	Control	0.6% calcium carbonate	1.0% calcium carbonate
Stride length (forepaw)	6.34 $\pm$ 0.85	6.95 $\pm$ 0.73	6.59 $\pm$ 0.78
Stride length (hind paw)	6.42 $\pm$ 0.78	6.98 $\pm$ 0.67	6.58 $\pm$ 0.70
Step length (forepaw)	1.49 $\pm$ 0.49	1.5 $\pm$ 0.39	1.29 $\pm$ 0.43*
Step length (hind paw)	2.90 $\pm$ 0.45	3.10 $\pm$ 0.31	3.00 $\pm$ 0.64
Overlap asymmetry	0.68 $\pm$ 0.27	0.81 $\pm$ 0.25	0.86 $\pm$ 0.39*

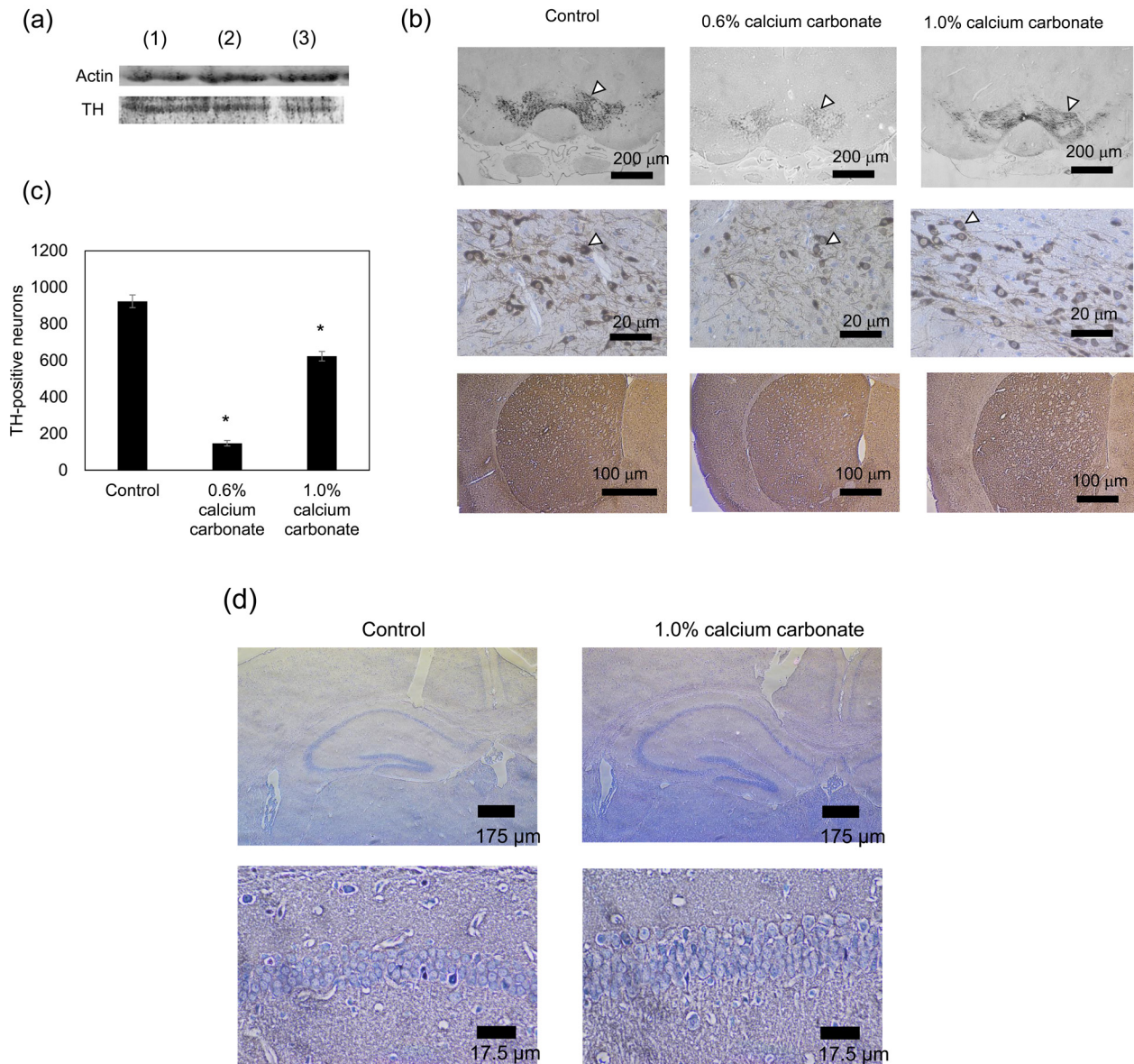
Values are given as means  $\pm$  SD for eight mice. Asterisks indicate statistically significant differences relative to the control ( $P<0.05$ ).

the calcium carbonate diets. Next, we investigated the locomotor activity using the open-field test. These results revealed no difference in locomotor activity between the mice fed the control and calcium carbonate diets (Fig. 3b).

Finally, we performed the buried pellet test to determine whether the intake of calcium carbonate causes olfactory impairment as in the MPTP-treated PD model. The calcium carbonate-fed mice showed a shorter latency to pellet detection than the control mice (Fig. 3c), suggesting that calcium carbonate intake does not cause damage to odor discrimination but conversely increases the sensitivity of it.

### Effect of calcium carbonate intake on dopaminergic neurons in the striatum and substantia nigra

We used western blotting and immunostaining to observe the effect of calcium carbonate intake on dopaminergic neurons. Western blotting showed a decrease in TH expression in the basal ganglia (Fig. 4a). Consistent with this result, immunostaining revealed a similar change in the TH expression levels (Fig. 4b). The number of TH-positive neurons in the substantia nigra was lower in the mice fed the calcium carbonate diets. The number of TH-positive neurons in the control mice was approximately 5.0-fold higher than that in the 0.6% calcium carbonate-fed mice (Fig. 4c). Loss of dopaminergic neurons was larger in the 0.6% calcium carbonate-fed



**Fig. 4.** Tyrosine hydroxylase (TH) expression in the striatum of the mice fed the control and calcium carbonate diets. (a) Western blotting of the basal ganglia extracted from the mice fed the control diet (1), 0.6% calcium carbonate diet (2), or 1.0% calcium carbonate diet (3). (b) Immunostaining of TH in the substantia nigra (upper and middle panels) and the striatum (lower panel) of the mice fed the control diet, 0.6% calcium carbonate diet, or 1.0% calcium carbonate diet. TH-positive neurons are indicated by an arrowhead. Scale bar=20 (middle panel) and 100  $\mu$ m (lower and upper panels). (c) The number of TH-positive neurons in the substantia nigra was calculated in five different fields for each mouse. (d) The neuronal cell morphology in the entire (upper panel) and CA1 regions (lower panel) in the hippocampus of the mice fed with the control diet and 1.0% calcium carbonate diet was observed with hematoxylin-eosin staining. Scale bar=17.5 and 175  $\mu$ m.

mice than in the 1.0% calcium carbonate-fed mice (Fig. 4b). The TH expression levels of neurites and cell bodies were also weaker in the 1.0% calcium carbonate-fed mice than in the control mice (Fig. 4b, middle panel). Intake of calcium carbonate also resulted in lower levels of TH expression in the striatum of the calcium carbonate-fed mice than in that of the control mice (Fig. 4b, lower panel). These results suggest that the intake of calcium carbonate causes the loss of dopaminergic neurons in the substantia nigra, resulting in motor dysfunction.

Conversely, the intake of calcium carbonate did not cause degeneration of hippocampal neurons (Fig. 4d).

#### Catecholamine content in the basal ganglia

To determine whether motor dysfunction in the mice fed the calcium carbonate diets was correlated with alterations in the content and metabolism of dopamine in the striatum, we assessed the dopamine, L-dopa, serotonin, and noradrenaline concentrations in basal ganglia homogenates. Intake of calcium carbonate decreased the

dopamine concentration in the basal ganglia. The dopamine concentration was approximately half that of the control mice (Fig. 5). In contrast, the noradrenaline and serotonin concentrations did not change.

### Effect of calcium carbonate intake on the mRNA expressions of dopamine receptors, calcium-binding proteins, and antioxidants

To further confirm that the intake of calcium carbonate affects brain function, we examined the change in mRNA expression in the basal ganglia using real-time PCR (Fig. 6). The intake of calcium carbonate increased the expression of the dopamine D1 receptor but did not change the expression of the D2 receptor, dopamine transporter, or vesicular monoamine transporter. The expression of calcium-binding proteins was also found to be affected. Although the expression of calretinin and calmodulin did not change, the expression of calbindin increased. To investigate whether the intake of calcium carbonate causes oxidative stress in the brain, we also investigated the MDA content and the expression of antioxidant enzymes. The MDA content did not differ between the control and calcium carbonate-fed mice, and the expression of antioxidant enzymes was not significantly different in the calcium carbonate-fed mice.

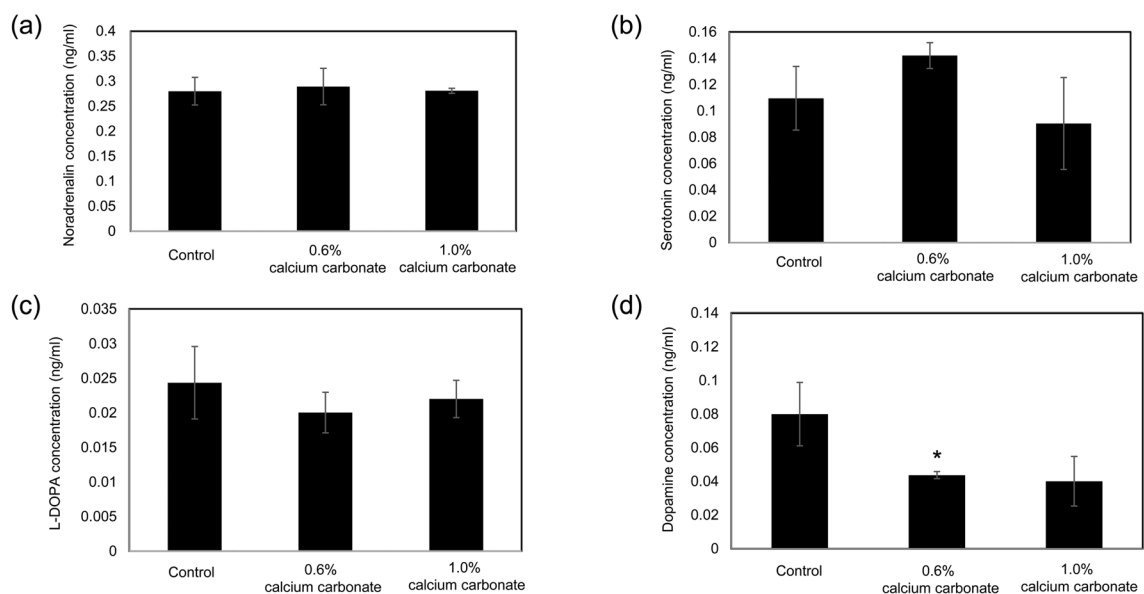
### Effect of L-dopa administration on calcium carbonate-induced motor dysfunction

Our results suggest that the intake of calcium carbonate causes the loss of dopaminergic neurons in the sub-

stantia nigra and a decrease in dopamine content, resulting in motor dysfunctions. To confirm this hypothesis, we investigated the effect of L-dopa administration on calcium carbonate-induced motor dysfunction using the catalepsy and pole tests (Fig. 7). An increase in catalepsy induced by the intake of calcium carbonate was counteracted by the administration of L-dopa. In addition, the administration of L-dopa resulted in a decrease in the time required to reorient with the pointed head downward in the pole test in the mice fed the calcium carbonate diets, although significant differences were not observed. These results support our hypothesis.

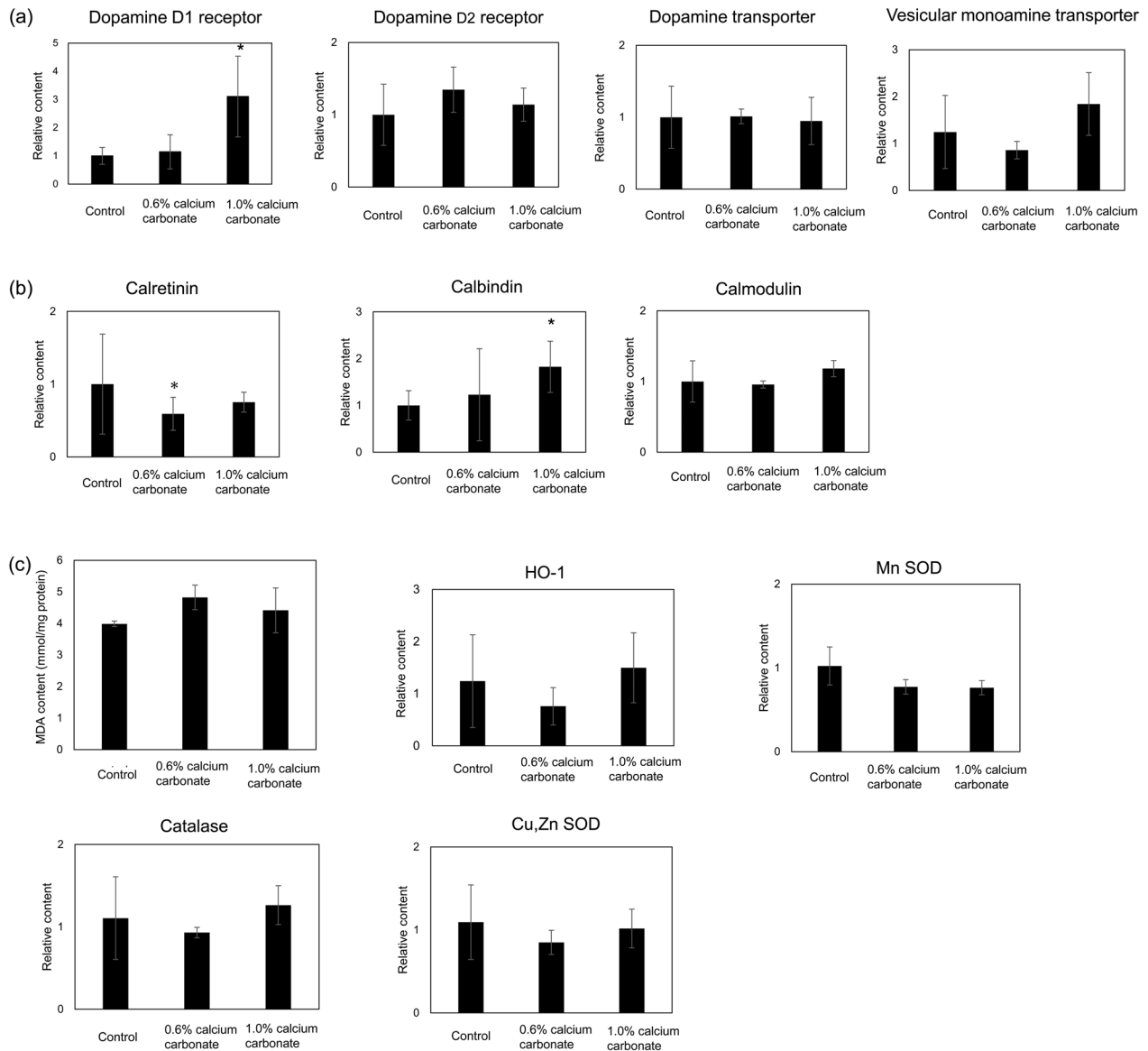
## Discussion

Some pharmacological models of PD, such as reserpine-treated, haloperidol-treated, 6-OHDA-treated, and MPTP-treated rodent models, have been widely used [29–32]. These animal models display Parkinsonian symptoms, such as akinesia, bradykinesia, rigidity, and changes like reductions in striatal dopamine and TH contents, and degeneration of dopaminergic neurons due to inhibition of the monoamine transporter, antagonism of dopamine D2 receptor, or disruption of the dopaminergic nigrostriatal pathway. Our results suggest that the intake of calcium carbonate also causes rigidity and bradykinesia through a decrease in dopamine content and TH expression in the basal ganglia, similar to other pharmacological models of PD. In addition, the calcium carbonate-fed mice showed memory impairment [14],

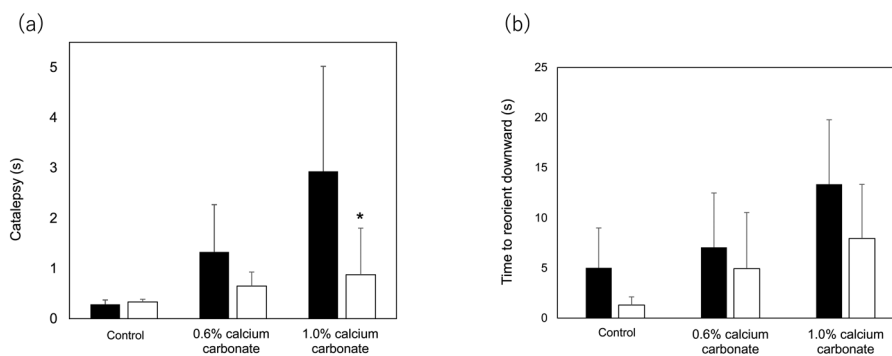


**Fig. 5.** Monoamine content in the striatum of the mice fed the control and calcium carbonate diets. The contents of noradrenaline (a), serotonin (b), L-dopa (c), and dopamine (d) in the striatum were measured as described in the Materials and Methods. The values of five mice are presented as means  $\pm$  SD ( $n=5$ ). Asterisks indicate statistically significant differences relative to the control ( $P<0.05$ ).





**Fig. 6.** Changes in the expression of dopamine-related proteins (a) and calcium-binding proteins (b) in the basal ganglia of the mice fed the control and calcium carbonate diets. The mRNA expression levels were measured using real-time polymerase chain reaction. (c) Oxidative stress in the brain of the mice fed the control and calcium carbonate diets was estimated on the basis of the malondialdehyde content and the expression levels of antioxidant enzymes. The values of five to eight mice are presented as means  $\pm$  SD. Asterisks indicate statistically significant differences relative to the control ( $P < 0.05$ ).



**Fig. 7.** Effect of L-dopa administration on calcium carbonate-induced motor dysfunction. The mice fed the control, 0.6% calcium carbonate, and 1.0% calcium carbonate diets for 2 months were administered L-dopa, and the catalepsy (a) and pole (b) tests were performed. Open and closed bars show the values of the treated and untreated mice, respectively. The values of five to eight mice are presented as means  $\pm$  SD. Asterisks indicate statistically significant differences relative to the control ( $P < 0.05$ ).

as reported previously in MPTP- and 6-OHDA-treated rodent models [33, 34]. However, some differences were observed between the groups. The spontaneous locomotor activity in mouse models of PD was reported to decrease in the open-field test [35, 36], and this was also observed for the stride length in a gait analysis [37]. Meanwhile, the mice fed the diets containing calcium carbonate in the present study did not show any distinct changes. Treatment of mice with morphine caused increases in locomotor activity and catalepsy [38]. Although administration of MPTP to BALB/c mice led to a reduction in locomotor activity in the open-field test, there was no change in bradykinesia in the pole test [39]. These results agree with our results that showing that locomotor activity is not correlated with catalepsy and bradykinesia.

The calcium carbonate-fed mice in the present study showed differences in oxidative stress in the brain compared with MPTP-treated mice. MPTP treatment has been reported to cause oxidative stress in the brain, to increase the MDA content, and to decrease the expression of antioxidant enzymes [40]. Conversely, calcium carbonate intake did not cause oxidative stress or change the expression of antioxidant enzymes in the brain. Smell impairment often appears earlier than motor dysfunction in PD [41, 42]. MPTP-treated mouse models have also been reported to exhibit smell impairment [43]. In contrast, calcium carbonate intake increased odor sensitivity. These results suggest that the motor dysfunction caused by calcium carbonate intake is due to a mechanism different from that of other pharmacological models of PD. The mechanisms that underlie odor sensitivity are not well understood. Calcium carbonate-fed mice may be useful for studying odor sensitivity.

In a previous cohort study, it was reported that the total intake of calcium derived from all daily foods was associated with a higher risk of Parkinson's disease [21]. Conversely, Park *et al.* reported that a higher intake of milk is associated with a risk of PD; however, the intake of calcium was not related to this risk [23]. Although the relationship between calcium intake and the risk of PD remains unclear, the results obtained from the mice fed the calcium carbonate diets might be useful for deciphering this relationship. The pathological hallmarks of PD are deposits of aggregated  $\alpha$ -synuclein, known as Lewy bodies, and the loss of dopaminergic neurons in the substantia nigra. Braak *et al.* proposed that the  $\alpha$ -synuclein pathology in PD emerges in the gastrointestinal tract during the early stages of PD and is transmitted to the brain through the vagus nerve, resulting in the loss of dopaminergic neurons in the substantia nigra [42]. In this study, we showed that calcium carbonate intake

decreased the number of TH-positive dopaminergic neurons in the substantia nigra. Therefore, it would be interesting to investigate whether  $\alpha$ -synuclein aggregation occurs in the gastrointestinal tract and brain of mice fed calcium carbonate diets in order to investigate the relationship between calcium intake and the risk of PD.

Hanahisa and Yamaguchi reported that oral administration of calcium transiently produced a significant increase in brain calcium content [44]. In SH-SY5Y neuroblastoma cells, excess extracellular calcium increased the intracellular calcium content through L-type calcium channels [45]. In this study, intake of calcium carbonate increased calbindin expression in the brain. An increase in extracellular calcium ion concentrations was reported to increase the expression of calbindin in kidney cells [46]. This result suggests that the intake of calcium carbonate may increase extracellular calcium concentrations in the brain. If successive intake of a diet containing calcium carbonate causes a higher extracellular calcium content in the brain, the intracellular calcium in the neuronal cells of the brain might increase and induce  $\alpha$ -synuclein aggregation [47] and neuronal cell death. In addition, dopaminergic neurons in the nigrostriatal system are known to indicate autonomous pacemaking activity caused by calcium influx through L-type calcium channels [48]. The amplitude of the calcium current was reported to increase with increasing extracellular calcium concentration in the sinoatrial node cells of rabbits with pacemaking activity [49]. Higher concentrations of extracellular calcium may also influence the activity and cause toxicity in dopaminergic neurons.

In this study, we used 0.6% and 1.0% calcium carbonate diets, and the mice were fed the diets with calcium carbonate for 8 weeks. The mice fed the 0.6% calcium carbonate diet showed a greater loss of dopaminergic neurons in the substantia nigra than the mice fed the 1.0% calcium carbonate diet. Song *et al.* showed that feeding a high calcium diet caused a decrease in the efficiency of intestinal calcium absorption [50]. The difference in the loss of dopaminergic neurons in the substantia nigra in the present study may be due to a difference in calcium absorption between the mice fed the 0.6% calcium carbonate diet and 1.0% calcium carbonate diet.

To investigate the influence of calcium carbonate supplementation on mice, we used young mice (5–7 weeks) in this study. Several studies have shown that aged mice show decreased motor performance in the pole test and a remarkable decrease in nigral dopaminergic neurons as compared with young mice in MPTP- and 6-OHDA-induced Parkinson's disease mouse mod-

els [51, 52]. It would be interesting to investigate whether calcium carbonate supplementation causes prominent effects in aged mice compared with young mice.

A limitation of this study was that it was unable to clarify the relationship between the intake of calcium carbonate and Parkinson's disease. It is necessary to further investigate the relationship between the calcium content in the diet and dopamine content in the basal ganglia, which causes motor dysfunction. In addition, we plan to investigate the relationship between the feeding period for calcium carbonate diets and motor dysfunction.

In this study, we showed that a diet containing calcium carbonate causes motor dysfunction. However, the mechanism by which calcium carbonate acts and causes motor dysfunction remains unclear. Further studies will be needed to clarify the cause of motor dysfunction.

### Conflicts of Interest

The authors declare no conflict of interest.

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