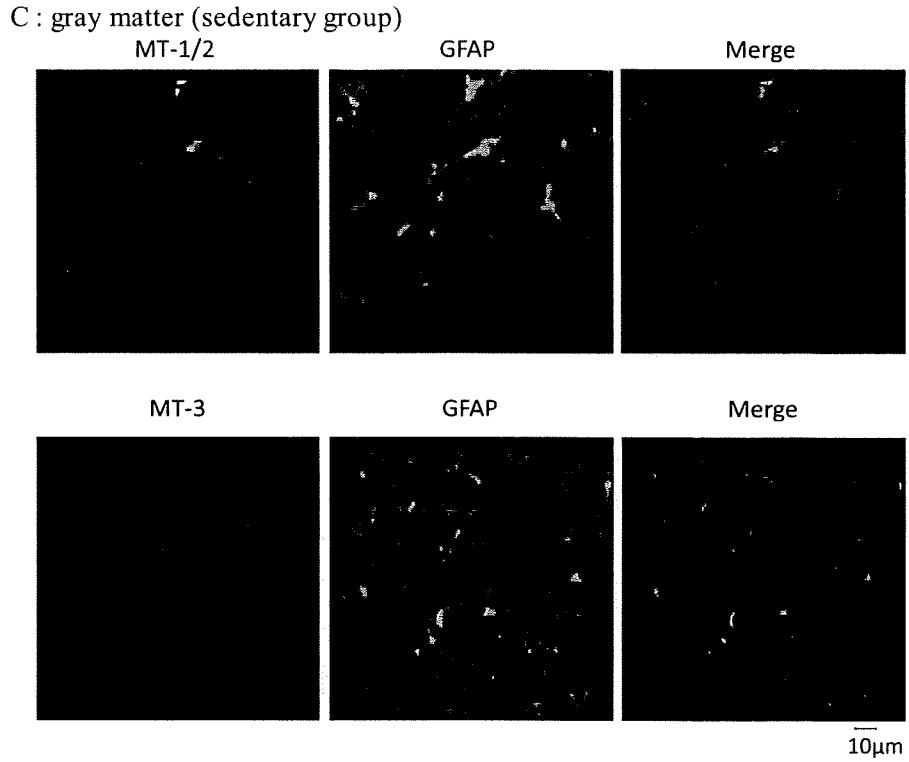


**Fig. 5.** (*Continued*).

al. (2002) showed the downregulation of MT-3 mRNA in the SALS spinal cord but did not recognize the change only in motor neurons (Jiang et al., 2005), which suggests the localization and importance of MT-3 in astrocytes. The mechanisms of induction of MTs in astrocytes remain to be elucidated.

MTs are considered to prevent neuronal death in some neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease in addition to ALS (Handschin and Spiegelman, 2008). MT-3 was found to be a protein that was dramatically diminished in the AD brain (Uchida et al., 1991). The adenovirus encoding MT-3 cDNA was found to prevent the degeneration of motor neurons following avulsion of a facial nerve (Sakamoto et al., 2003) and brain damage following a stab wound (Hozumi et al., 2006).

MT-3 is found to be induced by brain injury (Hozumi et al., 1995), sleep deprivation (Cirelli and Tononi, 2000) and eugenol (Irie et al., 2004), whereas MT-1/2 are induced by various stimuli such as heavy metals, cytokines and toxic agents (West et al., 2008). In this study, we demonstrated that exercise is one physiological method to induce MTs.

Exercise is reported to improve mental function in adults at risk of dementia, which might affect cognitive function (Burns et al., 2008). The study leads us to consider that exercise may also have beneficial effects on mental function via induction of MTs. The demonstration that MTs are induced by exercise provides a new clue to the development of treatments of neurodegenerative diseases such as ALS, AD and Parkinson's disease.

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*Toxicogenomics/proteomics Report*

# DNA microarray analysis of transcriptional responses of mouse spinal cords to physical exercise

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**ABSTRACT** — We present herein transcriptional changes in mouse spinal cords in response to physical exercise on a treadmill using a DNA microarray. By 30-min exercise, the expression of 3 genes was enhanced and expression of 29 genes reduced. By continuous 2-week exercise (30-min exercise per day), the expression of 1 gene was enhanced and expression of 13 genes reduced.

**Key words:** Exercise, Treadmill, Spinal cord, DNA microarray, Amyotrophic lateral sclerosis

## INTRODUCTION

Recent studies suggest that physical exercise has a beneficial effect on the progression of amyotrophic lateral sclerosis (ALS) (McCrate and Kaspar, 2008). In addition, regular exercise can increase the lifespan of a transgenic mouse model of familial ALS (FALS) (G93A SOD1) and delay the onset (Kirkinezos *et al.*, 2003). ALS is a progressive and fatal neurodegenerative disease, characterized by selective motor neuron degeneration. The majority of ALS cases are sporadic ALS (SALS), and approximately 10% of ALS cases are FALS, 10-20% of which are the result of mutations in the Cu/Zn superoxide dismutase (SOD1) gene. Although the etiology of SALS remains unclarified, several possible mechanisms of motor neuron degeneration in SALS have been proposed, including oxidative stress, heavy metal toxicity, the conformational disorder of proteins and dysfunction of neurotrophic factors.

However, the mechanism by which exercise affects motor neurons is poorly understood. Herein, we examined how acute or continuous physical exercise affects gene expression in the spinal cords of normal mice on the 1st day and the 14th day.

## MATERIALS AND METHODS

### Animals and exercise

C57BL/6J male mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Mice were housed in a temperature-controlled (23 ± 3°C) room with a 12-hr light/12-hr dark cycle. Food and water were provided *ad libitum* throughout this study. The treadmill running exercise program was administered as described previously (Kirkinezos *et al.*, 2003). Briefly, the male mice in the exercise groups ran on a treadmill machine (MK-680S, Muromachi, Tokyo, Japan) at 13 m/min and 30 min/day. The exercise program was carried out from 9 to 10 o'clock. A control group remained sedentary. The exercise was started at 7 weeks of age. For transcriptional change analysis following 30 min of exercise stimulation, mice were anesthetized and sacrificed, and their spinal cords removed 12 hr after exercise. On the other hand, for transcriptional change analysis following 2 weeks of 30-min daily exercise, mice were prepared 24 hr after the last exercise. The experimental designs and all procedures were in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the guidelines on the handling of experimental animals issued by the Japanese Association for Laboratory Animal Science and the Animal Experimental Committee of Gifu University. This study was approved by the Animal Experimental Committee of Gifu University.

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**Table 1.** Changes in gene expression after single 30-min exercise

| Accession No. | Gene Name | Difinition | Function (GO process) | -fold changes |
|---|---|---|---|---|
| NM_198417 | C030039L03Rik | RIKEN cDNA C030039L03 gene | regulation of transcription, DNA-dependent | 2.05 |
| NM_008760 | Ogn | osteoglycin | | 2.41 |
| NM_026172 | Decr1 | 2,4-dienoyl CoA reductase 1, mitochondrial | metabolic process | 2.02 |
| NM_133362 | Erdr1 | erythroid differentiation regulator 1 | biological process | 0.49 |
| NM_011176 | St14 | suppression of tumorigenicity 14 (colon carcinoma) | proteolysis, cell migration | 0.47 |
| NM_001081100 | Morn1 | MORN repeat containing 1 | DNA methylation | 0.49 |
| NM_029850 | Bcl7a | B-cell CLL/lymphoma 7A | biological process | 0.43 |
| NM_009405 | Tnni2 | troponin I, skeletal, fast 2 | hydrogen transport | 0.44 |
| NM_009606 | Acta1 | actin, alpha 1, skeletal muscle | muscle contraction, cytoskeleton organization and biogenesis | 0.37 |
| NM_015745 | Rbp3 | retinol binding protein 3, interstitial | proteolysis, lipid metabolic process, transport, visual perception | 0.46 |
| BC070477 | 201031E24Rik | RIKEN cDNA 2010317E24 gene | | 0.48 |
| NM_016886 | Gria3 | glutamate receptor, ionotropic, AMPA3 (alpha 3) | transport, ion transport | 0.46 |
| XM_357781 | Usp31 | ubiquitin specific peptidase 31 | ubiquitin-dependent protein catabolic process | 0.44 |
| NM_146176 | Cnot3 | CCR4-NOT transcription complex, subunit 3 | transcription | 0.47 |
| NM_010466 | Hoxc8 | homeo box C8 | transcription, regulation of transcription | 0.34 |
| NM_133818 | AI597479 | expressed sequence AI597479 | | 0.46 |
| NM_134142 | Tmem109 | transmembrane protein 109 | | 0.45 |
| NM_013868 | Hspb7 | heat shock protein family, member 7 | protein folding | 0.49 |
| NM_172283<br>NM_181666 | Fuk | fucokinase | metabolic process, phosphorylation | 0.47 |
| NM_030037 | Mospd3 | motile sperm domain containing 3 | heart development | 0.47 |
| X68364 | Pou2f1 | POU domain, class 2, transcription factor 1 | transcription | 0.45 |
| NM_178117 | BC035947 | cDNA sequence BC035947 | | 0.49 |
| NR_002843. 1<br>(ensenbl_refseq) | Rnu3b4 | U3B small nuclear RNA 4 | | 0.50 |
| NM_017379 | Tuba8 | tubulin, alpha 8 | microtubule cytoskeleton organization and biogenesis | 0.49 |
| NM_011119 | Pa2g4 | proliferation-associated 2G4 | transcription | 0.41 |
| NM_134077 | Rbm26 | RNA binding motif protein 26 | translational initiation | 0.43 |
| XM_140321 | EG225468 | predicted gene, EG225468 | | 0.45 |

**Table 1.** (Continued)

| Accession No. | Gene Name | Difinition | Function (GO process) | -fold changes |
|---|---|---|---|---|
| NM_026816 | Gtf2f2 | general transcription factor IIF, polypeptide 2 | transcription | 0.46 |
| BC048846 | Sypl | synaptophysin-like protein | | 0.48 |
| AC090431 | Dock5 | dedicator of cytokinesis 5 | | 0.45 |
| NM_177276<br>NM_011374 | St8sia1 | ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1 | protein amino acid glycosylation, positive regulation of cell proliferation | 0.45 |
| NM_144800 | Mtss1 | metastasis suppressor 1 | cell motility, actin filament organization | 0.50 |

Genes whose expression were altered more than two-fold are listed. The value indicates a ratio of the gene expression level in exercised mice to that in sedentary mice.

**Table 2.** Changes in gene expression after 2 weeks of 30-min daily exercise

| Accession No. | Gene Name | Difinition | Function (GO process) | -fold changes |
|---|---|---|---|---|
| NM_153457 | Rtn1 | reticulon 1 | biological process | 2.06 |
| NM_013590 | Lyz1 | lysozyme 1 | metabolic process, cell wall catabolic process | 0.41 |
| NM_022024 | Gmfg | glia maturation factor, gamma | | 0.46 |
| NM_010407 | Hck | hemopoietic cell kinase | protein amino acid phosphorylation, intracellular signaling cascade | 0.45 |
| NM_146248 | Cchcr1 | coiled-coil alpha-helical rod protein 1 | protein export from nucleus | 0.47 |
| XM_483944 | Atp2b1 | ATPase, Ca++ transporting, plasma membrane 1 | calcium ion transport | 0.50 |
| NM_009892 | Chi3l3 | chitinase 3-like 3 | carbohydrate metabolic process | 0.27 |
| NM_017372 | Lyz2 | lysozyme 2 | metabolic process, cell wall catabolic process | 0.41 |
| NM_030082 | Hist3h2ba | histone cluster 3, H2ba | nucleosome assembly | 0.41 |
| NM_019731 | Nme4 | non-metastatic cells 4, protein expressed in | GTP biosynthetic process | 0.47 |
| NM_001003960<br>NM_001003961<br>NM_001003963<br>NM_010068 | Dnmt3b | DNA methyltransferase 3B | DNA methylation | 0.49 |
| NM_011538 | Tbx6 | T-box 6 | mesoderm formation | 0.49 |
| NM_013650 | S100a8 | S100 calcium binding protein A8 (calgranulin A) | chemotaxis | 0.17 |
| NM_017379 | Tuba8 | tubulin, alpha 8 | microtubule cytoskeleton organization and biogenesis | 0.50 |

Genes whose expression were altered more than two-fold are listed. The value indicates a ratio of the gene expression level in exercised mice to that in sedentary mice.

### Preparation of RNAs for DNA microarray analysis

Total RNAs were isolated from each of 4 or 5 mice spinal cords per exercised or sedentary group using the RNeasy Lipid Tissue Mini Kit (Qiagen GmbH, Hilden, Germany). Pooled total RNA (5 μg each, 4 or 5 mice per group) was applied to OpArray™ Mouse V4.0 (Operon Technologies, Alameda, CA, USA), in which 35,852 genes are registered. We used Low RNA Fluorescent Linear Amp Kit PLUS (Agilent Technologies Inc., Santa Clara, CA, USA) to synthesize double-stranded (ds) cDNA from the total RNA and then synthesize cRNA from the ds cDNA as a template. Total RNA was reverse-transcribed with a T7 Promoter primer. After second-strand synthesis, we used the ds cDNA as a template for Cyanine (Cy) 3/Cy5-labeled cRNA synthesis. The OpArray slide was hybridized with 20-25 μg of each labeled cRNA and washed with the OpArray Hybridization Buffer Kit (Operon). A fluorescent image of the OpArray slide was then recorded with CRBIO (Hitachi Software Engineering, Tokyo, Japan). The digitized image data were processed with DNASIS Array software (Hitachi Software Engineering). The ratios of intensity of Cy5 to that of Cy3 were calculated. Information on each gene on the slide was obtained from the National Center for Biotechnology Information (NCBI) database.

## RESULTS AND DISCUSSION

This study demonstrated that single 30-min or 2-week exercise resulted in specific gene expression changes in mouse spinal cords using microarrays. By 30-min exercise, the expression of 3 genes was enhanced, and the expression of 29 genes reduced (Table. 1). By continuous 2-week exercise, the expression of 1 gene was enhanced, and the expression of 13 genes was reduced (Table. 2). Gria3 is glutamate receptor called GluR3, whose antisense peptide nucleic acid targeting GluR3 delayed disease onset and progression in the SOD1 G93A mouse model of familial ALS (Rembach *et al.*, 2004). The elimination of St8sia1 coding GD3 synthase improved memory and reduced amyloid-beta plaque load in Alzheimer disease model mice (Bernardo *et al.*, 2008). Reticulon family members including Rtn1 coding reticulon1 modulated BACE1 activity and amyloid-beta peptide generation (He *et al.*, 2004). This study will provide a valuable clue on the molecular basis of exercise-induced neuroprotective effects in spinal cords.

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ORIGINAL COMMUNICATION

# Is there a difference in gastric emptying between Parkinson's disease patients under long-term L-dopa therapy with and without motor fluctuations? An analysis using the $^{13}$C-acetate breath test

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**Abstract** The mechanism underlying the motor fluctuations that develop after long-term L-dopa therapy is not fully known. It has been speculated that malabsorption of L-dopa from the small intestine occurs. It was reported that gastric retention in Parkinson's disease (PD) patients with motor fluctuations is increased as compared with that in PD without fluctuations. Because L-dopa therapy may worsen the symptoms of delayed gastric emptying (GE), it was not clear whether the delayed GE of PD patients with motor fluctuation was affected by L-dopa therapy. We assessed GE in PD patients with and without motor fluctuations. We investigated GE in 40 patients with PD under long-term L-dopa therapy, 20 fluctuators with "delayed-on" and "no-on" phenomena, 20 nonfluctuators, and 20 healthy volunteers. GE was examined by the $^{13}$C-acetate breath test ($^{13}$C-ABT) [the half emptying time (HET), the peak time of the $^{13}$C-%-dose-excess curve ($T_{max}$)], with expirations collected for 4 h after a test meal and analyzed for $^{13}CO_2$ using an infrared (IR) spectrophotometer. The $T_{max}$ of GE as assessed using the $^{13}$C-ABT was significantly delayed in all PD patients as compared with controls ($P = 0.002$). The HET was significantly delayed in all PD patients as compared with controls ($P < 0.001$). The $T_{max}$ and HET were not significantly delayed in PD patients with motor fluctuations as compared with PD patients without motor fluctuations. These results demonstrated that GE is commonly delayed in PD patients with long-term L-dopa therapy. Delayed GE does not differ between PD patients with and without motor fluctuations. This finding demonstrated that the motor fluctuation in PD may not be influenced by GE.

**Keywords** Parkinson's disease · Gastric emptying · Motor fluctuations · L-Dopa therapy · $^{13}$C-acetate breath test

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## Introduction

Patients with Parkinson's disease (PD) often complain of gastrointestinal (GI) tract symptoms such as heartburn, nausea, vomiting, and abdominal full sensation. Some studies have reported on the dysfunction of the GI tract in PD patients [3, 4, 17, 19, 21].

Approximately 50% of PD patients, when treated with L-dopa for more than 5 years, experience motor fluctuations such as the "wearing-off" phenomenon or the "no-on" phenomenon [23]. The mechanism of these motor fluctuations has not been fully elucidated, but the blood concentration of L-dopa is decreased during off-periods despite sustained drug administration [16]. It has recently been reported that delayed gastric emptying (GE) may influence the absorption of L-dopa [18]. Djaldetti reported that delayed GE is common in PD patients and is more marked in those with response fluctuations [3]. Because L-dopa therapy itself may worsen the symptoms of delayed GE [2, 16], their interpretation of the results of their study is limited. It was not clear whether the delayed GE of PD

patients with response fluctuation was affected by L-dopa therapy.

Recently, the $^{13}$C-acetate breath test ($^{13}$C-ABT) has been widely recognized as useful for evaluating GE because it is less invasive than isotope or acetaminophen methods [8]. Braden reported that the $^{13}$C-ABT was a reliable and noninvasive tool for the analysis of GE rates of liquid phases without radiation exposure [1]. In the present study, we investigated GE in PD patients with and without motor fluctuations using the $^{13}$C-ABT.

## Methods

### Patients

Our study population consisted of 40 patients [14 men and 26 women; median age 67.0 years (range 42–86 years); disease duration 6.0 years (range 3.0–31.0 years)] with an initial diagnosis of PD on the basis of the UK Parkinson's Disease Society Brain Bank Clinical diagnostic criteria [9, 13] and 20 healthy volunteers (control group) [10 men and 10 women; median age 69.0 years (range 63–73 years)]. The PD patients were consecutively consulted at our hospital. Modified Hoehn and Yahr stages 3–4 were seen, according to the Unified Parkinson's Disease Rating Scale [6, 12]. All PD patients were being treated with antiparkinsonian medications (long-term L-dopa therapy). No patient was treated with drugs that might alter GE. None of the PD patients had basic diseases such as liver dysfunction, renal failure, cardiopulmonary disease, diabetes mellitus, GI disease or history of gastric surgery.

The patients were divided into two groups: 20 patients with motor fluctuations and 20 patients without motor fluctuations. All PD patients with motor fluctuations exhibited "delayed-on" and "no-on" phenomena. "Delayed on" was manifested by latencies longer than 30 min from ingestion of L-dopa to turning "on" for almost all daily doses. "No-on" phenomenon was manifested by episodic total unresponsiveness to single dose of L-dopa. Clinical characteristics (including age, gender, height, weight, body mass index, duration of disease, duration of therapy with L-dopa, daily dose of L-dopa, other drugs, and GI symptoms) were not significantly different between the PD groups with and without motor fluctuations.

The results of blood examinations were within normal range. There were no differences between the PD groups with and without motor fluctuations. In addition, there were no differences between the PD groups in terms of pepsinogen I, II, and serum gastrin levels, which might affect gastric motility [25]. The positive ratio of anti-HP antibody did not differ significantly between the PD groups.

Informed consent was obtained from each subject prior to participation in this study. The study protocol was approved by the Ethical Committee of Gifu University, and was carried out in accordance with the 1975 Declaration of Helsinki.

### Gastric emptying examination

The GE examination was carried out using the $^{13}$C-breath test according to Ghoos [8] with slight modifications. PD patients and healthy volunteers were tested after an overnight fast of 12 h. All PD patients did not take the antiparkinsonism drug over 24 h. Early in the morning, PD patients and healthy volunteers took the liquid test meal (Racol: TM, 200 kcal/200 ml; Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan) containing 100 mg $^{13}$C-sodium acetate. Thereafter, an expiration breath sample was collected every 10 min for 4 h and analyzed for $^{13}CO_2$ using an IR spectrophotometer (UBiT-IR300; Otsuka Electronics Co., Ltd., Tokyo, Japan). During the examination, all subjects were in a sitting position.

The principle of $^{13}$C-ABT is ingestion of a liquid test meal containing $^{13}$C-acetate, gastric emptying, absorption from the digestive tract, metabolism in the liver (production of $^{13}CO_2$), expiration from the lung, and increase of $^{13}CO_2$ in expired breath.

### Mathematical analysis

The data were used for mathematical curve fitting. A best-fit curve of expired $^{13}CO_2$ was constructed for each subject. The %-$^{13}CO_2$ cumulative excretion in the breath was assessed using a nonlinear regression formula: $y = m(1 - e^{-kt})^{\beta}$ to fit the curve of the cumulative $^{13}$C recovery [7, 10]. The %-$^{13}CO_2$ excretion per hour was fitted to the formula $mk\beta e^{-kt}(1 - e^{-kt})^{\beta - 1}$ [7, 10]. $T$ is time and $m$, $k$, and $\beta$ are constants. The value of $m$ represents the total cumulative $^{13}CO_2$ recovery when the time is infinite. The half emptying time (HET) was calculated using the formula: $\mathrm{HET} = -1/k \ln(1 - e^{-1/\beta})$ [7, 10]. $T_{max}$ is the peak time of the $^{13}$C-%-dose-excess curve (%-dose/h) based on a time profile of the $^{13}CO_2$ excretion rate. The parameters were estimated with Excel software (Microsoft Co., Ltd., Redmond, WA).

### Statistical analysis

Categorical variables were compared using Fisher's exact probability test. Other variables were expressed as median (range). Medians were compared using Mann-Whitney's $U$ test. All analyses were carried out on StatView statistical software, version 5.0 (Abacus Concepts, Inc., Berkeley, CA). A $P$ value less than 0.05 was considered significant.

## Results

Controls and PD patients (Figs. 1a, 2a, 3a)

The examinations were safely carried out in all PD patients and controls. In all PD patients, the blood concentration of L-dopa was below 25 ng/ml (below the detection level) at start of examination. The $T_{max}$ of GE using the $^{13}$C-ABT was significantly delayed in all PD patients (median 1.17 h, range 0.50–2.17 h) as compared with the controls (median 0.83 h, range 0.67–1.00 h) ($P = 0.002$). The HET was significantly delayed in all PD patients (median 2.09 h, range 1.60–3.30 h) as compared with the controls (median 1.44 h, range 1.30–1.64 h) ($P < 0.001$).

PD patients with and without motor fluctuations (Figs. 1b, 2a, 3a; Table 1)

$T_{max}$ was not significantly delayed in PD patients with motor fluctuations (median 1.17 h, range 0.50–2.17 h) as compared with those without motor fluctuations (median 1.17 h, range 0.67–2.17 h). HET was not significantly delayed in PD patients with motor fluctuations (median 2.09 h, range 1.73–3.30 h) as compared with those without motor fluctuations (median 2.14 h, range 1.60–3.07 h).

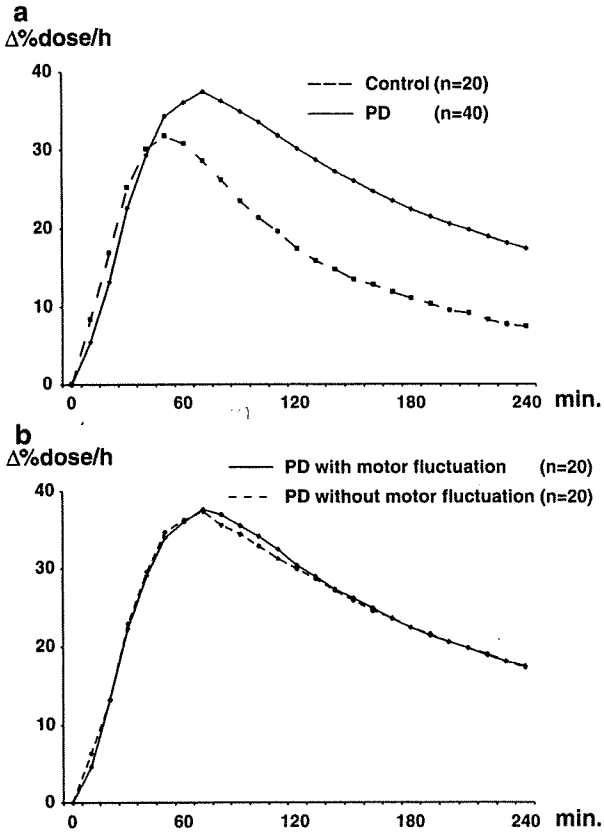


**Fig. 1** Mean percentage $^{13}$C expired per hour obtained by $^{13}$C-acetate breath test in **a** control and PD patients, and **b** PD patients with and without motor fluctuation

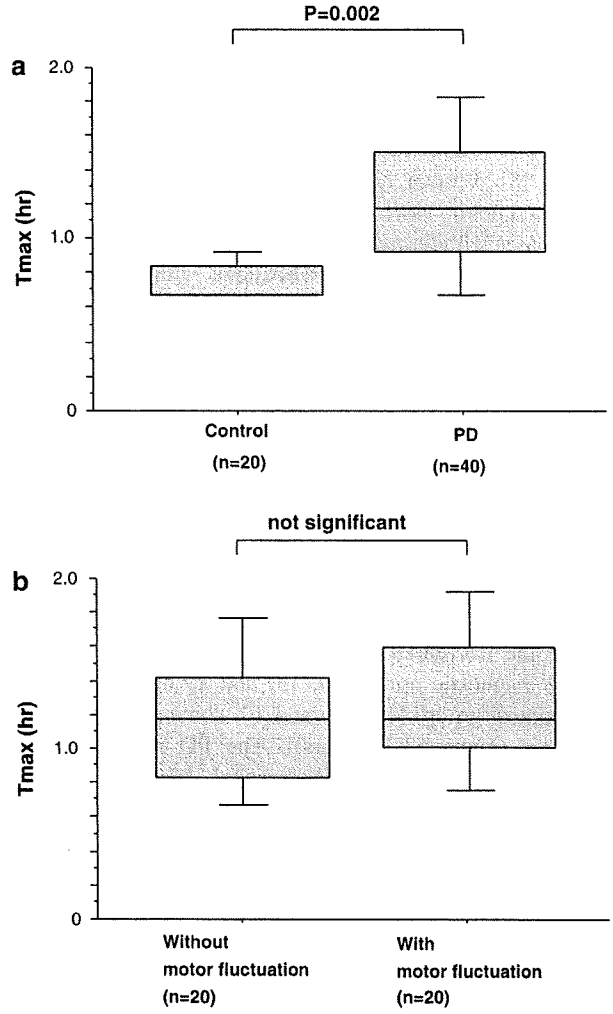


**Fig. 2** $T_{max}$ in **a** controls and PD patients, and **b** PD patients with and without motor fluctuation

## Discussion

In the present study we have shown two important points. First, GE was significantly delayed in PD patients as compared with controls. Secondly, GE was not significantly delayed in PD patients with motor fluctuations as compared with those without motor fluctuations.

About one-half of PD patients, when treated with L-dopa for a relatively long period, experience motor fluctuations such as "delayed-on" phenomenon or "no-on" phenomenon [24]. The mechanisms of these motor fluctuations are not fully known, but it has been speculated that

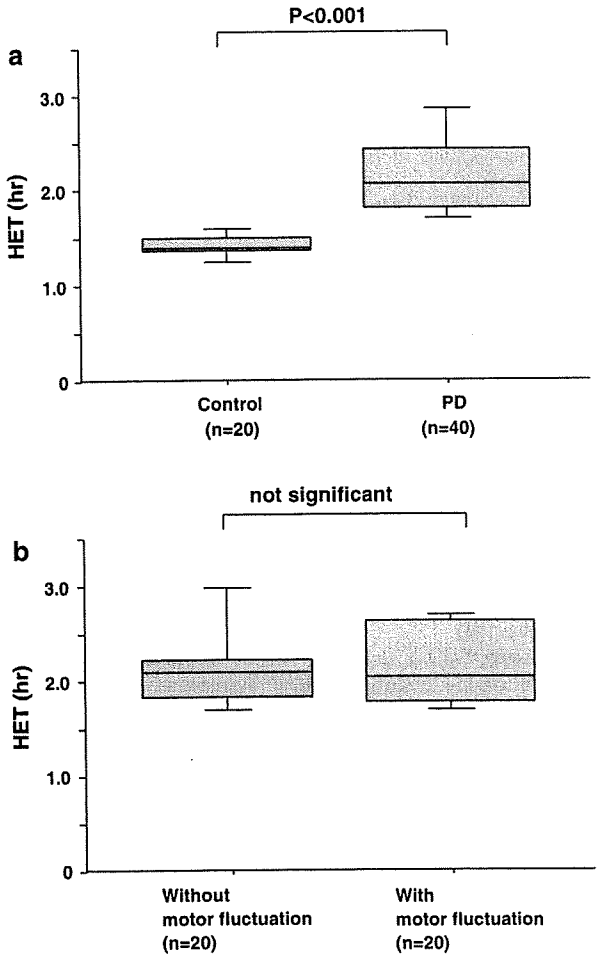


**Fig. 3** HET in **a** controls and PD patients, and **b** PD patients with and without motor fluctuation

malabsorption of L-dopa from the small intestine occurs [23]. In addition, PD patients sometimes complain of upper GI symptoms such as heartburn, nausea, vomiting, and abdominal full sensation [22]. It has been speculated that the delayed GE of PD patients with motor fluctuations was responsible for the low blood concentration of L-dopa. Djaldetti reported that GE in PD patients with motor fluctuations is increased as compared with those without fluctuations [3]. However, their interpretation of the results of their study is limited. Djaldetti divided the PD patients into two groups: patients with and without fluctuating symptoms [3]. However, these two groups differed in the percentage of patients receiving L-dopa and disease duration. Because L-dopa therapy itself may worsen the symptoms of delayed GE [2, 16], it was not clear whether the delayed GE of PD patients with motor fluctuation was affected by L-dopa therapy. There is evidence to suggest that these complications in PD patients may be attributed to peripheral, pharmacokinetic mechanisms, mainly delayed

**Table 1** Clinical characteristics of PD patients with and without motor fluctuation

| | PD without motor fluctuation ($n = 20$) | PD with motor fluctuation ($n = 20$) | *P* value |
|---|---|---|---|
| Age (years) | 68.0 (55–80) | 66.5 (42–86) | 0.66 |
| Gender (male/female) | 9/11 | 5/15 | 0.32* |
| BMI (kg $m^{-2}$) | 20.0 (16.8–26.7) | 20.7 (17.7–27.1) | 0.34 |
| Duration (years) | 5.4 (3–31) | 6.0 (3–12) | 0.78 |
| Duration with L-dopa (years) | 5.0 (3–30) | 5.7 (3–11) | 0.69 |
| L-dopa (mg) | 400 (100–600) | 425 (200–750) | 0.55 |
| GI symptoms | 6 | 5 | 0.99* |
| $CV_{R-R}$ | 1.46 (0.83–4.01) | 1.94 (0.67–5.14) | 0.24 |
| OH | 11 | 10 | 0.99* |
| MIBG (H/M ratio) | 1.50 (1.29–1.93) | 1.44 (1.27–1.95) | 0.87 |

Variables expressed as median (range). Medians were compared using Mann–Whitney's *U* test. Categorical variables were compared using Fisher's exact probability test

*PD* Parkinson's disease, *BMI* body mass index, *GI* gastrointestinal tract, *CV* coefficient of variation, *OH* orthostatic hypotension, *MIBG* I-[123]-metaiodobenzylguanidine scintigraphy, *H/M ratio* heart/mediastinum ratio

GE as a side-effect of L-dopa [5, 11, 14]. L-dopa itself or after decarboxylation to dopamine may have an effect on dopaminergic receptors present in the GI tract and particularly in the stomach [20]. Dopaminergic agents also activate the vomiting center in the medulla via the chemoreceptor trigger zone, resulting in nausea, abdominal bloating, and vomiting [5].

In this study, the PD patients were off drugs, including L-dopa, and the blood concentration of L-dopa was below the detection level at start of examination. PD patients were divided into two groups: those with and those without motor fluctuations. The two groups did not have significantly different backgrounds, including with respect to the daily dose of L-dopa and blood parameters of gastric function such as pepsinogen I/II, serum gastrin, immunoglobulin G (IgG) anti-*Helicobacter pylori* antibody, and hemoglobin A1c (HbA1c) [6]. Under these conditions, there was not a difference in GE of PD patients with and without motor fluctuations. Therefore, we speculate that the cause of motor fluctuation is not delayed GE.

With respect to the pathological background of PD, neuronal degeneration occurring in the dorsal nucleus of the vagus, in addition to the nigrostriatal tract, may be responsible for the degeneration of the gastrointestinal myenteric plexuses. In study of the enteric nervous system of PD, Wakabayashi reported Lewy bodies in the Auerbach's and Meissner's plexuses of the lower esophagus and stomach [26]. Cytoplasmic inclusions similar to

Lewy bodies were present in the ganglion cells of the colonic myenteric plexuses in PD patients [15]. These observations might indicate that the primary disease process itself plays a role in gastric movement disorder.

In conclusion, our study demonstrated that GE was commonly delayed in PD patients and that delayed GE did not differ between PD patients with and without motor fluctuations. From a pharmacokinetic viewpoint, it will be important to actually measure GE in PD patients in clinical settings. Our results demonstrated that the motor fluctuation of PD may not be caused by GE, but probably by absorption of L-dopa or a dosage form of L-dopa.

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Research Report

# Metallothionein-III knockout mice aggravates the neuronal damage after transient focal cerebral ischemia

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ABSTRACT

Metallothioneins (MTs) are metal-binding proteins and have four isoforms. MT-III was, at first, found in the brains of patients with Alzheimer's disease. MT-III exists mainly in the central nervous system, and the main effects are thought to be anti-oxidative and regulate zinc levels. In some previous reports, MT-III exhibited neuroprotective effects in various pathological situations, but its detailed effects are still unclear. In the present study, we examined neuronal damage after a middle cerebral artery occlusion (MCAO) in MT-III knockout (KO) mice to elucidate the relationship between MT-III and cerebral infarction. There was no significant difference in cerebral infarction after 24-h permanent MCAO between the wild-type and MT-III KO mice. On the other hand, after 2-h MCAO and 22-h reperfusion, cerebral infarction in the MT-III KO mice was aggravated compared with the wild-type mice. Furthermore, fatal rate of MT-III KO mice increased from 3 days after MCAO, and neurological deficits at 5 and 7 days after MCAO of MT-III KO mice were worse than those of wild-type. We examined terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and the immunostaining of an oxidative stress marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG), at 24 h after transient MCAO. In the penumbra lesion, the positive cell numbers in both staining assays were higher in the MT-III KO mice than those of the wild-type mice. These findings indicate that neuronal damage was aggravated by reperfusion injury in the MT-III KO mice compared with the wild-type mice, suggesting that MT-III plays anti-oxidative and neuroprotective roles in transient cerebral ischemia.



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*Abbreviations:* MT, metallothionein; GIF, growth inhibitory factor; CBF, cerebral blood flow; CNS, central nervous system; MCAO, middle cerebral artery occlusion; TTC, 2,3,5-triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; 8-OHdG, 8-hydroxy-2′-deoxyguanosine

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## 1. Introduction

Metallothioneins (MTs) are cysteine-rich and metal-binding proteins. They have multiple functions such as regulation of metal concentration, detoxification of heavy metal, and anti-oxidation (West et al., 2008). It has been reported that MTs have four isoforms known as MT-I, MT-II, MT-III, and MT-IV. MT-III, one of the family, is especially expressed in the central nervous system (CNS) (Hidalgo et al., 2001). MT-III expression decreases in the brains of patients with Alzheimer's disease and is known to be a growth inhibitory factor (GIF) (Erickson et al., 1994). MT-III increases in a variety of CNS abnormalities such as brain injuries due to stab wounds (Hozumi et al., 1995). MT-III overexpression and KO mice are very resistant and susceptible to the toxicity of kainic acid, respectively (Erickson et al., 1997). Furthermore, an adenoviral vector encoding MT-III cDNA prevents neuronal cell death after a brain injury (Hozumi et al., 2006). These data indicate that MT-III may exhibit neuroprotective effects *in vivo*. However, to our knowledge, there are no reports regarding the effects on MT-III KO mice after cerebral ischemia.

Yanagitani et al. (1999) have reported that MT-III was expressed in neuronal cell bodies in the rat CA1 to CA3 regions of the hippocampus, dentate gyrus, cerebral cortex, olfactory bulb, and Purkinje cells in the cerebellum. The authors also reported that MT-III increased in the cerebrum after transient global ischemia in the rat. Hence, MT-III may play a neuroprotective role in transient cerebral ischemia.

In the present study, to examine the roles of MT-III against cerebral ischemia in a murine MCAO model, we evaluated the effects of MT-III on permanent and transient focal cerebral ischemia using MT-III KO mice. Furthermore, we examined terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining and the immunostaining of an oxidative stress marker, 8-hydroxy-2′-deoxyguanosine (8-OHdG).

## 2. Result

### 2.1. Physiological parameter

There were no significant differences in cerebral blood flow (CBF), mean arterial blood pressure, heart rate, $PaCO_2$, or $PaO_2$ between the wild-type and MT-III KO mice groups (Table 1). Surface CBF was reduced to approximately 30% of the baseline value immediately after MCAO in both groups (Table 1), and increased to approximately 80% of the baseline value after reperfusion in the transient ischemia model (data not shown).

### 2.2. Cerebral infarction in permanent ischemia model

In the permanent ischemic mice, an ischemic zone was consistently identified in the cortex and subcortex of the left cerebral hemisphere at 24 h after MCAO. According to the 2, 3, 5-triphenyltetrazolium chloride (TTC) staining results, there was no clear difference between the wild-type and MT-III KO mice (Fig. 1A). Measuring the infarction showed no significant differences in both the infarct area and volume (Figs. 1B and C). Moreover, there was no difference in neurological deficits (Fig. 1D). No wild-type mice had died at 24 h after MCAO, but one MT-III KO mouse died (Fig. 1D).

**Table 1 – Physiological parameters before and after MCAO in wild-type and MT-III KO mice.**

| Parameters | Wild-type | MT-III KO |
|---|---|---|
| Mean blood pressure (mm Hg) | | |
| Before ischemia | 69.3±4.8 | 71.9±3.4 |
| After ischemia | 77.3±2.6 | 78.0±3.7 |
| pH | | |
| Before ischemia | 7.35±0.04 | 7.29±0.09 |
| After ischemia | 7.22±0.06 | 7.20±0.10 |
| $PaCO_2$ | | |
| Before ischemia | 37.1±3.1 | 31.3±2.5 |
| After ischemia | 40.6±3.1 | 32.2±2.6 |
| $PaO_2$ | | |
| Before ischemia | 147.4±12.2 | 151.0±10.6 |
| After ischemia | 169.4±9.5 | 174.5±15.1 |
| Regional CBF (%) | 31.7±3.2 | 31.1±3.4 |

Blood pressure was monitored via the femoral artery. Arterial blood samples were taken at 30 min before and 30 min after MCAO, and pH, $PCO_2$, and $PO_2$ were measured. Regional CBF values were measured by laser-Doppler flowmetry. There were no significant differences between the wild-type and MT-III KO mice groups. Data are shown as mean±S.E.M. ($n=3$).

### 2.3. Cerebral infarction in transient ischemia model

Contrary to the results of the MT-III KO mice in the permanent model, there was a significant difference between the wild-type and MT-III KO mice in terms of neuronal damage after transient MCAO, according to the TTC staining results (Fig. 2A). According to the measurements of the infarction, the area and volume were significantly aggravated in MT-III KO mice compared to the wild-type mice (Figs. 2B and C). Neurological deficits tended to be worse in the MT-III KO mice than in the wild-type mice, but not significant (Fig. 2E).

In addition, we evaluated survival rate and neurological score during 7 days after MCAO. Survival rate was significantly less in MT-III KO mice than in wild-type mice (Fig. 2D). Fifty percent of MT-III KO mice could not survive for 3 days, and only 30% mice survived at 7 days after MCAO. On the other hand, 80% wild-type mice survived at 7 days. Neurological score was greater in MT-III KO mice than that in wild-type mice (Fig. 2E). Especially at day 5 and day 7, MT-III KO mice showed worse neurological score significantly.

### 2.4. TUNEL staining in transient ischemia model

The morphological features of TUNEL stained cells (indicative of the ischemic damage and apoptotic cell death induced by 2-h MCAO and 22-h reperfusion) are shown in Fig. 3B. In the penumbra lesion, TUNEL-positive cells were fewer in number in the wild-type mice group than those in the MT-III KO mice group in both sections at 4 and 6 mm from olfactory bulb (Fig. 3C).

### 2.5. Immunostaining of an oxidative stress marker, 8-OHdG

The guanine base in DNA is oxidized by reactive oxygen species (ROS) and changes to 8-OHdG. Thus, "8-OHdG-

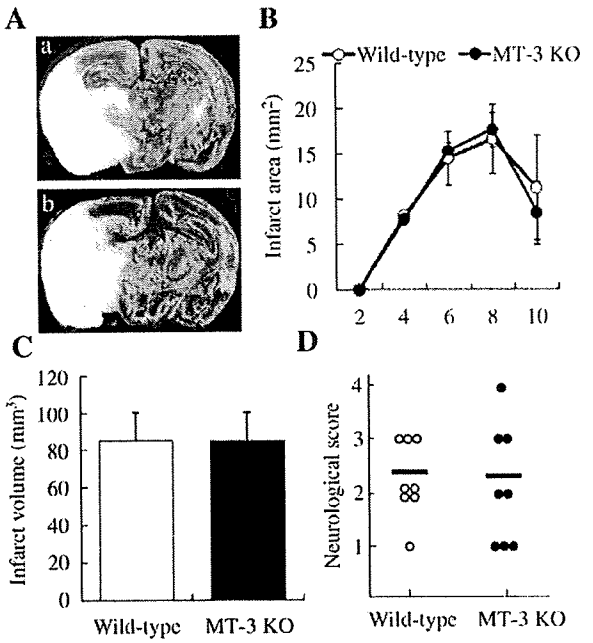


**Fig. 1 – Evaluation of cerebral infarction at 24 h after MCAO (permanent model) in MT-III KO mice. (A) TTC staining of coronal brain sections (4 mm from the olfactory bulb) [(a) Wild-type mouse, (b) MT-III KO mouse]. The white and red areas represent infarction and no infarction (normal), respectively. There was no difference in infarction after MCAO between the wild-type and MT-III KO mice. (B) Infarct area and (C) volume at 24 h after the permanent MCAO. There were no significant differences between wild-type and MT-III KO mice in both the infarct area and volume. $n=8$. (D) Neurological scores at 24 h after the permanent MCAO. There was no significant difference between the wild-type and MT-III KO mice. $n=8$.**

positive" means that neuronal cells are damaged by ROS. We performed 8-OHdG immunostaining in the brains of reperfusion mice (at 22 h after 2-h MCAO). The number of 8-OHdG-positive cells in the penumbra lesion in the MT-III KO mice was higher than the number in the wild-type mice at 4 mm from olfactory bulb (Figs. 4B and C).

## 3. Discussion

In the present study, we investigated neuronal damage after focal cerebral ischemia using MT-III KO mice. In the permanent model, the cerebral infarction in the MT-III KO mice was the same as in the wild-type mice, but in the transient ischemia model, that of MT-III KO mice was more aggravated than in the wild-type mice. Furthermore, the number of both TUNEL and 8-OHdG-positive cells was higher in the MT-III KO mice than in the wild-type mice.

Neuronal damage after ischemia was aggravated by transient, but not permanent, MCAO. Although the underlying mechanism is still unclear, the aggravation of the infarction after transient, but not permanent, ischemia in the MT-III KO mice may be due to deficient scavenging effects of radicals increased by reperfusion. This is the first report about the relationship between a deficiency of MT-III and cerebral infarction. Uchida et al. (2002) have reported that MT-III scavenges hydroxyl radicals in cultured neurons under hydrogen peroxide-induced oxidative stress. Furthermore, we have reported that MT-III exhibited a neuroprotective effect in brain stab wounds *in vivo* (Hozumi et al., 2006).

In the present study, survival rate of MT-III KO mice at 7 days was significantly less than that of wild-type. Yanagitani et al.(1999) reported that the protein level of MT-III increased at 3 days after cerebral ischemia, and peaked at 4 days. MT-III may protect neuronal cell damage not only in early phase of ischemic insult, but in late phase.

One of the remarkable results of the present study is the difference between permanent and transient ischemia. The major difference between permanent and transient ischemia is ischemia/reperfusion (I/R) injury. I/R injury is mainly caused by ROS. Oxygen transported by reperfused cerebral blood flow is changed to ROS by mitochondrial permeability transition and xanthine oxidase (Warner et al., 2004). Excess of ROS after transient ischemia causes lipid peroxidation, protein oxidation, DNA damage, and neuronal cell death. On the other hand, You et al. (2002) have reported that MT-III was able to remove the superoxide anion, which was generated from the xanthine/xanthine oxidase system *in vitro*. In the present study, the number of 8-OHdG-positive cells in the MT-III KO mice was higher than that in the wild-type mice. Taken together, MT-III may play a protective role partly via anti-oxidation of DNA on the increased ROS after transient MCAO.

In the present study, we performed TUNEL staining to evaluate apoptotic cell death. Lei et al. (2004) have reported that the number of TUNEL-positive cells at 24 h after the transient MCAO in rats increased in the penumbra rather than in the ischemic core. Moreover, Linnik et al. (1995) have reported that apoptotic cell death occurs mainly in the penumbra area rather than the core. Thus, we counted the TUNEL-positive cells in the penumbra to evaluate the effect of MT-III on apoptosis induced by transient MCAO. In the result, the number of TUNEL-positive cells in the MT-III KO mice was higher than that in the wild-type mice (Fig. 3). Fragmentation of DNA is caused by various reasons such as influx of calcium (Bano and Nicotera, 2007), toxic metals (Linder, 2001), or oxidative stress. MT-III has a metal-binding effect (Palumaa et al., 2002). Therefore, the neuroprotective effect of MT-III may come from not only the anti-oxidative effect but also the metal-binding effect. However, further studies will be needed to clarify the neuroprotective mechanism of MT-III.

In conclusion, MT-III KO mice demonstrated aggravated neuronal damage after transient, but not permanent, focal ischemia, suggesting that MT-III may play a pivotal role in the neuroprotection mechanism after reperfusion injury. The effects of MT-III exerted not only in early phase of ischemia, but in late phase.

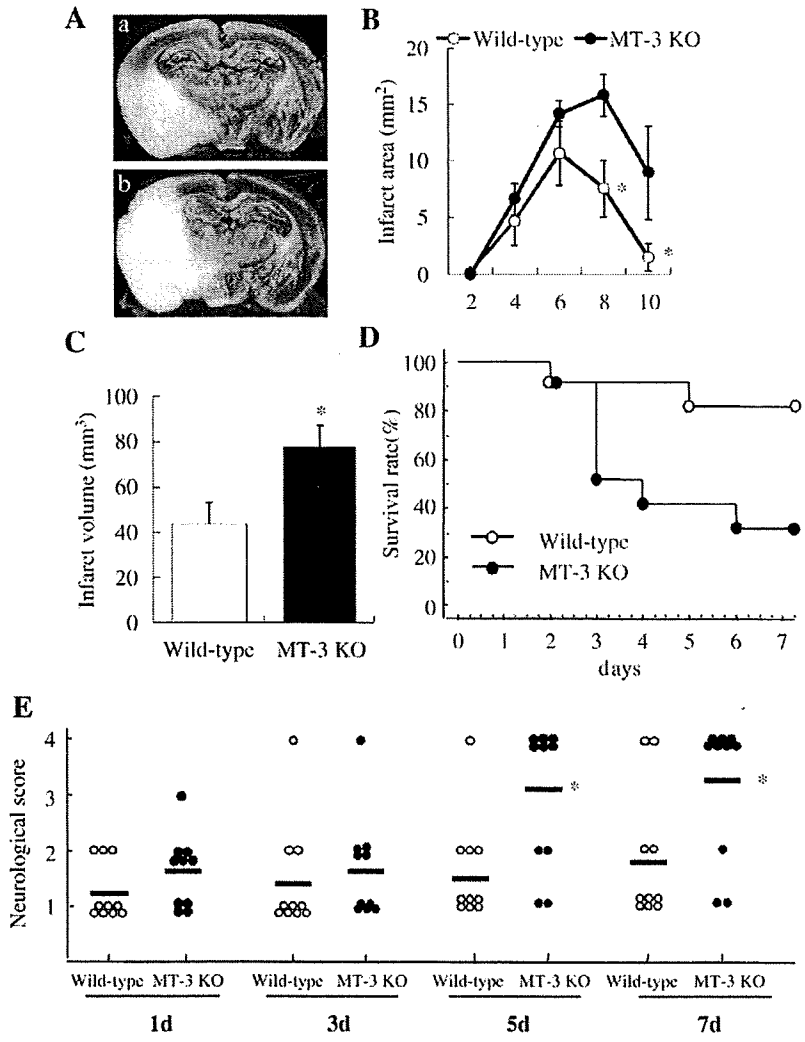


**Fig. 2 – Evaluation of cerebral infarction after 2-h MCAO (reperfusion model) in MT-III KO mice. (A) TTC staining of coronal brain sections at 22-h after reperfusion (4 mm from the olfactory bulb) [(a) Wild-type mouse, (b) MT-III KO mouse]. The infarction area was greater in the MT-III KO mice than in the wild-type mice. (B) At 22-h after reperfusion, the infarct area and (C) volume were significantly larger in the MT-III KO mice. *$p<0.05$ (Dunnet test), $n=8$. (D) Survival rate of the MT-III KO mice and the wild-type mice after MCAO. From day 3, the MT-III KO mice died more than the wild-type mice. Logrank $p<0.05$, $n=10$. (E) Neurological scores at 1, 3, 5, and 7 days after MCAO. The MT-III KO mice showed more neurological deficits than the wild-type mice. *$p<0.05$ (Mann–Whitney U-test), $n=10$.**

## 4. Experimental procedures

### 4.1. *Animals*

The experimental designs and all procedures were in accordance both with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and with the Animal Care Guidelines issued by the Animal Experimental Committee of Gifu Pharmaceutical University. Every effort was made to minimize the number of animals used and their suffering.

MT-III KO mice and 129/Sv mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and routinely bred in the laboratory animal facility of Gifu Pharmaceutical University. MT-III KO mice were developed by Erickson et al. (1997) and were of the 129/Sv strain genetic background. Age- and sex-matched 129/Sv mice were used as wild-type controls. All mice (10–15 weeks old) were housed in a room with a 12-h light/dark cycle (light on at 8:00 a.m.) and had ad libitum access to food and water.

### 4.2. *Focal cerebral ischemia model*

Mice were anesthetized with isoflurane 2% to 3% (for induction) and maintained with 1.0% to 1.5% isoflurane in 70% $N_2O$ and 30% $O_2$ via a face mask (Soft Lander; Sin-ei Industry, Saitama, Japan). Focal cerebral ischemia was induced [using an 8-0 nylon

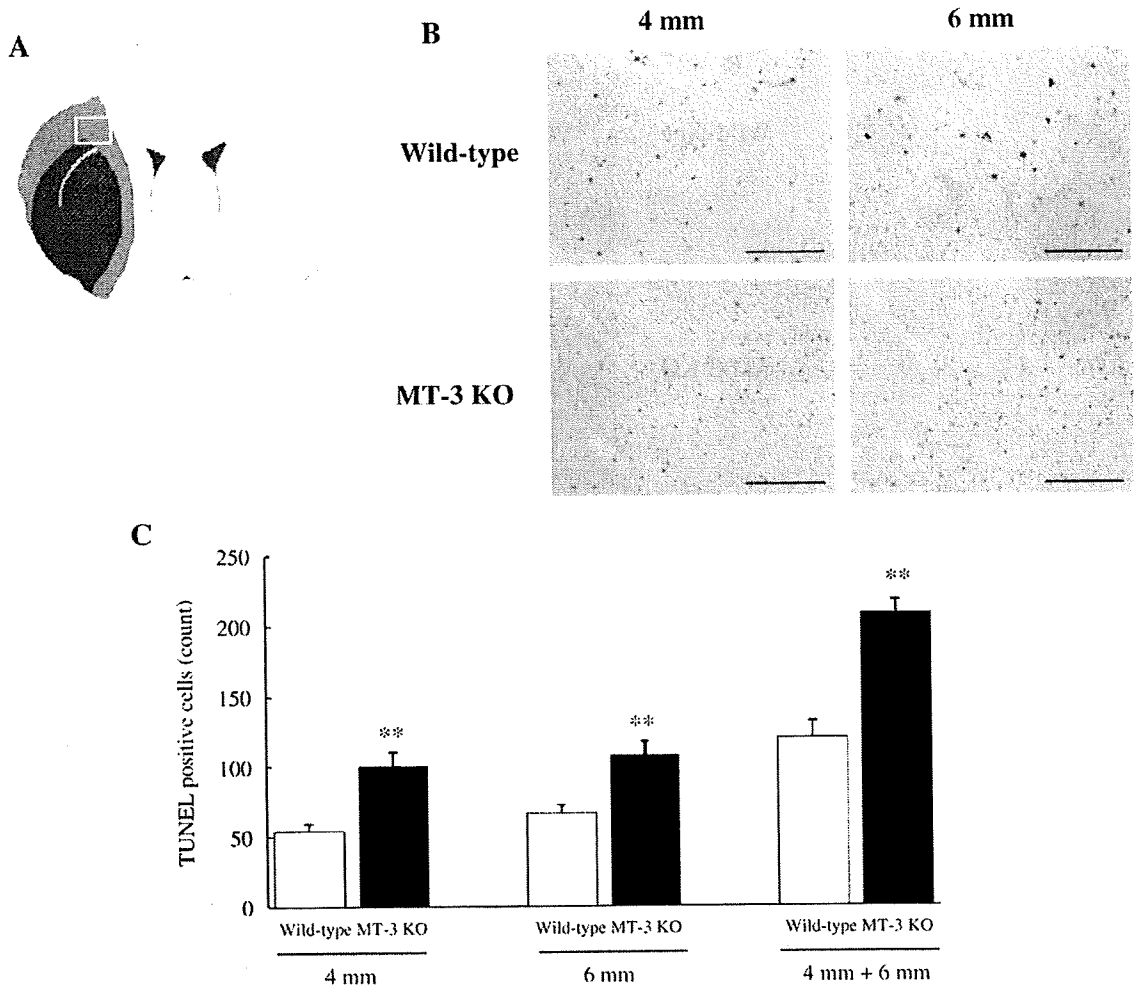


**Fig. 3 – TUNEL staining of coronal brain sections at 22 h after 2-h MCAO (reperfusion model) in the MT-III KO mice. (A) Schema of brain sections and penumbra. White square means penumbra. (B) Brain sections show 4 mm from the olfactory bulb, 20 μm thickness. Scale bar: 50 μm. (C) The number of TUNEL-positive cells in the penumbra in the MT-III KO mice was higher than that in the wild-type mice. **$p<0.01$ (Dunnet test).**

monofilament (Ethicon, Somerville, NJ, USA) coated with silicone hardener mixture (Xantpren; Bayer Dental, Osaka, Japan)] via the internal carotid artery, as described previously (Hara et al., 1996). Briefly, the coated filament was introduced into the left internal carotid artery through the common carotid artery, and then advanced up to the origin of the anterior cerebral artery via the internal carotid artery, so as to occlude the middle cerebral artery and posterior communicating artery. At the same time, the left common carotid artery was occluded. Anesthesia did not exceed 10 min. To examine the reperfusion injury, some mice were reanesthetized with isoflurane, and the monofilament was removed at 2 h after MCAO.

### 4.3. *Measurement of regional cerebral blood flow and physiological parameters*

In all animals during surgery and ischemia, the body temperature was maintained between 37.0 and 37.5 °C with the aid of a heating lamp and heating pad. In randomly selected animals, the left femoral artery was cannulated, and blood pressure was measured during the preparation, with mean systemic arterial blood pressure (Power Laboratory; AD Instrument, Nagoya, Japan) measured for 3-min periods starting 10 min before and ending 30 min after MCAO. Arterial blood samples taken 30 min before and 30 min after the induction of ischemia were analyzed for pH and the partial pressures of oxygen ($PaO_2$) and carbon dioxide ($PaCO_2$) (i-STAT 3G; Abbott Point-of-Care Inc., East Windsor, NL, USA). Regional cerebral blood flow (CBF) was determined by laser-Doppler flowmetry (Omegaflow flo-N1; Omegawave Inc., Tokyo, Japan) using a flexible 0.5-mm fiber-optic extension to the master probe. The tip of the probe was fixed to the intact skull over the ischemic cortex (2 mm posterior and 6 mm lateral to bregma). Steady-state values obtained after occlusion were expressed as a percentage of the baseline value (obtained at 30 min before MCAO).

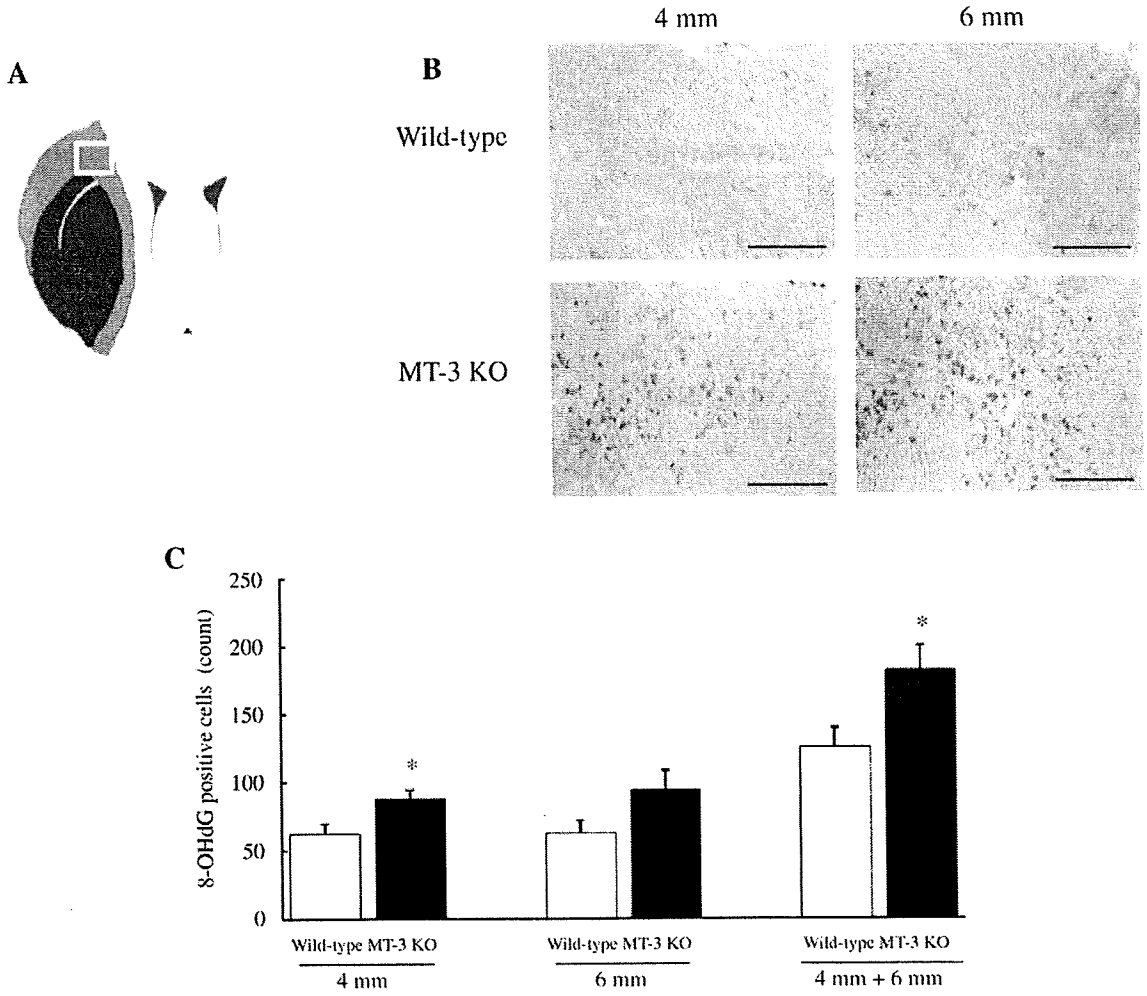


**Fig. 4 – Immunostaining of coronal brain sections at 22 h after 2-h MCAO (reperfusion model) in MT-III KO mice. (A) Schema of brain sections and penumbra. White square means penumbra. (B) Brain sections show 4 mm from the olfactory bulb, 20 μm thickness. Scale bar: 50 μm. (C) The number of 8-OHdG-positive cells in the penumbra at 4 mm sections and amount of 4 mm and 6 mm in the MT-III KO mice were higher than that in the wild-type mice. *$p<0.05$ (Dunnet test).**

### 4.4. *Evaluation of infarct size and neurological deficits in focal cerebral ischemia model*

At 24 h after MCAO, the mice were given an overdose of pentobarbital sodium, and then decapitated. The forebrain was divided into five coronal 2-mm sections using a mouse brain matrix (RBM-2000C; Activational Systems, Warren, MI, USA). These slices were immersed for 20 min in a 2% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, MO, USA) in normal saline at 37 °C, and then fixed in 10% phosphate-buffered formalin at 4 °C. TTC reacts with intact mitochondrial respiratory enzymes to generate a bright red color that contrasts with the pale color of the infarction. The caudal face of each slice was photographed. The area of the infarction (unstained) in the left cerebral hemisphere was traced and measured using Image J (http://rsb.info.nih.gov/ij/download/), and the infarction volume per brain ($mm^3$) was calculated from the measured infarction area.

Mice were tested for neurological deficits at each 24 h after MCAO. These were scored as described in our previous study (Hara et al., 1996): 0, no observable neurological deficits (normal); 1, failure to extend the right forepaw (mild); 2, circling to the contralateral side (moderate); 3, loss of walking or righting reflex (severe); and 4, dead.

### 4.5. *TUNEL staining and 8-OHdG immunostaining*

In preparation for TUNEL staining and 8-OHdG immunostaining, the mice were deeply anesthetized with pentobarbital sodium at 24 h after MCAO (at 22 h after the reperfusion), and then perfused with 4% paraformaldehyde. Coronal sections (thickness: 20 μm) were obtained from the frozen brains by serial sectioning. Sections at 4 or 6 mm from olfactory bulb were chosen for staining. The TUNEL assay was performed according to the manufacturer's instructions (Roche Molecular Biochemicals Inc., Mannheim, Germany). The number of TUNEL-positive cells in

the ischemic penumbra was counted in randomly chosen areas within a high-power field (×400). For 8-OHdG immunostaining, the sections were rinsed three times in PBS and blocked with 1% mouse serum for 30 min. A monoclonal antibody against 8-OHdG (anti-8-hydroxy-deoxyguanosine monoclonal antibody; JAICA, Shizuoka, Japan) was applied to sections overnight at 4 °C. Secondary antibody (M.O.M. biotinylated anti-mouse) was applied for 10 min. The avidin/biotinylated horseradish peroxidase complex (ABC Elite kit; Vector Laboratories, UK) was applied for 30 min, and the sections were allowed to develop Chromagen in 3, 3-diaminobenzidine plus nickel solution (Sigma-Aldrich) for 2 min. The histologists (A.K. and J.H.) were blind as to the group to which each mouse belonged.

### 4.6. *Statistical analysis*

Data are presented as the means ± S.E.M. Statistical comparisons were made using a one-way ANOVA followed by Dunnet test and Mann–Whitney U-test, with $p<0.05$ being considered to indicate statistical significance.

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# Metallothionein-3 deficient mice exhibit abnormalities of psychological behaviors

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ABSTRACT

Metallothioneins (MTs) are metal binding proteins and have four isoforms. MT-3, known as growth inhibitory factor (GIF), exists mainly in the central nervous system. It regulates zinc levels and exhibits a neuroprotective effect in the various types of brain diseases. However, the reports demonstrate that the relation between MT-3 and psychiatric disorder is still unknown. In the present study, the authors carried out behavioral tests on MT-3 knock-out (KO) mice. The duration of the MT-3 KO mice's social interactions were significantly shorter than that of the wild-type (WT) mice. The acoustic startle response of the MT-3 KO mice showed diminished prepulse inhibition (PPI) at all prepulse intensities. However, the locomotor activity tests of the MT-3 KO mice displayed normal circadian rhythm, activity, and habituation to a novel environment. In the novel object recognition test, the MT-3 KO mice exhibited normal memory. These findings indicate that abnormalities of psychological behavior were observed in the MT-3 KO mice. Further experiments will be needed to clarify the involvement of MT-3 in higher brain function.



Metallothioneins (MTs) are small and cysteine-rich proteins. They bind metal ions such as zinc, and have functions in the regulation of metal concentration and detoxification [21]. It has been reported that MTs have four isoforms. MT-1 and MT-2 are expressed in all tissues, and MT-3 and MT-4 are expressed in the central nervous system (CNS) and keratinizing epithelia, respectively [10]. MT-1 and MT-2 are upregulated by various drugs, metals, or diseases and show neuroprotective effects [20]. On the other hand, little is known about MT-3's regulation and effect.

At first, MT-3 was found in the brains of Alzheimer's disease (AD) patients and characterized as growth inhibitory factor (GIF). MT-3 can inhibit the outgrowth of embryonic cortical neurons *in vitro* [3]. In contrast, MT-3 expression decreases in the brains of AD patients [5], and increases variety of CNS abnormality such as cerebral ischemia [22] and brain injuries by stab wound [11]. Furthermore, an adenoviral vector encoding MT-3 cDNA prevents neuronal cell death from damage caused by brain injury [13]. MT-3 inhibits neuron outgrowth *in vitro*, but it exhibits neuroprotective effects *in vivo*. These detailed mechanisms are still not fully understood. MT-3 may relate not only to acute brain damage, but to chronic change and congenital abnormality.

In this report, the authors examined various behavioral tests using MT-3 KO mice. Erickson et al. have reported that they used the open field locomotor activity test, the Morris water maze, and the passive avoidance task, revealing that MT-3 deficient mice exhibited normal learning and memory [4]. In the present study, the authors examined behavior during locomotor activity, social interaction, and prepulse inhibition (PPI) tests. Reportedly, abnormal behavior exhibited during these tests may correlate with psychological disorders such as depression, schizophrenia, and autism [9,19,8]. There have not been any reports about the relation between MT-3 and psychological disorders; thus, this is the first report about this topic.

MT-3 KO mice and 129/Sv mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and routinely bred in the vivarium of the Gifu Pharmaceutical University. MT-3 KO mice were developed by Erickson et al. [4] and their genetic background was of the 129/Sv strain. Age- and sex-matched 129/Sv mice were used as WT controls. Between WT mice and MT-3 KO mice, there was no change in their fertility, litter size, or litter survival [4]. Additionally, the maternal behavior of female MT-3 KO mice is normal.

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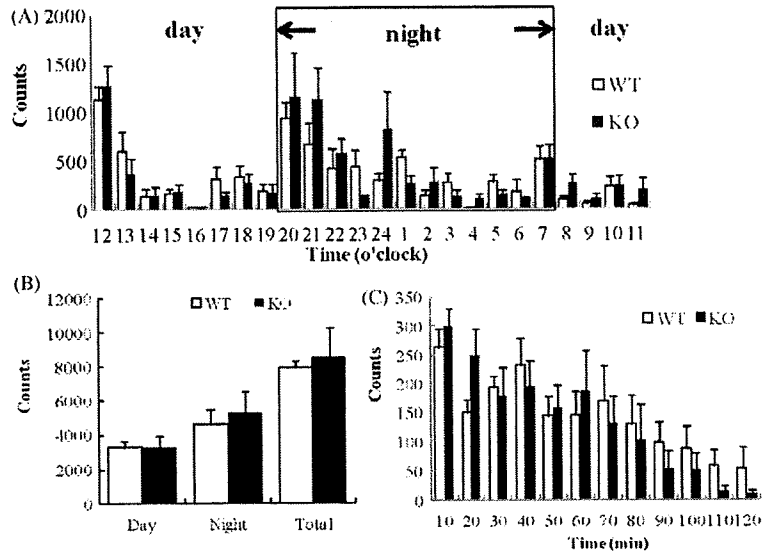


**Fig. 1.** Locomotor activity test of WT and MT-3 KO mice. WT ($n = 8$) and MT-3 KO ($n = 8$) mice were placed in individual home cages, and their locomotion was assessed every hour for 24 h. (A) Locomotor activity throughout the 24-hr period and (B) locomotor activity was analyzed separately during the day and night. There was no significant difference between WT and MT-3 KO mice. (C) Detailed analysis of the first 120 min. There was no significant difference in habituation to a novel environment between the two groups.

All mice (10–15 weeks old) were housed in a transparent plastic cage (length 17.5 × width 24.5 × height 12.5 cm) with sawdust bedding on the floor with a 12-h light/dark cycle (light on at 8:00 a.m.) and had ad libitum access to food and water. Five or six mice were housed in the same cage separated by their sex and genotypes. Mice were weaned at 4 weeks old, and from that point, handling was conducted by the experimenter once a week. Behavioral tests were performed using male mice between 9:00 a.m. and 5:00 p.m., except for the 24-h home-cage locomotor activity test. We used totally 23 WT mice and 25 KO mice got from 8 litters each. Each mouse was subjected one or two behavioral tests consecutively with adequate interval. All procedures relating to animal care and treatment conformed to the Animal Care Guidelines of the Animal Experiment Committee of Gifu Pharmaceutical University.

To measure locomotor activity in a novel environment, a mouse was placed in a transparent plastic cage (length 17.5 × width 24.5 × height 12.5 cm) with sawdust bedding on the floor. "Home cage" in this draft means the same cage the mice usually spend time in, i.e. the same size (length 17.5 × width 24.5 × height 12.5 cm) and same color. Locomotion was measured every hour for 1 day using a digital counter with an infrared sensor (NS-ASS01; Neuroscience Inc., Tokyo, Japan). Movement of the mice was detected using the infrared ray sensor on the basis of released infrared rays associated with the mice's temperature. When objects (mice) emitting infrared rays moved under the sensor, the sensor detected the action as locomotor counts. Animals were placed in the cages at 12:00 p.m. and left there for a 24-h period. The room light was on from 8:00 a.m. to 8:00 p.m.

The social interaction test in a novel environment was performed in a manner similar to a published method [17]. Two mice of identical genotypes that had previously been housed in different cages were placed in a box together (length 17.5 × width 24.5 × height 12.5 cm) and allowed to explore freely for 10 min. Social behavior was monitored by means of a video camera (Everio; Victor, Yokohama, Japan). The number and mean duration of contacts were measured at 10 min after the start of the test.

Acoustic startle responses were measured in a startle chamber (SR-LAB; San Diego Instruments, San Diego, CA, USA) using standard methods described previously [12]. Mice were presented with a series of four discrete trials: pulse-alone trials, prepulse-plus-pulse trials, prepulse-alone trials, and trials in which no discrete stimulus, other than the constant background noise, was presented. A reduction in startle magnitude in the prepulse-plus-pulse trials relative to that in the pulse-alone trials constitutes PPI. The pulse stimulus employed was 120 dB in intensity and 40 ms in duration. Prepulses of various intensities were employed: 73, 76, and 82 dB. The duration of the prepulse stimuli was 20 msec. The stimulus onset asynchrony of the prepulse and pulse stimuli in the prepulse-plus-pulse trial was 100 ms. A session began with the animals being placed into a Plexiglass enclosure. They were acclimatized to the apparatus for 5 min before the first trial began. The mice were presented with 6 blocks of discrete test trials. Each block consisted of one trial of each of the following trial types: pulse-alone trials, prepulse-plus-pulse trials with each of the three levels of prepulse, prepulse-alone trials with each of the three levels of prepulse, and no stimulus trials (i.e. background noise alone). The interval between successive trials was variable, the mean being 30 s (range, 20–40 s).

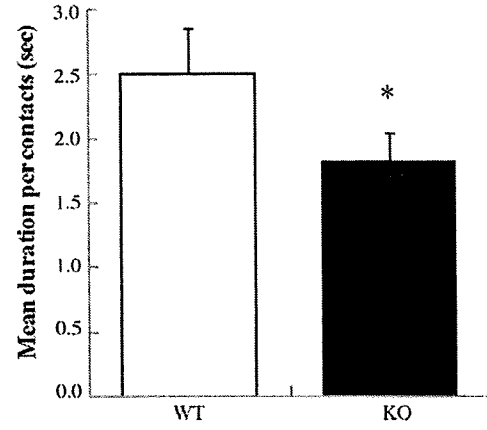


**Fig. 2.** Social interaction test of WT and MT-3 KO mice in a novel environment. We used 11 pairs of MT-3 KO mice and WT mice. Two genetically identical mice that had been housed separately were placed in the same cage. Their social interaction was then monitored for 10 min. Mean duration per contact of MT-3 KO mice was significantly shorter than that of WT mice ($p < 0.05$, $t$-test), $n = 11$ pairs.