Effects of Yokukansan, a Traditional Japanese Medicine, on Memory Disturbance and Behavioral and Psychological Symptoms of Dementia in Thiamine-Deficient Rats

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Effects of yokukansan (TJ-54) on memory disturbance and behavioral and psychological symptoms of dementia (BPSD) were investigated in thiamine-deficient (TD) rats which were produced by feeding a TD diet for 37 d. Daily oral administration of TJ-54 (0.5, 1.0 g/kg) ameliorated the memory disturbance, anxiety-like behavior, the increase in aggressive behaviors, the decrease in social behaviors, and several neurological symptoms including opisthotonos observed in TD rats, in a dose-dependent manner. In addition, histopathological examinations showed that TJ-54 inhibited the degeneration of neuronal and astroglial cells in the brain stem, hippocampus and cortex in TD rats. Microdialysis experiments showed that TJ-54 inhibited extracellular glutamate rise in the ventral posterior medial thalamus in TD rats. These results suggest that TJ-54 possesses the preventive or progress inhibitive effect against the development of memory disturbance and BPSD-like behaviors induced by the degeneration of neuronal and astroglial cells resulting from TD. TJ-54 may inhibit glutamate-mediated excitotoxicity as one of mechanisms.

Key words yokukansan; thiamine; memory; aggression; anxiety; astrocyte

Behavioral and psychological symptoms of dementia (BPSD), including anxiety, depression, excitement, anger, hallucination, and roaming, are seen in patients with Alzheimer’s disease and other forms of senile dementia. To date, although atypical or conventional antipsychotic medications are used to treat BPSD, drug-induced extrapyramidal symptoms and other adverse events are seen. In addition, the Food and Drug Administration warned in 2005 that the antipsychotic medications increase mortality among elderly patients. Therefore, new remedies without adverse effects have been sought.

Yokukansan (TJ-54) is a traditional herbal medicine called a ‘kampo medicine’ in Japan. The Ministry of Health, Labor and Welfare in Japan has approved it as a remedy for neurosis, insomnia, and irritability in children. Recently, TJ-54 has been reported to ameliorate excitement, anger, and hallucination in BPSD in patients with Alzheimer’s disease, dementia with Lewy bodies, and other forms of senile dementia. However, there is limited research on this compound and the mechanism by which it alters the symptoms of dementia is unknown.

Up to now, various dementia models including β-amyloid protein precursor (APP) or α-synuclein transgenic mice, or scopolamine-treated animals have been used for research in the pathogenesis and therapy of dementia. However, because most studies focused on deficits of the functions of learning and memory that are the main symptoms of dementia, or because only the abnormalities of learning and memory functions are observed in the most models, information regarding BPSD was few in the animal models. Thus, animal models covering peripheral symptoms like BPSD observed in patients with dementia have little been reported. However, recently, it has been reported that not only impairment of learning and memory but also BPSD-like behaviors such as anxiety, depression, muricide, attacking, and startle responses are observed in thiamine-deficient (TD) rats and mice, i.e., the data about BPSD-like behaviors are more abundant than other dementia models. TD is a critical factor in the etiology of Wernicke–Korsakoff’s syndrome, which is characterized by a decrease in thiamine pyrophosphate (biologically active form of thiamine)-dependent enzymes involved in cellular glucose and energy metabolism in the brain. Thus, although the pathogenesis (or an induction factor) in each dementia model including TD animals is different, there is a common point that memory dysfunction is observed in each model. Furthermore, similar deficiencies in thiamine pyrophosphate-dependent enzyme activities are reported in postmortem brain tissues of patients with Alzheimer’s disease. TD has been also reported to induce selective neuronal loss, cholinergic deficits, and accumulations of the abnormal tau isoforms and APP that are involved in Alzheimer’s disease. These findings suggest that TD animals may be a valuable tool for evaluation of pharmacotherapy for BPSD as well as dysfunction of learning and memory which is a core symptom of dementia. In the present study, therefore, we investigated the effects of TJ-54 on memory disturbance and BPSD-like behaviors observed in TD rats.

MATERIALS AND METHODS

Animals Three-week-old male Wistar rats weighing 35—45 g were obtained from Charles River Laboratories (Yokohama, Japan). The animals were housed individually in stainless steel cages (RBC-12 type, 260×380×200 mm, Ishihara Co., Ltd., Tokyo, Japan) at a temperature of 23±2°C, relative humidity of 55±10%, and a 12-h light/dark cycle with lights on from 07:00 to 19:00 daily, and allowed free access to water and standard laboratory food (MF, Oriental Yeast Co., Ltd., Tokyo, Japan). After habituation for 1 week, 4-week-old rats weighing 79—98 g at the beginning of the experiment were used in the present study.

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Conspecific male rats were also obtained from Charles River to evaluate aggression of subject rats in a social interaction test. They were group-housed (five rats in a cage) in the same breeding environment until the animals were used in the tests.

The TD and AIN-93G control diets used in the present study were purchased from Oriental Yeast Co., Ltd. The TD diet was the AIN-93G control diet without thiamine. The AIN-93G control diet as well as the standard laboratory chow (MF) contained 0.5 mg thiamine per 100 g of diet.

All experimental procedures were performed according to the “Guidelines for the care and use of laboratory animals” approved by the Laboratory Animal Committee of Tsumura & Co.

Drugs and Reagents The TJ-54 used in the present study, a dry powdered extract from a mixture of Atractylodes Lancea rhizome (4.0 g, the rhizome of *Atractylodes lancea* De Candolle), Hoelen (4.0 g, the sclerotium of *Poria cocos* Wolf), Cnidii Rizoma (3.0 g, the rhizome of *Cnidium officinale* Makino), Japanese Angelica root (3.0 g, the root of *Angelica acutiloba* Kitagawa), Bupleurum root (2.0 g, the root of *Bupleurum falcatum* Linne), Glycyrrhiza root (1.5 g, the root and stolon of *Glycyrrhiza uralensis* Fischer) and Uncaria thorn (3.0 g, the thorn of *Uncaria rhynchophylla* Miqul), was supplied by Tsumura & Co. (Tokyo, Japan). Dosages of TJ-54 (0.5 and 1.0 g/kg body weight) in the present experiment were prepared by dissolving it in 10 ml of distilled water.

Other reagents used for analysis were purchased from commercial sources.

**Thiamine Content of TJ-54** TJ-54 (35 mg/ml) was dissolved in distilled water and centrifuged at 3000 rpm for 10 min; then the supernatant was passed through a 0.45-μm membrane filter. An aliquot (20 μl) of the filtrate was injected into a HPLC (LC-10V system, Shimadzu Co., Kyoto, Japan) for determination of thiamine. The chromatographic conditions were column: TSK gel ODS-80TM (4.6×250 mm long, Tosoh Corporation, Tokyo, Japan), mobile phase: 20 mM phosphate solution containing 5 mM sodium heptane-sulfonate and 9% acetonitrile, flow rate: 1.0 ml/min at 40 °C, and detector: UV254 nm. The thiamine peak was not detected in the extract.

**Experimental Design** TD rats were produced by feeding rats a formulated diet that was thiamine deficient. Male Wistar rats were divided into four groups: control (n=10), TD (n=11), TD+0.5 g/kg TJ-54 (n=10), and TD+1.0 g/kg TJ-54 (n=11). All rats were initially trained to avoid electric shock while still receiving the normal MF diet. For 37 d after the acquisition of avoidance memory, the animals in the TD and TD+TJ-54 (0.5, 1.0 g/kg) groups were given a TD diet ad lib. In control group, the amount of control diet (AIN-93G) given to animals was determined on the basis of the average amount eaten by animals on the TD diet, because the food intake of animals fed with a TD diet is known to decrease significantly.

Distilled water (10 ml/kg) was orally administered once every day to the rats in the control and TD groups as a vehicle for TJ-54. TJ-54 at 0.5 g/kg and 1.0 g/kg was also orally administered once every day to the rats in the TD+0.5 g/kg TJ-54 and TD+1.0 g/kg TJ-54 groups, respectively.

Open-field tests to evaluate anxiety were performed on Days 14 and 28, and memory retention tests were performed on Days 15 and 29, respectively. A social interaction test to evaluate aggressive and social behaviors was performed on Day 21. The incidence of neurological symptoms was examined on Day 37, and then all rats were sacrificed for pathological examination.

In another set of experiments, basal levels of extracellular glutamate in the ventral posterior medial thalamus (VPM), which is a vulnerable region of animals in the control (n=6), TD (n=6), and TD+1.0 g/kg TJ-54 (n=6) groups were evaluated by microdialysis experiments on Day 28.

**Step-Through Passive-Avoidance Test to Evaluate Memory Disturbance** The apparatus (Neuroscience, Inc., Tokyo, Japan) for step-through passive avoidance test consisted of two compartments, one illuminated (200 mm×100 mm×190 mm with the light at the top of compartment set at 27 W and 3000 lux) and the other dark (200 mm×230 mm×190 mm). The compartments were separated by a guillotine door. In the learning process to acquire avoidance memory from electric shock, a rat was placed in the illuminated compartment. While the compartment was lit, the rat stepped through the open guillotine door into the dark compartment. The time spent in the illuminated compartment was defined as the latency period. Three seconds after the rat entered the dark compartment, a foot-shock (0.3 mA for 3 s) was delivered to the floor grid in the dark compartment. The rat could escape from the shock only by stepping back into the illuminated compartment. Such acquisition trials were performed once a day for 5 d. We judged that the rat had acquired avoidance memory from foot shock if the rat remained in the illuminated compartment for 300 s after being placed there. Retention trials without the foot shock were performed on Days 15 and 29. We judged that the rat retained the avoidance memory when it stayed in the illuminated safe compartment over 300 s.

**Open-Field Test to Evaluate Anxiety** An open-field apparatus (Neuroscience, Inc.) for evaluation of anxiety or anxiolytic effects consisted of an illuminated circular arena (120—130 lux) of 75 cm diameter enclosed by a wall 35 cm high. The floor was divided into five areas by drawing four concentric circles at 15 cm intervals. A rat was placed in the center of the apparatus and monitored with a video camera (video tracking system) for 5 min, and the data were saved on a computer. From the saved data, the total distance traveled (cm), total number of line crossings in five areas, and total number of entries into the central part (30 cm diameter) of the open field for 5 min were determined by using the analysis software LimeLight (Neuroscience Inc.).

**Social Interaction Test to Evaluate Aggressiveness and Sociability** Aggressive and social behaviors in rats were evaluated by a social interaction test. A subject rat and a non-treated (group-housed) control rat were placed together in an open-field apparatus (90 cm L×90 cm W×40 cm H). Interactive behaviors between the two animals were monitored with a video camera for 10 min, and the data were saved on a computer. Later, the total number and duration (s) of aggressive behaviors (such as tail rattling, chasing and attacking) as the index of aggressiveness, or normal social behaviors (such as sniffing, following and contacting) as the index of sociability, of the subject rat toward the control rat for 10 min were measured by two observers blind to the treatment. The total
distance traveled (cm) of the subject animal in the field was analyzed by using software (analyzing behavior system, Viewer II: Bioserve, Bonn, Germany) as motor activity.

Pathological Examination All rats were anesthetized with diethyl ether and perfused transcardially with 0.9% saline for a few minutes on Day 37. Subsequent perfusion was performed with 10% phosphate-buffered formalin. Thereafter, the brain was removed and used for light-microscopic and electron-microscopic examinations.

For light-microscopic examination, the brain was embedded in paraffin, and coronal paraffin sections were stained with hematoxylin and eosin for light microscopy, according to conventional procedure.

For electron-microscopic examination, some of small pieces in the cerebral cortex, hippocampus and brain stem were cut and rinsed with phosphate buffer. The tissue blocks were post-fixed with 1% osmium tetroxide solution for 2h, dehydrated through graded alcohols and embedded in Epon, according to the conventional procedure. Ultrathin sections were stained with uranyl acetate and lead nitrate for examination under an electron microscope (model 1010, JEOL Ltd., Tokyo, Japan).

Microdialysis Experiments In another set of experiments, basal levels of extracellular glutamate in VPM of rats were evaluated by microdialysis experiments. In brief, on Day 24, animals (n=6) in each group (control, TD and TD+1.0 g/kg TJ-54) were anesthetized with 50 mg/kg sodium pentobarbital, intraperitoneally, and a guide cannula (CMA/12, CMA, Solna, Sweden) was implanted into the VPM (coordinates: anterior −3.3 mm and right lateral 2.8 mm from the bregma, and ventral 7.0 mm from the dura) according to rat brain atlas of Paxinos and Watson.16) On Day 28, a vertical-type microdialysis probe (membrane: 0.5 mm o.d.×2.0 mm long, CMA) was inserted into the implanted guide cannula of unanesthetized freely-moving rats. The probe was perfused at a constant flow rate of 2.0 μl/min with Ringer’s solution (147 mM Na+, 4 mM K+, 1.2 mM Ca2+, 153.4 mM Cl−). The perfusates through the probe membrane were discarded for 3 h after the insertion of the probe, and then sampled at 20-min interval for 60 min (three samples) to obtain the basal level. When glutamate levels in the perfusates were stable, we judged it as the basal level. Thus, eighteen perfusate-samples from six animals in each group were obtained (54 samples in total). The glutamate level in the perfusate was determined by a HPLC with electrochemical detection with o-phthalaldehyde derivatization.17) The derivatization was carried out by mixing 30 μl of perfusate and 10 μl of 4 mM o-phthalaldehyde-2-mercaptopoethanol at 10°C for 10 min. The chromatographic conditions were column: EICOMPAK SC-5ODS (3.0Φ×150 mm long, Eicom), mobile phase: 0.1 m phosphate buffer, pH 6.0, containing 30% methanol and 13.4 μM EDTA-2Na, flow rate: 0.5 ml/min, column temperature: 30°C, an applied potential to an Ag/AgCl reference electrode: +600 mV, and working electrode: graphite electrode.

Statistical Analysis All values are represented as the mean±S.E.M. The statistical significance was evaluated by a one-way ANOVA followed by Fisher’s protected least significant difference (PLSD) test or Bonferroni multiple comparison procedure. The incidence of neurological symptoms was evaluated by a Fisher’s exact probability test, because some expected value was less than 5 in a 2×2 contingency table. The significance level in each statistical analysis was accepted at p<0.05.

RESULTS

Effects of TJ-54 on Food Intake and Body Weight in TD Rats The effects of TJ-54 on food intake (A) and body weight (B) in TD rats are shown in Fig. 1. Both food intake and body weight of the control, TD, and TD+TJ-54 groups gradually increased until Days 12—14. However, both food intake and body weight of TD and TD+TJ-54 (0.5, 1.0 g/kg) groups gradually decreased thereafter. On the terminal Day 37, the food intake and body weight of these groups had dramatically decreased to 4—6 g and 150—170 g, respectively. In both parameters, no statistically significant changes were observed among control, TD, and TD+TJ-54 (0.5, 1.0 g/kg) groups throughout the experimental period.

Effect of TJ-54 on Memory Disturbance The results of memory acquisition trials (learning process) of the four groups of rats while still receiving a normal diet are shown in Fig. 2A. In the first acquisition trial, all rats in each group entered the dark compartment within 30 s after being placed in the illuminated compartment. Repeating the acquisition trial increased the latency times in all groups. All rats in all groups acquired the avoidance memory, staying in the illuminated compartment over 300 s on the fifth day. No statistically significant differences were observed in the mean latency times among the four groups during the acquisition trials.

Memory retention tests were performed on Days 15 and 29 after TD feeding was started. On Day 15, most animals in each group stayed in the illuminated compartment, and the latency times remained at 288—300 s. No significant differences were observed among these groups (data not shown here). However, in the memory retention test on Day 29, 90% (9/10 rats) in the control group stayed in the illuminated compartment over 300 s, whereas only 9% (1/11) of the animals in the TD group stayed in the illuminated compartment over 300 s. The TD-induced decrease in the number of animals that stayed in the illuminated area increased to 70%
(7/10 rats) in the TD + 0.5 g/kg TJ-54 group and 55% (6/11 rats) in the TD + 1.0 g/kg TJ-54 group. The mean latency times of the animals in each group are shown in Fig. 2B. One-way ANOVA revealed significant difference of group factor \(F_{3,38} = 5.513, p < 0.01\). Post-hoc analysis revealed that the latency time (155 ± 25 s) of the TD group was significantly shorter \(p < 0.001\) than that (280 ± 20 s) of the control group. The shorter latency induced by TD was significantly inhibited or prolonged by treatment with TJ-54 in a dose-dependent manner.

**Effect of TJ-54 on Anxiety-Like Behavior** Open-field tests were performed on Days 14 and 28, respectively. On Day 14, abnormal behaviors by all rats in all groups were not observed (data not shown here). However, on Day 28, significant changes were observed in the locomotor patterns of animals among groups. The typical behavioral traces of rats in control (A), TD (B), TD + 0.5 g/kg TJ-54 (C), and TD + 1.0 g/kg TJ-54 (D) groups are shown in Fig. 3. The control rat actively moved over all in the circular arena (Fig. 3A), whereas the locomotion of TD rats seemed to be restricted the periphery of the apparatus; the rats walked close to the wall and did not enter the central area (Fig. 3B). Such behavioral abnormality in the TD rat seemed to be ameliorated in the TJ-54-treated groups in a dose-dependent manner (Figs. 3C, D).

From the behavioral tracing data, horizontal locomotion activities (total distance traveled in cm, total number of line crossings in five areas, and total number of entries into the central part) in the open field were quantitatively analyzed, and the results are shown in Fig. 4. Both the distance traveled \(F_{3,38} = 16.052, p < 0.0001\) (Fig. 4A) and the number of line crossings \(F_{3,38} = 15.976, p < 0.0001\) (Fig. 4B) of the TD group were decreased significantly more than those of the control group. In addition, the numbers of entries into the central part \(F_{3,38} = 3.076, p < 0.05\) of the TD group were also significantly decreased compared with that of the control group (Fig. 4C). The decreases in these parameters in the TD group were significantly ameliorated in the TJ-54-treated groups. These quantitative data supported the qualitative behavioral tracing data.

**Effects of TJ-54 on Aggression and Social Behaviors** The effects of TJ-54 on aggression and social behaviors were examined by social interaction tests on Day 21. The results are shown in Fig. 5. The total number \(F_{3,38} = 13.068, p < 0.0001\) and duration \(F_{3,38} = 10.650, p < 0.0001\) of aggressive behaviors in the TD group significantly increased more than those in the control group. The TD-induced increases in both parameters were significantly inhibited by treatment with 1.0 g/kg TJ-54 (Figs. 5A, B). The total number \(F_{3,38} = 9.023, p < 0.0001\) and duration \(F_{3,38} = 25.662, p < 0.0001\) of social behaviors in the TD group decreased significantly more than those in the control group. The TD-induced decreases in both parameters of social behaviors also were significantly inhibited by treatment with 1.0 g/kg TJ-54 (Figs. 5C, D). No significant differences of the motor activities in TD, TD + 0.5 g/kg TJ-54 and TD + 1.0 g/kg TJ-54 groups were observed compared with the control group (Fig. 5E).

**Effect of TJ-54 on Neurological Symptoms** From Day 30, several neurological symptoms including opisthotonus...
and convulsion were observed in a few rats in the TD group, and the incidence of these symptoms gradually increased and reached 73% (8/11 rats) on the terminal Day 37 (Fig. 6). In the control group, the neurological symptoms were entirely absent. TJ-54 significantly inhibited the TD-induced increase in the incidence of neurological symptoms in a dose-dependent manner (40% in the TD/H11001 0.5 g/kg TJ-54 group and 18% in the TD/H11001 1.0 g/kg TJ-54 group).

**Histopathological Changes** Light microscopic examinations of all animals in each group were performed on Day 37. In the TD animals, a marked sponge-like degeneration characterized by various sized vacuolations was observed in the brain stem (medulla), as shown in Fig. 7A. In addition, neuronal degeneration and loss were observed in the hippocampus (Fig. 7B), and the temporal and basalis regions of the cerebral cortex (Fig. 7C). The incidences of these cerebral degenerations are summarized in Table 1. The high TD-induced incidences of neuropathological degeneration in the brain stem (9/11 rats=81.8%), hippocampus (5/11 rats=45.5%), and cerebral cortex (5/11 rats=45.5%) were decreased by treatment with TJ-54 in a dose-dependent manner.

The ultrafine structural changes were examined electron microscopically in the brain stem, hippocampus, and cortex. Degeneration of astrocytes was more severe than degenera-

![Figure 4](image1.png)  
Fig. 4. The Total Distance Traveled (A), the Total Number of Line Crossings (B) and the Total Number of Entries in the Central Part (C) of the Open Field for 5 min on Day 28.

Value represents the mean±S.E.M. (n=10—11). Significance by Fisher’s PLSD test following one-way ANOVA is indicated as **p<0.01 and ***p<0.001 vs. Control, †p<0.05 and ††p<0.01 vs. TD.

![Figure 5](image2.png)  
Fig. 5. The Frequency (A) and Duration (B) of Aggressive Behaviors, the Frequency (C) and Duration (D) of Social Behaviors, and the Motor Activity (E) in Social Interaction Test on Day 21.

Value represents the mean±S.E.M. (n=10—11). Significance by Fisher’s PLSD test following one-way ANOVA is indicated as ***p<0.001 vs. Control, and ††p<0.01 and †††p<0.001 vs. TD.

![Figure 6](image3.png)  
Fig. 6. Incidence of Neurological Symptoms on Day 37.

Value represents the mean±S.E.M. (n=10—11). Significance by a Fisher’s exact probability test is indicated as **p<0.01 vs. Control, †p<0.05 vs. TD.
tion of neuronal cells in all areas of the TD rats. Figure 8 shows typical electron micrographs of astrocytes and their dendrites in the cortex of control (A, D), TD (B, E), and TD+TJ-54 (C, F) groups. In TD rats, severe vacuolated degeneration was observed in the cytoplasm of astrocytes, and the swollen dendrites showed signs of edematous degeneration (Fig. 8B). In addition, disintegration of mitochondria (mitochondrial swelling and disappearance of the cristae)
Stress induces long-lasting memory facilitation or long-fixed foot-shock (0.3 mA for 3 s). The electric foot-shock experienced by difference of individual pain threshold against a experiment, and also that the learning process was not influ-
nanced learning ability of all four groups was similar at start of the day of the learning process. These results suggest that the luminated compartment to avoid electric shock by the fifth threshold, all animals in each group learned to stay in the il-
though we did not examine about the individual’s pain
ments, basal levels of extracellular glutamate in VPM of ani-
were inhibited in TJ-54-treated rats (Figs. 8C, F). The TD-induced vacuolated degeneration in astrocytes and mitochondrial disintegration were inhibited in TJ-54-treated rats (Figs. 8C, F).
Effect of TJ-54 on TD-Induced Increase in Extracellu-
level of Glutamate in VPM In another set of experiments, basal levels of extracellular glutamate in VPM of ani-
mals in control, TD and TD+1.0 g/kg TJ-54 groups were evaluated by microdialysis experiments. As shown in Fig. 9, the extracellular glutamate level in the TD group significantly increased more than that in the control group (F_{2,51} = 14.473, p < 0.0001). The TD-induced increase in glutamate level was significantly inhibited by treatment with TJ-54 (1.0 g/kg).

DISCUSSION
Learning process, i.e., memory acquisition trial, in the step-through passive avoidance task has been reported to be influenced by changes in individual difference of pain threshold against electric shock with treatment of drug. In the present study, the acquisition trials were performed in rats still receiving the normal diet before drug-treatment. Although we did not examine about the individual’s pain threshold, all animals in each group learned to stay in the illuminated compartment to avoid electric shock by the fifth day of the learning process. These results suggest that the learning ability of all four groups was similar at start of the experiment, and also that the learning process was not influenced by difference of individual pain threshold against a fixed foot-shock (0.3 mA for 3 s). The electric foot-shock stress induces long-lasting memory facilitation or long-
term memory. In fact, we judged that control rats retained the avoidance memory on Day 29, because the latency (280±20 s) was retained for 29 d. However, the latency in TD rats significantly decreased on Day 29. This result suggests that the memory in rats is impaired by TD feeding. These results are consistent with the previous report that the avoidance learning is impaired on the 25th day of TD feeding in rats. Amnesia in TD rats also has been demonstrated by other tests, such as the water maze task and the T-maze task, for evaluation of learning and memory. Taken together, these findings suggest that memory disturbance occurs in TD animals. Treatment with TJ-54 recovered the decrease in latency time in TD rats (Fig. 2B). As the retention test in the present study was evaluated in the condition that an electric foot-shock did not deliver to floor grid, the data of TJ-54 is thought to be not due to the reduction of pain threshold against the electric shock, i.e., these changes were selective to memory function. Therefore, we judged that TJ-54 improved the memory disturbance induced by TD.
The open field test is a widely used to evaluate anxiety in rodents. In this test, exploratory locomotor activity decreases in rodents experiencing anxiety. In particular, the decrease of locomotion in the central area is considered to be anxiety-related. These evaluations are based on the assumption that the arena center is more threatening for rodents than the periphery, and an increase in center occupation is seen after administration of anxiolytic drugs. In the present study, exploratory locomotion activity (total distance traveled and total number of line crossings) and central locomotion activity (total number of entries in the central part) in the open field decreased in TD rats. Therefore, we judged that the behaviors observed in TD rats were anxiety-like behaviors. The anxiety-like behaviors were ameliorated in TJ-54-treated animals. These results suggest the possibility that TJ-54 possesses an anxiolytic effect. The possibility is supported by the results of previous study using an improved plus-maze test in mice treated with TJ-54.
Aggressiveness such as muricide (mouse-killing behavior) has been observed in TD rats. In the present study, the aggressiveness of TD rats was evaluated by a social interaction test because allowing muricide is not ethical. As shown in Fig. 5, the number and duration of aggressive behaviors increased in TD rats, and those of social behaviors, which are considered to be forms of inter-animal communication or recognition, decreased in TD rats. Because the TD-induced increase in aggressive behaviors and decrease in social behaviors were inhibited by treatment with TJ-54, TJ-54 is suggested to be an effective to treat aggression and decreased sociability. These effects by TJ-54 are thought to be selective, i.e., these are not due to the changes in motor activity.

### Table 1. Effects of TJ-54 on Incidences of Cerebral Degeneration in Brain Stem, Hippocampus, and Cerebral Cortex of TD Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of animals</th>
<th>Medulla (brain stem)</th>
<th>Hippocampus</th>
<th>Cerebral cortex</th>
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<td></td>
<td></td>
<td>Sponge-like degeneration</td>
<td>Neuronal loss</td>
<td>Neuronal loss</td>
</tr>
<tr>
<td>Control†+DW</td>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TD+DW</td>
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<td>9</td>
<td>81.8</td>
<td>5</td>
</tr>
<tr>
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<td>4</td>
<td>40.0</td>
<td>1</td>
</tr>
<tr>
<td>TD+1.0 g/kg TJ-54</td>
<td>11</td>
<td>2</td>
<td>18.2</td>
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</tbody>
</table>

* Fig. 9. Extracellular Concentrations of Glutamate in VPM of Animals in Control, TD and TD+1.0 g/kg TJ-54 Groups

On Day 28, basal levels (three samples/animal) of extracellular glutamate in VPM of rats (six animals/group) were evaluated by microdialysis experiments. Value represents the mean±S.E.M. (eighteen samples/six animals/group). Significance by Bonferroni multiple comparison procedure following one-way ANOVA is indicated as ***p<0.001 vs. Control, †p<0.01 vs. TD.
because the treatment of control, TD or TD+TJ-54 did not affect motor activity of animals. The effectiveness of TJ-54 against aggression and sociability obtained in the present study may also be supported by a clinical study reporting that TJ-54 ameliorated excitement and anger and improved activities of daily living in patients with Alzheimer’s disease.  

At the termination of the present experimental period, neurological symptoms, including opisthotonus and convulsions, that are characteristic of TD rats were observed. TJ-54 significantly inhibited the TD-induced increase in the incidence of the symptoms in a dose-dependent manner. These results may also suggest that TJ-54 inhibits excessive excitability.

As described above, the present study suggests that TJ-54 possesses the preventive or progress inhibitory effect against the development of memory disturbance and BPSD-like behaviors such as anxiety-like behavior, aggressive or social behavior, and neurological symptoms observed in TD rats, because TJ-54 was administered together with TD breeding. It is unclear whether TJ-54 possesses curative effect against these symptoms, in the present study. However, we recently demonstrated that three-week-administration of TJ-54 significantly ameliorated aggressiveness induced by intracerebroventricular injection of amyloid β into mice in a dose-dependent manner, while a single administration of TJ-54 did not ameliorate the aggressiveness, suggesting that chronic treatment with TJ-54, at least, exhibits curative effects against aggressiveness.  

Several symptoms, for example, decreases in daily food intake, a decline in the growth rate and the development of neurological symptom so on have been reported to be improved by giving thiamine to TD rats. If thiamine were contained in TJ-54, the food intake and body weight in TJ-54-treated rats would have increased. However, no significant changes were observed in TD and TJ-54 groups. Furthermore, we have confirmed that thiamine was not detected in the extract of TJ-54 as described in Materials and Methods. Taken together, the pharmacological effects of TJ-54 on TD rats are, at least, not achieved by the effects of supplemental thiamine. On the other hand, it has been reported that TD-induced muricide is suppressed by 5-hydroxytryptophan, a serotonin precursor, chlorimipramine, a selective inhibitor of serotonin reuptake, L-threo-dihydroyxphenylserine, a direct precursor of norepinephrine, and desmethyli mipramine, an inhibitor of norepinephrine reuptake. TD diet-induced depressive behavior in mice are reduced by chronic administration of the tricyclic antidepressant imipramine, which is an inhibitor of reuptake of serotonin and noradrenaline. In addition, cerebral choline acetyltransferase activity is reported to decrease in the TD rat, and donepezil, an acetylcholinesterase inhibitor, reversed a significant impairment of cognition in patients with Wernicke–Korsakoff’s syndrome.  

These results suggest that the developmental mechanism of TD diet-induced behavioral and psychological symptoms may be involved in the degeneration of central serotonergic, noradrenergic, and cholinergic neurons. Therefore, the prevention of abnormal behavioral and psychological symptoms by TJ-54 observed in TD rats also may involve these neurons, although the hypothesis should be clarified in future studies.

Thus, several factors are proposed as the mechanism in the background of the symptoms caused by TD. Although there is not yet the established theory, the glutamate excitotoxicity is proposed as one of the convincing hypotheses. In pathological examination of the present study, neuronal cell loss and degeneration were observed in the brain stem, hippocampus and cortex. In particular, hippocampal or cortical neuronal loss and degeneration agree with those observed in dementia models such as ischemia and APP transgenic animals. Selective cell death is common in many aging-related neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease and progressive supranuclear palsy. The neurological disorder that is most clearly associated with TD in humans is Wernicke–Korsakoff syndrome, which is characterized by severe memory loss, cholinergic deficits and selective cell death in specific brain regions. These feature of the TD model are thought to be suitable for investigating the cellular mechanism of neurodegeneration.

Electron-microscopic examination in the present study showed that edematous degeneration in astrocytes was more severe than those in neuronal cells in TD brain regions. These results were consistent with those reported by Hazell et al. Collins has also demonstrated that astrocytes are among the first cells to be affected by thiamine deficiency in advance of neuronal cell death. Disintegration of mitochondria (mitochondrial swelling and disappearance of the cristae) in the TD astrocytes as shown in Fig. 8 agrees with the findings reported by Parker et al. The disruption of mitochondria implies disturbance of the energy metabolism necessary to maintain cell function. Thiamine pyrophosphate, active form of thiamine, is cofactor for three major enzymes (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase and transketolase) involved in cerebral glucose and energy metabolism which is related to TCA cycle of mitochondria. Therefore, dysfunction in the mitochondrial energy metabolism under TD condition may be related to the cell degeneration and the disintegration of mitochondria.

Alzheimer’s disease also is reported to significantly decrease cerebral glucose utilization. The enzymes related to cerebral glucose and energy metabolisms decrease in Alzheimer’s disease as well as TD. , i.e., the cell function may be impaired, which in turn causes cell degeneration.

Astrocytes perform many important functions in the brain, one of which is the efficient removal of glutamate from the extracellular space via the glutamate transporters GLAST and GLT-1. Recently, extracellular glutamate concentrations have been demonstrated to be increased in vulnerable regions of the brain such as the thalamus in TD. A selective down-regulation of the astrocyte glutamate transporters GLT-1 and GLAST provides a rational explanation for the increase in interstitial glutamate levels. In addition, the extent of cell death in the TD brain is reported to be reduced by treatment with the non-competitive N-methyl-d-aspartic acid receptor antagonist MK-801. These lines of evidence suggest that neuronal degeneration and cell death are closely related to the glutamate excitotoxic mechanism. Elevation of the extracellular concentration of glutamate is also reported in ischemia model and epilepsy model. Amyloid β peptide which is considered as a causative substance of Alzheimer’s disease is also reported to inhibit glutamate uptake into astrocytes. In the present study, TJ-54 inhibited the TD-induced neuronal degeneration and, in particular, severe degeneration of astrocytes. The elevation of extracellular-
lar glutamate concentration in the VPM of TD rats was inhibited by treatment with TJ-54. These results suggest that extracellular glutamate rise may be due to the dysfunction of astrocytes. In order to clarify the hypothesis, more recently, we demonstrated in cultured astrocytes that not only the up-take of glutamate into astrocytes but also the levels of proteins and mRNA expressions of GLAST significantly decreased under TD condition, and these decreases were ameliorated by treatment with TJ-54. In addition, TJ-54 inhibited glutamate-induced PC12 cell death. These results suggest that TJ-54 may exert a neuroprotective effect against the glutamate excitatory neurotoxicity not only by amelioration of dysfunction of astrocytes but also by direct protection of neuronal cells.

TJ-54 is a dry powdered extract from a mixture of Atractylodes Lancea rhizome, Hoelen, Cnidii Rizoma, Japanese Angelica root, Bupleurum root, Glycyrrhiza root, and Uncaria rhizome. In order to clarify the hypothesis, more recently, we demonstrated in cultured astrocytes that not only the up-take of TJ-54 has to be investigated more in detail, in future.

In conclusion, the present study suggests, at least, that TJ-54 possesses the preventive or progress inhibitive effect against the development of memory disturbance and BPSD-like behaviors induced by the degeneration of neuronal and astroglial cells resulting from TD, although additional studies are required to clarify the underlying mechanisms of TJ-54.

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