Original Article

Behavioral and biochemical characterization of rats treated chronically with thioacetamide: proposal of an animal model for hepatic encephalopathy associated with cirrhosis

Hiroshi Kawai[1], Takuya Ishibashi[1], Naomi Kudo[2], Yoichi Kawashima[2] and Atsushi Mitsumoto[1]

[1]Faculty of Pharmaceutical Sciences, Josai International University, 1 Gumyo, Togane, Chiba 283-8555, Japan
[2]Faculty of Pharmaceutical Sciences, Josai University, 1-1 Keyakidai, Sakado, Saitama 350-0295, Japan

ABSTRACT — Hepatic encephalopathy (HE) is a syndrome observed in patients with liver dysfunction such as hepatitis and cirrhosis, and is characterized by cognitive impairment, personality changes, and a depressed level of consciousness. The detailed mechanism underlying the pathogenesis of HE remains unclear. In the present study, our aim was to establish an animal model for HE with cirrhosis. Therefore, we carried out behavioral and biochemical analysis of cirrhotic rats after treatment with thioacetamide (TAA) for 20 weeks. The rats subjected to chronic TAA treatment (TAA rats) showed reduction of cognitive scores in the novel object recognition test (NOR), and a decrease in immobility and an increase in swimming in the forced swim test (FST). In biochemical analyses, the TAA rats exhibited elevated blood levels of ammonia, and increased metabolic activities of serotonergic and noradrenergic neurons in the brain, while the levels of Glu and GABA were not affected. Post-oral treatment of lactulose, a clinically utilized drug for HE, effectively reduced the elevated blood ammonia levels, and restored the reduced cognitive scores and the decreased immobility, without any effects on neurotransmitter contents in the brain, compared with the control. These results indicated lactulose-restorable memory disturbance and irritated mood in the TAA rats. In other words, rats treated chronically with TAA are a potential model for cirrhosis-HE, and the combination of NOR and FST in TAA rats may be useful as a simple assay system for the screening and development of anti-HE agents.

Key words: Thioacetamide, Cirrhosis, Hepatic encephalopathy, Psychiatric deficit, Ammonia, 5HT

INTRODUCTION

Hepatic encephalopathy (HE) is a syndrome observed in patients with liver dysfunction such as hepatitis and cirrhosis, and is characterized by cognitive impairment, personality changes, and a depressed level of consciousness. HE is classified into types A, B and C depending on the underlying cause (Ferenci *et al.*, 2002). Type A describes HE associated with *A*cute liver failure, typically associated with cerebral edema, resulting from severe inflammatory and/or rapid onset of liver disease. Type B is caused by portal-systemic *B*ypass without associated parenchymal liver disease. Type C occurs in patients with *C*irrhosis and portal hypertension or portal-systemic shunts, and is subdivided into episodic, persistent and minimal encephalopathy.

The development of hepatic encephalopathy is explained, to some extent, by the effects of neurotoxic substances, which occur in the setting of cirrhosis and portal hypertension. Ammonia has multiple neurotoxic effects. It can alter the transit of amino acids, water, and electrolytes across astrocytes and neurons, and can also impair amino acid metabolism and energy utilization in the brain. Ammonia can also inhibit the generation of excitatory and inhibitory postsynaptic potentials. Normally, ammonia is detoxified in the liver by conversion to urea, and also consumed in the conversion of glutamate to glutamine. In the case of cirrhosis, a decreased mass of functioning hepatocytes and portosystemic shunting contribute to the hyperammonemia. The ammonia hypothesis is also supported by the clinical observation that treatments that decrease blood ammonia levels can improve

Correspondence: Hiroshi Kawai (E-mail: hkawai@jiu.ac.jp)

hepatic encephalopathy symptoms.

Lactulose is a nonabsorbable disaccharide that has been in common clinical use since the early 1970s. It is thought to improve the generation of ammonia by bacteria, and to render the ammonia (NH_3) inabsorbable by converting it to ammonium (NH_4^+). A 2004 review by the Cochrane Collaboration concluded that there was insufficient evidence to determine whether lactulose is of benefit for all types of HE, but it remains the first-line treatment for type C HE.

Animal models for HE have been developed by several methods, including hepatotoxicant treatments and surgical operations. Acute administration of hepatotoxicants at a very high dose induces severe hepatitis or fulminant hepatic failure (FHF), which finally induces HE and hepatic coma (type A HE) (Zimmerman *et al*., 1989; Yurdaydin *et al*., 1996). Portacaval shunting (PCS) leads to HE symptoms without inducing liver damage (type B) (Steindl *et al*., 1996; Watanabe *et al*., 1997). Bile duct ligation (BDL) induces obstruction of bile flow, which leads to biliary cirrhosis and HE (type C) (Jover *et al*., 2006). Since there are a wide variety of etiologies and symptoms in human liver disease, it is not possible to reproduce all aspects of HE in a single animal model. Indeed, the International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) has suggested that there is a dearth of satisfactory animal models of HE, and an urgent need to develop such models in order to clarify the pathogenesis of HE and develop effective therapeutic treatments (Butterworth *et al*., 2009). HE models with PCS or BDL are useful for such investigations, but other animal models will be necessary for the investigation of type C HE with chronic liver dysfunction, such as cirrhosis.

Administration of hepatotoxicants provides a stable animal model of liver disease. Various toxicants such as acetoaminophen, carbontetrachloride, D-galactosamine, azoxymethane, and thioacetamide (TAA) are known to induce liver damage (Rahman *et al*., 2002; Yamamoto and Sugihara, 1987; Bassett *et al*., 1987; Matkowskyj *et al*., 1999; Zimmerman *et al*., 1989). Among them, TAA is useful for induction of both acute and chronic liver damage. Acute administration of TAA at a high dose to rats induces HE, and this model meets the majority of criteria for type A HE (Zimmerman *et al*., 1989; Butterworth *et al*., 2009). On the other hand, chronic TAA treatment at a low dose to rats induces cirrhosis and hepatic tumors (Müller *et al*., 1988; Fontana *et al*., 1996; Kawai *et al*., 2009). The characteristic features of TAA-induced hepatotoxicity in rats resemble those of human cirrhosis (Müller *et al*., 1988; Dashti *et al*., 1989; Sato *et al*., 2000), and

rats chronically treated with TAA exhibit memory impairment (Méndez *et al*., 2008a). Therefore, we hypothesized that chronic TAA treatment of rats might induce HE-like neuropsychiatric symptoms, such as memory impairment and mental disturbance. In the present study, our aim was to establish an animal model for HE with cirrhosis. To this end, we carried out behavioral and biochemical analysis of cirrhotic rats after treatment with TAA for 20 weeks.

MATERIALS AND METHODS

Chemicals

Lactulose (Monilac 65% syrup) was obtained from Chugai Seiyaku (Tokyo, Japan). TAA and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Animals

Male adult Wistar-Hannover rats (150-200 g) were obtained from CLEA Japan Inc. (Tokyo, Japan), and were maintained in an air conditioned room at $24 \pm 2°C$ with a 12/12 hr light/dark cycle (lights on at 07:00). The rats had free access to food and water.

The experimental schedules are illustrated in Fig. 1. Induction of liver damage was performed by continuous administration of TAA in drinking water (0.3 or 0.5 g/l) for 20 weeks. The dose and the duration of TAA treatment was determined according to our previous study that rats developed cirrhosis by TAA treatment at 0.5 g/l for 16 weeks (Kawai *et al*., 2009). In the case of lactulose treatment, 2 g/kg of lactulose was orally administered once a day between 17:00 and 19:00 for 2 weeks. On the 21st and 22nd week, behavioral tests were conducted between 08:00 and 13:00. Administration of TAA in the drinking water was continued during behavioral tests. After the behavioral tests, rats were sacrificed, and blood and brain samples were collected.

Animal maintenance and treatments were in accordance with the general recommendations of Japanese animal protection legislation. All procedures were approved by the Institutional Animal Care and Use Committee of Josai International University.

Novel object recognition test

The novel object recognition (NOR) test, which is used to evaluate the non-spatial visual memory in rodents (Ennaceur and Delacour, 1988), is a non-invasive method that does not require punishment or reward and that can be implemented quickly and simply.

In this test, on the first day, rats were habituated for 5 min to the circular field (80 cm diameter) on which

An animal model for TAA (chemical)-induced hepatic encephalopathy

(A)

(B)
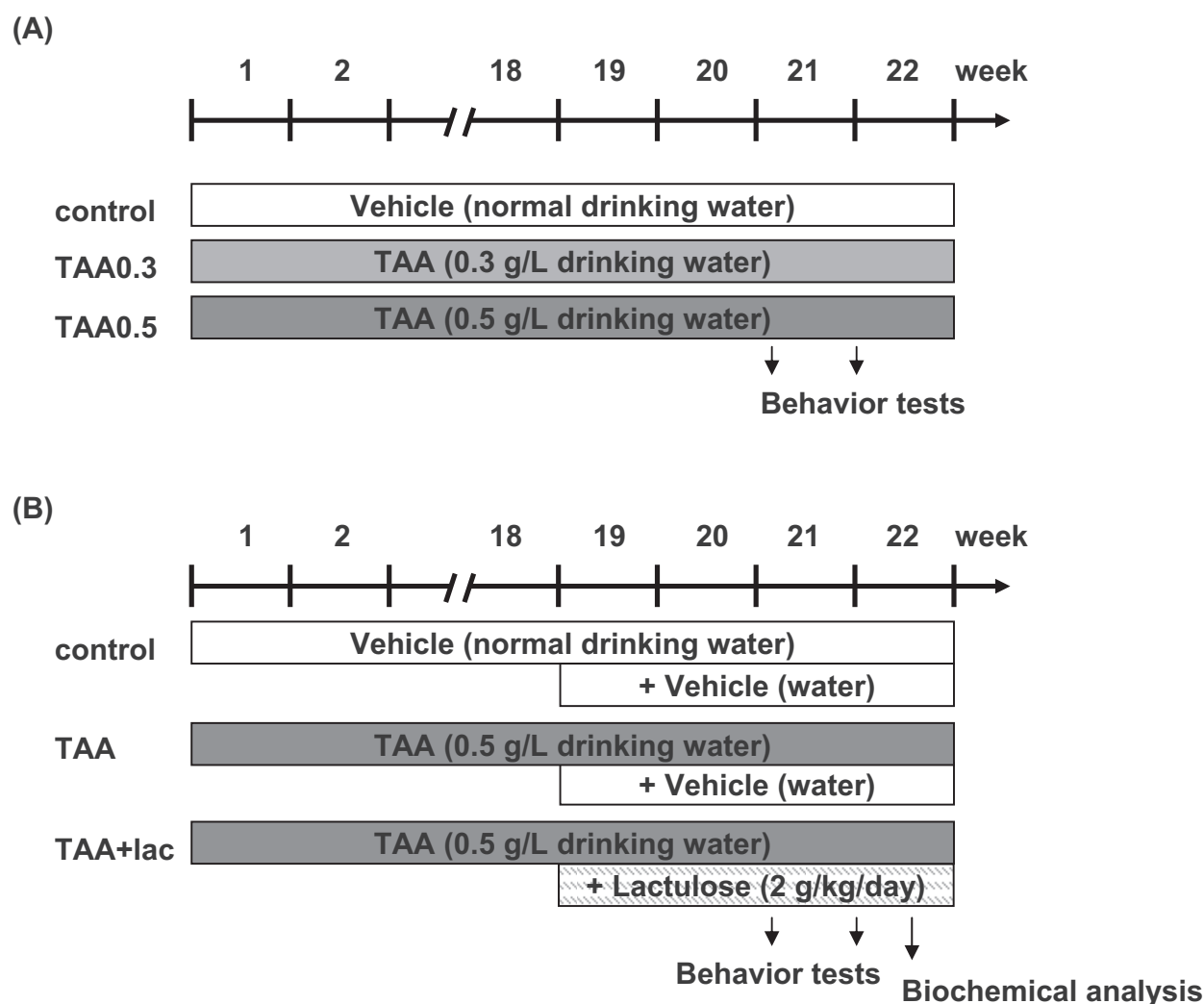
Fig. 1. Experimental schedules. (A) In the first set of experiments, rats were administered 0.3 g/l or 0.5 g/l of TAA in drinking water throughout the experimental period (TAA0.3 and TAA0.5 groups, respectively). Behavioral tests were carried out after 20 weeks of TAA administration. The exploring activity test, home-cage activity test, NOR, and FST were carried out within 2 weeks. (B) In another set of experiments, the TAA group and TAA+lac group were presented with 0.5 g/l of TAA in drinking water. After 18 weeks, vehicle or 2 g/kg of lactulose were perorally administered once a day. TAA and lactulose treatment were continued until the end of the experiments. Behavioral tests were carried out after 20 weeks of exposure to TAA. Blood and brain samples were removed after behavioral tests, and were used in the biochemical analysis.

the test was performed. On the second day, the rats were exposed to two identical objects in the field for 2 min. The rats were allowed to freely explore these objects (training session). On the third day, 24 hr after the training session, the rats were again placed in the field for 2 min with one object having been replaced by a novel object, and the time that the rat spent exploring each object was recorded (test session). The ratio of the time spent exploring the novel object to the time spent exploring both objects was calculated as the novel object preference. The reduction in the novel object preference was assessed as memory impairment. All objects and the test field were thoroughly cleaned with 70% ethanol and dried before each use.

Forced swim test

We used a modified forced swim test (FST) according to the method of Detke *et al*. (1995). On the day before measurement, rats were individually placed in plastic cyl-

inders (24 cm diameter, 50 cm height) filled with water at room temperature (24 ± 2°C) to a depth of 30 cm. Rats were exposed to the apparatus for 10 min, then removed from the cylinder, allowed to dry in a clean cage, and returned to their home cages. Twenty-four hours after the first exposure, the rats were again placed in the apparatus, and their behaviors were analyzed for 5 min. At regular 5-sec intervals, a trained observer recorded which of the following three behaviors was predominant: immobility, swimming, or climbing. The total counts for each behavior over the 5 min-test session were analyzed as the FST score.

Blood chemistry

Blood samples were removed from the postcaval vein with a 0.70 × 32 mm needle attached to a 5-ml syringe. One ml of blood samples was mixed with sodium tungstate, and centrifuged at 1,200 × g for 10 min to obtain deproteinized blood samples. The remaining blood samples were centrifuged at 1,200 × g for 10 min to obtain serum. The deproteinized blood samples were analyzed to determine the blood ammonia levels, and serum samples were analyzed to measure the serum activities of alkaline phosphatase (ALP) and alanine aminotransferase (ALT) and the serum contents of albumin (Alb), the latter of which was used to evaluate the liver damage (Mitsubishi Chemical Medience Corporation, Tokyo, Japan). The molar ratio of the serum content of branched-amino acids to that of tyrosine (BTR) was also measured to evaluate the changes in the amino acids-balance in blood (Mitsubishi Chemical Medience Corporation). All biochemical assays were performed according to the standard methods.

Sample preparation for analysis of neurotransmitters

Rats were anesthetized with diethylether and sacrificed by decapitation. The brains were rapidly dissected out and divided into 7 sections: the cerebral cortex, striatum, hippocampus, midbrain/thalamus, hypothalamus, pons/medulla, and cerebellum. The sectioned brain tissues were immediately frozen in liquid nitrogen and kept at -80°C until use.

The divided brain tissues were homogenized in a Polytron (Kinematica, Switzerland) with 5 volumes of 0.2 M perchloric acid containing 0.1 mM EDTA. Isoproterenol at 1 nmol/sample and β-alanine at 1 μmol/sample were added as an internal standard for the analysis of monoamines and amino acids (Glu and GABA), respectively. The homogenate was centrifuged at 20,000 × g for 15 min at 4°C, and the supernatant was adjusted to pH 3

with 1 M sodium acetate, and then filtered through a 0.45-μm filter. A portion of the filtrate was subjected to HPLC analysis as described below.

Analysis of monoamine neurotransmitters

Monoamine neurotransmitters and their metabolites were analyzed with HPLC-ECD. Serotonin (5-hydroxytryptamine, 5HT), 5-hydroxyindolacetic acid (5HIAA), noradrenaline (NA), 3-methyoxy-4hydroxyphenylethylene glycol (MHPG), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanilic acid (HVA) were analyzed. The Shimadzu LC-10AD / Eicom ECD-700 system was used and the conditions were as follows: column, SC-5ODS (3.0 mm i.d. × 150 mm, Eicom) with a precolumn (PREPAK-AC, Eicom); mobile phase, 87% 0.1 M sodium acetate / 0.1 M citric acid buffer (pH 3.5), 13% methanol, containing 5 mg/l EDTA and 190 mg/l sodium octanesulfonate; flow, 0.5 ml/min; electrode, Eicom WE-3G graphite; reference, Eicom RE-100 Ag-AgCl; applied voltage, 700 mV vs. Ag-AgCl.

Analysis of glutamate and GABA

Glu and GABA were derivatized with *o*-phthalaldehyde (OPA) and analyzed with HPLC-FD. The OPA stock solution (40 mM) was prepared by dissolving 54 mg OPA with 1 ml methanol, and then mixing with 9 ml of potassium carbonate buffer (pH 9.5). The OPA stock solution was kept at -20°C until use. The derivatizing reagent (OPA-ME) was prepared daily by mixing 250 μl of the OPA stock solution, 1 μl of 2-mercaptoethanol, and 2.249 ml of potassium carbonate buffer (pH 9.5). The filtered sample solution was diluted with phosphate-buffered saline (1:1,000). Derivatization was carried out by mixing 10 μl of diluted sample solution and 30 μl of OPA-ME solution at room temperature for 2.5 min. A portion of the mixed solution was subjected to HPLC-FD analysis. The Shimadzu LC-10AD HPLC / RF-10A XL fluorescence detector system was used and the conditions were as follows: column, Gemini-ODS (2.0 mm i.d. × 150 mm; Phenomenex) with a precolumn (SecurityGuard C18 cartridge; Phenomenex); mobile phase, 85% 0.1 M sodium acetate buffer (pH 6.0) and 15% acetonitrile containing 100 mg/l EDTA; flow, 0.2 ml/min; excitation wave length, 360 nm; emission wave length, 450 nm.

Statistical analysis

Data are expressed as the means ± S.E.M. In the behavioral analysis (Figs. 2, 3), statistically significant differences between the experimental and control groups were analyzed by a two-tailed Dunnett's test. In the analysis of the effect of lactulose on the symptoms observed in TAA

An animal model for TAA (chemical)-induced hepatic encephalopathy

(A)

(B)

(C)
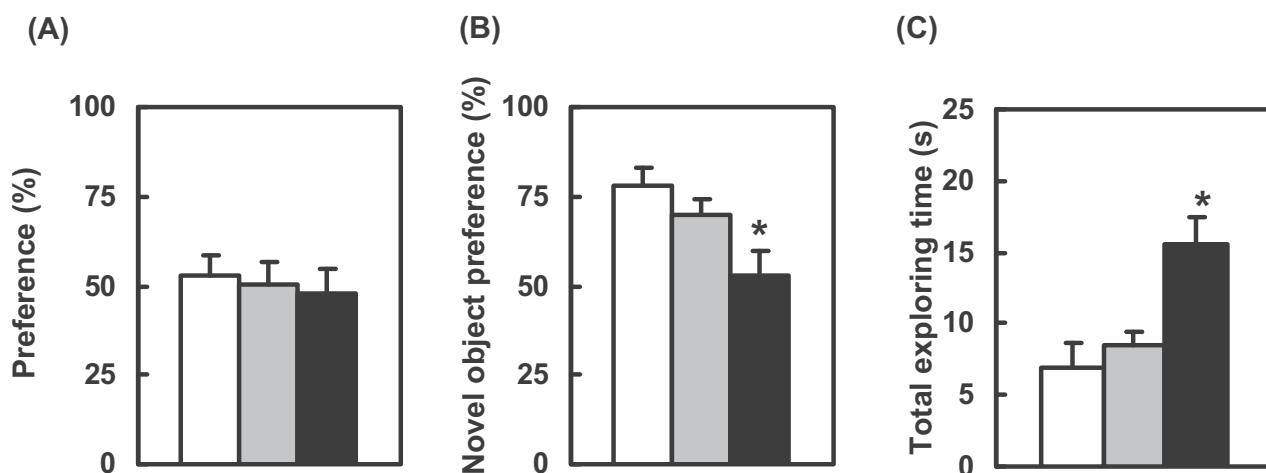
Fig. 2. Cognitive activity of TAA rats. An NOR test was carried out as described in the Materials and Methods. (A) The preference for one object in the training session. The ratio of the time spent exploring one object to the time spent exploring both objects was calculated. (B) NOR in test session. The ratio of the time spent exploring the novel object to the time spent exploring both objects was calculated as the novel object preference (%). (C) The total exploring time of two objects in test session is shown. Open column: control (n = 11); gray column: TAA0.3 (n = 12); closed column: TAA0.5 (n = 9). * p < 0.05 vs control by Dunnett's test.

rats (Figs. 3 and 4, Tables 1 and 2), statistical significance was determined by a two-tailed Tukey's test. A two-tailed Student's *t*-test was used in the analysis of Glu and GABA contents (Table 3). The significance level was set at 0.05.

RESULTS

Psychiatric assessment of rats with TAA-induced cirrhosis

In order to evaluate the neuropsychological state of the TAA rats, a novel object recognition test (NOR) and forced swim test (FST) were conducted. In the training phase of the NOR test, no difference was observed in preference for the novel object between the TAA rats and controls (Fig. 2A). In the test phase, no novel object preference was observed in the rats administered 0.5 g/l TAA (TAA0.5 rats), while the control rats exhibited a novel object preference of more than 75% (Fig. 2B). The effect of TAA on recognition of the novel object was dose-dependent. The TAA0.5 rats spent a longer time exploring than the control rats (Fig. 2C).

Figure 3 indicates the results of the FST in TAA-treated rats. TAA dose-dependently reduced the scores in immobility and increased the scores in swimming, while no effect was observed in the scores in climbing. The TAA rats sometimes showed aggressive behavior in their cages, and often bit the fingers of the experimenters (data
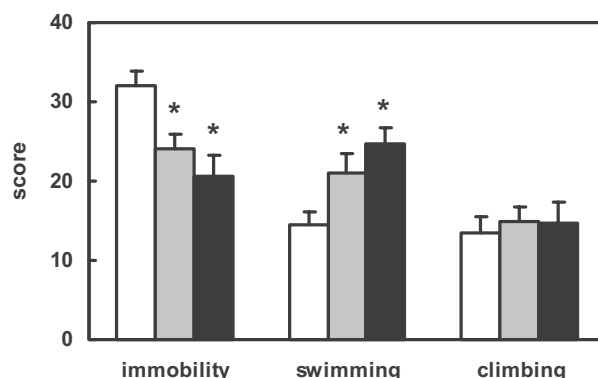
Fig. 3. Mood state of TAA rats. FST was carried out as described in the Materials and Methods. Immobility, swimming, and climbing scores are shown. Open column: control (n = 17); gray column: TAA0.3 (n = 18); closed column: TAA0.5 (n = 12). * p < 0.05 vs the respective control by Dunnett's test.

not shown). These findings suggest that the TAA rats may show irritated mood.

Effects of lactulose on the neuropsychiatric symptoms in TAA0.5 rats

Next we evaluated the effects of lactulose on the neu-

H. Kawai *et al.*

Table 1. Blood biochemical analysis of TAA rats

	ALP (IU/l)	ALT (IU/l)	Alb (mg/dl)	BTR	NH$_3$ (µg/dl)
Control	397 ± 39	49.9 ± 3.6	4.8 ± 0.1	3.7 ± 0.2	82 ± 13
TAA	722 ± 101 *	35.3 ± 7.3 *	4.2 ± 0.1 *	2.7 ± 0.1 *	162 ± 30 *
TAA+lac	702 ± 56 *	29.7 ± 3.1 *	4.3 ± 0.1 *	2.5 ± 0.1 *	117 ± 19

Values are presented as the mean ± S.E.M. (n = 6-8).
* $p < 0.05$ vs control by Tukey's test. No statistically significant difference was detected between TAA and TAA+lac.

Table 2. Brain contents of monoamine neurotransmitters and their metabolites

	Cor.	Str	Hipp	Mid	Hypoth	Pons	Cerebe
5HT							
Control	9.3 ± 1.0	8.8 ± 0.4	5.6 ± 0.2	16.0 ± 1.2	10.7 ± 1.3	11.5 ± 0.5	0.47 ± 0.04
TAA	10.3 ± 0.4	8.9 ± 0.4	5.2 ± 0.3	18.5 ± 1.6	11.2 ± 0.7	13.8 ± 0.6 *	0.58 ± 0.03
TAA+lac	10.1 ± 0.7	8.1 ± 0.3	4.8 ± 0.3	16.2 ± 0.9	9.2 ± 0.9	12.5 ± 0.5	0.59 ± 0.10
5HIAA							
Control	9.1 ± 1.3	13.2 ± 0.5	8.3 ± 0.3	18.6 ± 1.5	15.2 ± 0.8	11.8 ± 0.5	0.9 ± 0.1
TAA	14.1 ± 0.4 *	20.9 ± 1.0 *	12.3 ± 0.4 *	34.1 ± 2.2 *	22.8 ± 1.6 *	22.6 ± 1.4 *	1.4 ± 0.1 *
TAA+lac	12.9 ± 0.6 *	19.2 ± 1.0 *	11.6 ± 0.7 *	30.6 ± 2.6 *	21.1 ± 1.1 *	19.4 ± 1.3 *	1.5 ± 0.1 *
NA							
Control	3.6 ± 0.6	1.2 ± 0.2	3.3 ± 0.2	4.1 ± 0.5	11.6 ± 1.3	3.9 ± 0.2	1.5 ± 0.1
TAA	3.9 ± 0.1	1.3 ± 0.1	3.6 ± 0.1	4.9 ± 0.4	15.1 ± 1.0	4.8 ± 0.2 *	1.5 ± 0.1
TAA+lac	3.9 ± 0.4	1.5 ± 0.1	3.4 ± 0.4	4.5 ± 0.6	16.2 ± 1.3 *	4.8 ± 0.3 *	2.0 ± 0.3
MHPG							
Control	2.3 ± 0.5	2.2 ± 0.2	2.0 ± 0.2	1.7 ± 0.2	2.8 ± 0.1	1.6 ± 0.2	2.4 ± 0.2
TAA	5.1 ± 0.4 *	3.6 ± 0.2 *	3.4 ± 0.2 *	3.1 ± 0.3 *	3.8 ± 0.3 *	3.0 ± 0.4 *	3.8 ± 0.3 *
TAA+lac	5.1 ± 0.6 *	3.8 ± 0.4 *	3.7 ± 0.2 *	3.4 ± 0.5 *	4.2 ± 0.2 *	3.7 ± 0.4 *	5.4 ± 0.6 *†
DA							
Control	6.8 ± 0.9	91.3 ± 5.1	0.59 ± 0.04	1.4 ± 0.2	3.0 ± 0.4	0.47 ± 0.02	0.09 ± 0.01
TAA	5.1 ± 1.1	92.2 ± 4.2	0.60 ± 0.04	1.3 ± 0.1	3.2 ± 0.4	0.56 ± 0.04	0.10 ± 0.01
TAA+lac	5.3 ± 1.1	89.9 ± 7.8	0.53 ± 0.05	1.2 ± 0.1	2.4 ± 0.4	0.52 ± 0.03	0.13 ± 0.03
DOPAC							
Control	1.4 ± 0.2	30.4 ± 4.1	0.36 ± 0.03	0.44 ± 0.09	2.9 ± 1.1	0.24 ± 0.03	0.11 ± 0.01
TAA	1.3 ± 0.1	35.5 ± 3.3	0.53 ± 0.06	0.43 ± 0.04	2.9 ± 0.5	0.35 ± 0.06	0.12 ± 0.01
TAA+lac	1.6 ± 0.2	35.8 ± 2.5	0.43 ± 0.04	0.39 ± 0.03	2.1 ± 0.2	0.28 ± 0.02	0.15 ± 0.02
HVA							
Control	0.9 ± 0.2	7.5 ± 0.5	0.14 ± 0.01	0.36 ± 0.06	0.70 ± 0.21	0.19 ± 0.03	0.09 ± 0.01
TAA	1.1 ± 0.1	8.9 ± 0.4	0.20 ± 0.02	0.38 ± 0.03	0.91 ± 0.15	0.27 ± 0.03	0.10 ± 0.01
TAA+lac	0.9 ± 0.1	8.0 ± 0.6	0.18 ± 0.02	0.32 ± 0.04	0.58 ± 0.11	0.21 ± 0.03	0.14 ± 0.01 *†

Brain contents are shown in nmol/g tissue. Values are presented as the mean ± S.E.M. (n = 6-8).
* $p < 0.05$ vs control, † $p < 0.05$ vs TAA by Tukey's test.

An animal model for TAA (chemical)-induced hepatic encephalopathy

Table 3. Brain contents of Glu and GABA

	Cor.	Str	Hipp	Mid	Hypoth	Pons	Cerebe
Glu							
Control	11.1 ± 1.3	5.8 ± 0.7	8.4 ± 0.5	7.1 ± 0.4	6.2 ± 0.3	5.6 ± 0.1	5.9 ± 0.4
TAA	10.9 ± 0.6	6.7 ± 0.7	8.5 ± 0.4	7.9 ± 0.2	5.6 ± 0.5	4.9 ± 0.3	5.7 ± 0.8
GABA							
Control	2.2 ± 0.1	2.1 ± 0.2	2.0 ± 0.1	2.8 ± 0.3	4.4 ± 0.2	1.2 ± 0.1	1.2 ± 0.1
TAA	1.8 ± 0.2	2.5 ± 0.1	2.1 ± 0.1	3.1 ± 0.2	4.4 ± 0.2	1.3 ± 0.1	1.4 ± 0.1

Brain contents are shown in μmol/g tissue. Values are presented as the mean ± S.E.M. (n = 6-8).
No statistically significant difference was detected between control and TAA by Student's *t*-test.
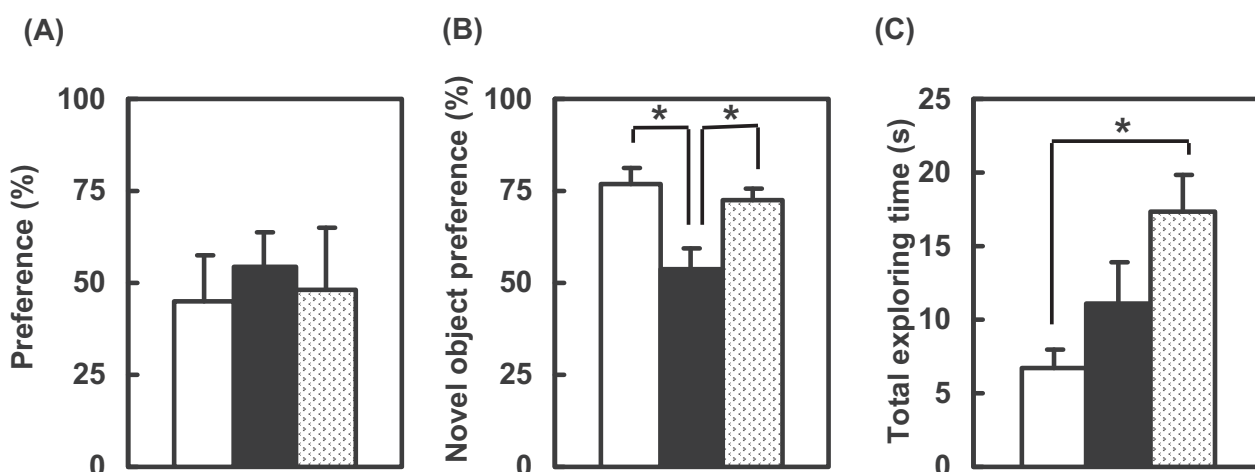
Fig. 4. Effects of lactulose on the memory function of TAA rats. The NOR test was carried out as described in the Materials and Methods. (A) The preference for one object in the training session. The ratio of the time spent exploring one object to the time spent exploring both objects was calculated. (B) NOR in test session. The ratio of the time spent exploring the novel object to the time spent exploring both objects was calculated as the novel object preference (%). (C) The total exploring time of two objects in test session is shown. Open column: control (n = 8); closed column: TAA (n = 8); hatched column: TAA+lac (n = 6). * p < 0.05 by Tukey's test.

ropsychiatric symptoms in the TAA0.5 rats. In the training phase of NOR tests, no difference was observed between groups (Fig. 4A). In the test phase, lactulose restored the reduced novel object preference in the TAA0.5 group (Fig. 4B). Rats treated with TAA and lactulose spent more time examining the novel object than the TAA0.5 rats (Fig. 4C). Fig. 5 shows the effects of lactulose on the FST. Lactulose significantly recovered the reduction in immobility of TAA0.5 rats, but had no significant effects on the increased swimming score in this group (Fig. 5). These results indicated that lactulose ameliorated the cognitive impairment and the irritated mood of TAA0.5 rats.

Blood biochemical changes in rats with TAA-induced cirrhosis

We analyzed biochemical indicators for liver damage in blood in the TAA rats (Table 1). Compared with those of the control animals, the ALP activities and NH$_3$ levels were significantly increased in TAA0.5 rats, while the ALT activities, Alb contents and BTR were significantly reduced in TAA0.5 rats. These results indicate impairment of hepatic function with cholestasis (Kawai *et al.*, 2009). In the group of lactulose-treated TAA rats, the activities of ALP and ALT, Alb contents, and BTR were significantly different from those of both the control and TAA0.5 groups, while the level of blood NH$_3$ tended to
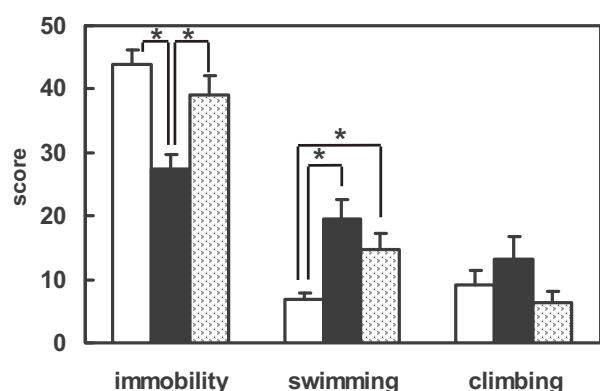
Fig. 5. Effects of lactulose on the mood state of TAA rats. FST was carried out, and immobility, swimming, and climbing scores are shown. Open column: control (n = 8); closed column: TAA (n = 8); hatched column: TAA+lac (n = 6). * p < 0.05 by Tukey's test.

be restored in lactulose-treated rats, but not to a statistically significant degree.

Neurochemical changes in the brains of rats with TAA-induced cirrhosis

Finally, we analyzed neurotransmitters in the rat brain. The contents of 5HT metabolite (5HIAA) and NA metabolite (MHPG) were significantly increased in various regions of the brains of TAA0.5 rats (Table 2). On the other hand, no alterations were detected in DA, Glu, and GABA (Tables 2, 3). Lactulose treatment slightly restored the 5HIAA contents in TAA0.5 rats, but not to a statistically significant degree, while it had little effect on the brain contents of other neurotransmitters, except MHPG and HVA in the cerebellum.

DISCUSSION

In this study, analysis of the neuropsychiatric behaviors of rats with cirrhosis induced by chronic administration of TAA revealed a reduction in the preference for a novel object in the NOR test (Fig. 2) and a decrease of immobility in the FST (Fig. 3). The results of the NOR test indicated that non-spatial memory in the TAA rats was impaired. The TAA rats also showed the elongation of total exploration time in the NOR test (Fig. 2C). We speculate that the elongation is due to the irritated mood of the TAA rats. The TAA rats were so aggressive that they were sniffing or biting the objects for a longer duration than the control rats. Lactulose could not improve

this behavior (Fig. 4C). The mean value of the total exploration time of the TAA+lac rats was longer than that of the TAA rats in Fig. 4C. But the total exploration time of the TAA+lac rats was similar to that of the TAA rats in Fig. 2C. The results of the TAA+lac rats should be within the margin of experimental error of the TAA rats. We considered that the TAA rats and the TAA+lac rats showed similar behavior in the total exploration time. Further studies are necessary to determine whether lactulose has a deteriorative effect on the behavior of the TAA rats.

The FST is a test suitable for evaluation of antidepressant activity, and immobility is reduced by administration of antidepressant (Porsolt *et al.*, 1978; Cryan *et al.*, 2002). The FST is also used to assess mood disorder in rats and mice: an increase of immobility is considered to reflect a depressive mood, and is used as an index for depression or negative symptom of schizophrenia (Noda *et al.*, 1995; 1997). Several studies have reported a reduction of immobility in animal models of mania (Roybal *et al.*, 2007; Mukherjee *et al.*, 2010; Scotti *et al.*, 2011), and the reduction of immobility was prevented by treatment with a mood stabilizer, Li (Roybal *et al.*, 2007). Reduction of immobility is also considered an index for mania or an irritated mood state. From these lines of evidence, we consider that the reduction of immobility in the TAA rats may have been at least partly associated with symptoms of mania or irritated mood states, which would correspond to some of the personality changes observed in HE patients.

Various neuronal systems including Glu, GABA, NA, and 5HT affect the NOR score (Dere *et al.*, 2007). Since the contents of 5HT, NA, and their metabolites were increased in the brain of the TAA rats (Table 2), the memory impairment could be due to the activation of NA and/or 5HT systems. Activation of NA system has been shown to enhance the memory consolidation in NOR (Dornelles *et al.*, 2007; Nirogi *et al.*, 2012). On the other hand, activation of 5HT systems reduced NOR score (Dere *et al.*, 2007). We considered that the activation of the 5HT systems rather than the NA systems induced the memory impairment in the TAA rats. Since antagonists of 5HT1A, 5HT2C, and 5HT6 receptors have been shown to improve NOR scores, 5HT neurotransmission has been negatively associated with learning and memory abilities (Schiapparelli *et al.*, 2006; Pitsikas and Sakellaridis, 2005; King *et al.*, 2004). In the TAA rats in the present study, the elevation of brain contents of 5HT and its metabolite may have increased the function of 5HT neuronal systems (Table 2), which could have led to memory deficit, one of the characteristic symptoms in humans with HE.

Several reports have suggested that it is possible to dif-

ferentiate between neuronal systems involved in swimming and those involved in climbing by using the modified FST (Detke *et al*., 1995; Cryan *et al*., 2002). That is, increases in swimming and climbing correlate with the activation of serotonergic and noradrenergic neuronal systems, respectively. The results shown in Fig. 5 indicate that serotonergic system might be activated in the TAA-treated rats, which is consistent with the results of our biochemical analysis that 5HT and 5HIAA contents were increased in the brain (Table 2). Treatment with lactulose reduced the 5HT and 5HIAA contents slightly but not significantly. Therefore, the decreases of BTR and neurotransmitters had little influence on neuropsychiatric behavior in the cirrhotic rats.

The analysis of biochemical changes revealed an elevation of blood ammonia levels in the TAA rats (Table 1). In both rats and humans with HE and cirrhosis, an inability for the liver to perform its usual detoxification functions may result in an elevation of ammonia. Ammonia appeared to play a major role in the abnormal behavior, since treatment with lactulose decreased the blood ammonia levels, and also improved the neuropsychiatric behaviors in the cirrhotic rats.

In HE patients, dysfunction of the liver results in disturbances of amino acid metabolisms and subsequent elevation of plasma and brain contents of aromatic amino acids such as Trp and Tyr (Bergeron *et al*., 1989). The elevation of these aromatic amino acids leads to increased synthesis of 5HT, DA, and NA in the brain (Cascino *et al*., 1982; Bernardini and Fischer, 1982; Fanelli *et al*., 1986). This process may play a role in the pathogenesis of HE (Lozeva-Thomas, 2004). In a previous study, we observed decreased BTR in TAA-induced cirrhotic rats (Kawai *et al*., 2009; Table 1 in the current study). The decrease of BTR may be involved in the elevation of 5HT, NA, and their metabolites (Table 2); however, treatment with lactulose had no effect on BTR.

Lactulose improved some symptoms such as memory impairment and mood disturbance (Figs. 3 and 4), whereas, lactulose was not effective to the other symptoms as shown in Tables 1 and 2. Lactulose ameliorates HE symptoms by modifying the metabolism of ammonia in intestines (Prasad *et al*., 2007; Mortensen, 1992). Since lactulose could not prevent liver damage, the indices of the liver damage were not improved by lactulose treatment in the TAA rats (Table 1). The contents of 5HT and 5HIAA tended to be decreased by lactulose, but these alterations were not sufficient to explain the behavioral improvement observed in NOR and FST. We speculate that HE symptoms were induced by various mechanisms including disruption of 5HT systems and direct neurotoxicity

of ammonia. The reduction of c-Fos activity, the alteration of cytochrome oxidase activity, and the up-regulation of acetylcholinesterase activity in cirrhotic rats have been reported (Méndez *et al*., 2008b, 2009 and 2011). These mechanisms could relate to the action of lactulose. Further investigations are necessary to clarify the relationships between lactulose and neuronal systems.

The TAA rats in this study meet three important criteria of animal models: face validity, construct validity, and predictive validity. Behavioral abnormalities in TAA rats, such as memory impairment and unstable mood state corresponding to those in HE patients, satisfy face validity (Figs. 2 and 3). Development of liver diseases such as cirrhosis (Kawai *et al*., 2009, and Table 1), and elevation of blood ammonia levels in TAA rats satisfy construct validity (Table 1). Amelioration of several symptoms by lactulose treatment satisfies predictive validity (Figs. 4 and 5). These lines of evidence support our proposal of this experimental rat as a model for HE with cirrhosis.

The pathogenesis of HE is still unclear. Further investigation will be required to clarify the exact mechanisms in the pathogenesis of HE. However, at present, this study indicates that lactulose-restorable memory disturbance and irritated mood are two characteristics of the TAA-treated cirrhotic rats. In other words, rats treated chronically with TAA are a potential model for cirrhosis-HE. The combined use of NOR and FST with the TAA rat model may be a simple but effective system for the screening and development of anti-HE agents.

REFERENCES

Bassett, M.L., Mullen, K.D., Skolnick, P. and Jones, E.A. (1987): Amelioration of hepatic encephalopathy by pharmacologic antagonism of the GABAA-benzodiazepine receptor complex in a rabbit model of fulminant hepatic failure. Gastroenterology, **93**, 1069-1077.

Bergeron, M., Reader, T.A., Layrargues, G.P. and Butterworth, R.F. (1989): Monoamines and metabolites in autopsied brain tissue from cirrhotic patients with hepatic encephalopathy. Neurochem. Res., **14**, 853-859.

Bernardini, P. and Fischer, J.E. (1982): Amino acid imbalance and hepatic encephalopathy. Annu. Rev. Nutr., **2**, 419-454.

Butterworth, R.F., Norenberg, M.D., Felipo, V., Ferenci, P., Albrecht, J., Blei, A.T. and Members of the ISHEN Commission on Experimental Models of HE (2009): Experimental models of hepatic encephalopathy: ISHEN guidelines. Liver Int., **29**, 783-788.

Cascino, A., Cangiano, C., Fiaccadori, F., Ghinelli, F., Merli, M., Pelosi, G., Riggio, O., Rossi Fanelli, F., Sacchini, D., Stortoni, M. and Capocaccia, L. (1982): Plasma and cerebrospinal fluid amino acid patterns in hepatic encephalopathy. Dig. Dis. Sci., **27**, 828-832.

Cryan, J.F., Markou, A. and Lucki, I. (2002): Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol. Sci., **23**, 238-245.

Dashti, H., Jeppsson, B., Hagerstrand, I., Hultberg, B., Srinivas, U., Abdulla, M. and Bengmark, S. (1989): Thioacetamide and carbon tetrachloride-induced liver cirrhosis. Eur. Surg. Res., **21**, 83-91.

Dere, E., Huston, J.P. and De Souza Silva, M.A. (2007): The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. Neurosci. Biobehav. Rev., **31**, 673-704.

Detke, M.J., Rickels, M. and Lucki, I. (1995): Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. Psychopharmacology (Berl), **121**, 66-72.

Dornelles, A., de Lima, M.N.M., Grazziotin, M., Presti-Torres, J., Garcia, V.A., Scalco, F.S., Roesler, R. and Schröder, N. (2007): Adrenergic enhancement of consolidation of object recognition memory. Neurobiol. Learn. Mem., **88**, 137-142.

Ennaceur, A. and Delacour, J. (1988): A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav. Brain Res., **31**, 47-59.

Fanelli, R.F., Cangiano, C., Capocaccia, L., Cascino, A., Ceci, F., Muscaritoli, M. and Giunchi, G. (1986): Use of branched chain amino acids for treating hepatic encephalopathy: clinical experiences. Gut, **27 Suppl. 1**, 111-115.

Ferenci, P., Lockwood, A., Mullen, K., Tarter, R., Weissenborn, K. and Blei, A.T. (2002): Hepatic encephalopathy--definition, nomenclature, diagnosis, and quantification: final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998. Hepatology, **35**, 716-721.

Fontana, L., Moreira, E., Isabel Torres, M., Isabel Fernandez, M., Rios, A., De Medina, F.S. and Gil, A. (1996): Serum amino acid changes in rats with thioacetamide-induced liver cirrhosis. Toxicology, **106**, 197-206.

Jover, R., Rodrigo, R., Felipo, V., Insausti, R., Saez-Valero, J., Garcia-Ayllon, M.S., Suarez, I., Candela, A., Compan, A., Esteban, A., Cauli, O., Auso, E., Rodriguez, E., Gutierrez, A., Girona, E., Erceg, S., Berbel, P. and Perez-Mateo, M. (2006): Brain edema and inflammatory activation in bile duct ligated rats with diet-induced hyperammonemia: A model of hepatic encephalopathy in cirrhosis. Hepatology, **43**, 1257-1266.

Kawai, H., Kudo, N., Kawashima, Y. and Mitsumoto, A. (2009): Efficacy of urine bile acid as a non-invasive indicator of liver damage in rats. J. Toxicol. Sci., **34**, 27-38.

King, M.V., Sleight, A.J., Woolley, M.L., Topham, I.A., Marsden, C.A. and Fone, K.C.F. (2004): 5-HT6 receptor antagonists reverse delay-dependent deficits in novel object discrimination by enhancing consolidation--an effect sensitive to NMDA receptor antagonism. Neuropharmacology, **47**, 195-204.

Lozeva-Thomas, V. (2004): Serotonin Brain Circuits with a Focus on Hepatic Encephalopathy. Metab. Brain Dis., **19**, 413-420.

Matkowskyj, K.A., Marrero, J.A., Carroll, R.E., Danilkovich, A.V., Green, R.M. and Benya, R.V. (1999): Azoxymethane-induced fulminant hepatic failure in C57BL/6J mice: characterization of a new animal model. Am. J. Physiol., **277**, G455-62.

Méndez, M., Méndez-López, M., López, L., Aller, M.Á., Árias, J., Cimadevilla, J.M. and Árias, J.L. (2008a): Spatial memory alter-

ations in three models of hepatic encephalopathy. Behav. Brain Res., **188**, 32-40.

Méndez, M., Méndez-López, M., López, L., Aller, M.Á., Árias, J. and Árias, J.L. (2008b): Working memory impairment and reduced hippocampal and prefrontal cortex c-Fos expression in a rat model of cirrhosis. Physiol. Behav., **95**, 302-307.

Méndez, M., Méndez-López, M., López, L., Aller, M.Á., Árias, J. and Árias, J.L. (2009): Basal and learning task-related brain oxidative metabolism in cirrhotic rats. Brain Res. Bull., **78**, 195-201.

Méndez, M., Méndez-López, M., López, L., Aller, M.Á., Árias, J. and Árias, J.L. (2011): Acetylcholinesterase activity in an experimental rat model of Type C hepatic encephalopathy. Acta Histochem., **113**, 358-362.

Mortensen, P.B. (1992): The effect of oral-administered lactulose on colonic nitrogen metabolism and excretion. Hepatology, **16**, 1350-1356.

Mukherjee, S., Coque, L., Cao, J.L., Kumar, J., Chakravarty, S., Asaithamby, A., Graham, A., Gordon, E., Enwright, J.F., 3rd, Dileone, R.J., Birnbaum, S.G., Cooper, D.C. and McClung, C.A. (2010): Knockdown of Clock in the Ventral Tegmental Area Through RNA Interference Results in a Mixed State of Mania and Depression-Like Behavior. Biol. Psych., **68**, 503-511.

Müller, A., Machnik, F., Zimmermann, T. and Schubert, H. (1988): Thioacetamide-induced cirrhosis-like liver lesions in rats--usefulness and reliability of this animal model. Exp. Pathol., **34**, 229-236.

Nirogi, R., Abraham, R., Jayarajan, P., Medapati, R.B., Shanmuganathan, D., Kandikere, V., Irappanavar, S., Saralaya, R., Benade, V., Bhyrapuneni, G. and Muddana, N. (2012): Difference in the norepinephrine levels of experimental and non-experimental rats with age in the object recognition task. Brain Res., **1453**, 40-45.

Noda, Y., Yamada, K., Furukawa, H. and Nabeshima, T. (1995): Enhancement of immobility in a forced swimming test by subacute or repeated treatment with phencyclidine: a new model of schizophrenia. Br. J. Pharmacol., **116**, 2531-2537.

Noda, Y., Mamiya, T., Furukawa, H. and Nabeshima, T. (1997): Effects of antidepressants on phencyclidine-induced enhancement of immobility in a forced swimming test in mice. Eur. J. Pharmacol., **324**, 135-140.

Pitsikas, N. and Sakellaridis, N. (2005): The 5-HT2C receptor antagonist RO 60-0491 counteracts rats' retention deficits in a recognition memory task. Brain Res., **1054**, 200-202.

Porsolt, R.D., Anton, G., Blavet, N. and Jalfre, M. (1978): Behavioural despair in rats: a new model sensitive to antidepressant treatments. Eur. J. Pharmacol., **47**, 379-391.

Prasad, S., Dhiman, R.K., Duseja, A., Chawla, Y.K., Sharma, A. and Agarwal, R. (2007): Lactulose improves cognitive functions and health-related quality of life in patients with cirrhosis who have minimal hepatic encephalopathy. Hepatology, **45**, 549-559.

Rahman, T.M., Selden, A.C. and Hodgson H.J.F. (2002): A novel model of acetaminophen-induced acute hepatic failure in rabbits. J. Surg. Res., **106**, 264-272.

Roybal, K., Theobold, D., Graham, A., DiNieri, J.A., Russo, S.J., Krishnan, V., Chakravarty, S., Peevey, J., Oehrlein, N., Birnbaum, S., Vitaterna, M.H., Orsulak, P., Takahashi, J.S., Nestler, E.J., Carlezon, W.A., Jr. and McClung, C.A. (2007). Mania-like behavior induced by disruption of CLOCK. Proc. Natl. Acad. Sci. USA, **104**, 6406-6411.

Sato, M., Kakubari, M., Kawamura, M., Sugimoto, J., Matsumoto, K. and Ishii, T. (2000): The decrease in total collagen fibers in

An animal model for TAA (chemical)-induced hepatic encephalopathy

the liver by hepatocyte growth factor after formation of cirrhosis induced by thioacetamide. Biochem. Pharmacol., **59**, 681-690.

Schiapparelli, L., Simon, A.M., Del Rio, J. and Frechilla, D. (2006): Opposing effects of AMPA and 5-HT1A receptor blockade on passive avoidance and object recognition performance: correlation with AMPA receptor subunit expression in rat hippocampus. Neuropharmacology, **50**, 897-907.

Scotti, M.A., Lee, G., Stevenson, S.A., Ostromecki, A.M., Wied, T.J., Kula, D.J., Gessay, G.M. and Gammie, S.C. (2011): Behavioral and pharmacological assessment of a potential new mouse model for mania. Physiol. Behav., **103**, 376-383.

Steindl, P.E., Coy, D.L., Finn, B., Zee, P.C. and Blei, A.T. (1996): A low-protein diet ameliorates disrupted diurnal locomotor activity in rats after portacaval anastomosis. Am. J. Physiol., **271**, G555-560.

Watanabe, M., Ozaki, T., Mushiroi, T., Ukai, Y., Ueda, F., Kimura, K., Katoh, M., Matsumoto, A., Kotani, E., Itoh, S., Yamaguchi, K. and Kyuki, K. (1997): Behavioral and electroencephalographic studies of beagles with an Eck's fistula: suitability as a model of hepatic encephalopathy. Pharmacol. Biochem. Behav., **57**, 367-375.

Yamamoto, H. and Sugihara, N. (1987): Blood ammonia levels and hepatic encephalopathy induced by CCl4 in rats. Toxicol. Appl. Pharmacol., **91**, 461-468.

Yurdaydin, C., Herneth, A.M., Puspok, A., Steindl, P., Singer, E. and Ferenci, P. (1996). Modulation of hepatic encephalopathy in rats with thioacetamide-induced acute liver failure by serotonin antagonists. Eur. J. Gastroenterol. Hepatol., **8**, 667-671.

Zimmermann, C., Ferenci, P., Pifl, C., Yurdaydin, C., Ebner, J., Lassmann, H., Roth, E. and Hortnagl, H. (1989). Hepatic encephalopathy in thioacetamide-induced acute liver failure in rats: characterization of an improved model and study of amino acid-ergic neurotransmission. Hepatology, **9**, 594-601.