Article

Arq Neuropsiquiatr 2010;68(4):597-602

# A thioacetamide-induced hepatic encephalopathy model in C57BL/6 mice

## A behavioral and neurochemical study

Aline Silva de Miranda[1], David Henrique Rodrigues[1], Luciene Bruno Vieira[2], Cristiano Xavier Lima[1], Milene Alvarenga Rachid[1], Paula Vieira Teixeira Vidigal[3], Marcus Vinicius Gomez[4], Helton José dos Reis[2], Cristina Guatimosim[3], Antônio Lúcio Teixeira[1]

**ABSTRACT**

**Objective:** Hepatic encephalopathy (HE) is a neuropsychiatric syndrome resulting from liver failure. In the present study, we aimed to standardize an animal model of HE induced by thioacetamide (TAA) in C57BL/6 mice evaluating behavioral symptoms in association with liver damage and alterations in neurotransmitter release. **Method:** HE was induced by an intraperitoneal single dose of TAA (200 mg/kg, 600 mg/kg or 1,200 mg/kg). Behavioral symptoms were evaluated using the SHIRPA battery. Liver damage was confirmed by histopathological analysis. The glutamate release was measured using fluorimetric assay. **Results:** The neuropsychiatric state, motor behavior and reflex and sensory functions were significantly altered in the group receiving 600 mg/kg of TAA. Biochemical analysis revealed an increase in the glutamate release in the cerebral cortex of HE mice. **Conclusion:** HE induced by 600mg/kg TAA injection in C57BL/6 mice seems to be a suitable model to investigate the pathogenesis and clinical disorders of HE.

**Key words:** hepatic encephalopathy, thioacetamide, behavioral changes, SHIRPA, glutamate.

**Modelo de encefalopatia hepática induzida por tioacetamida em camundongos C57BL/6: estudo comportamental e neuroquímico**

**RESUMO**

**Objetivo:** A encefalopatia hepática (EH) é uma síndrome neuropsiquiátrica resultante da falência hepática. O objetivo do presente estudo foi estabelecer um modelo de EH induzida por tioacetamida (TAA) em camundongos C57BL/6 avaliando transtornos comportamentais, falência hepática e alterações na liberação de neurotransmissores. **Método:** A EH foi induzida por meio de uma única dose intraperitoneal de TAA (200 mg/kg, 600 mg/kg, 1.200 mg/kg). As alterações comportamentais foram avaliadas utilizando a bateria SHIRPA. A falência hepática foi confirmada através de análises histopatológicas e a liberação de glutamato medida, por ensaio fluorimétrico. **Resultados:** Foram encontradas alterações significativas no estado neuropsiquiátrico, comportamento motor e função reflexa e sensorial no grupo que recebeu 600 mg/kg de TAA. Análises bioquímicas revelaram aumento na liberação de glutamato no córtex cerebral dos camundongos com EH. **Conclusão:** A EH induzida por 600 mg/kg de TAA em camundongos C57BL/6 parece ser um modelo apropriado para a investigação da patogênese e dos transtornos clínicos da EH.

**Palavras-chave:** encefalopatia hepática, tioacetamida, alterações comportamentais, SHIRPA, glutamato.

**Correspondence**
Antonio Lucio Teixeira
Departamento de Clínica Médica,
Faculdade de Medicina, UFMG
Avenida Alfredo Balena 190
30130-100 Belo Horizonte MG - Brasil
E-mail: altexr@gmail.com

**Support**
This work was supported by Rede Instituto Brasileiro de Neurociência (IBN Net/Finep), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Brazil

Received 27 January 2010
Received in final form 23 March 2010
Accepted 07 April 2010

Federal University of Minas Gerais, Belo Horizonte MG, Brazil: [1]Laboratory of Immunopharmacology, Department of Biochemistry and Immunology, Institute of Biological Sciences; [2]Laboratory of Neuropharmacology, Department of Pharmacology, Institute of Biological Sciences; [3]Department of Morphology, Institute of Biological Sciences; [4]Laboratory of Molecular Medicine (INCT), School of Medicine.

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome resulting from acute or chronic liver failure[1]. HE affects a considerable number of patients worldwide with a mortality index ranging from 50 to 90%[2]. This condition can cause a wide range of clinical manifestations which include psychomotor dysfunction, sensory abnormalities, poor concentration, impaired memory and increased reaction time. In its most severe form, patients may develop stupor, coma and death[1].

The pathogenesis of HE is still not well understood. The main hypothesis suggests a state of hyperammonemia which is responsible for both direct and indirect alterations in cerebral metabolism[3]. Hyperammonemia leads to increased levels of glutamine in astrocytes from amidation of glutamate by glutamine synthetase[4]. The accumulation of glutamine in the astrocytes produces osmotic stress and causes the astrocytes to swell. This could determine cerebral edema and intracranial hypertension which are ascribed to be the main pathophysiological features of HE[5]. Other mechanisms have been proposed to explain the pathogenesis of HE, including dysfunction of the immune[5] and neurotransmitter[6] systems. Therefore, it is important to clarify the mechanisms by which factors associated with liver failure affect cerebral function and animal models are of relevance in the investigation of the underlying mechanisms of HE[7,8].

Many drugs have been used to induce liver failure in experimental models, such as thioacetamide (TAA), D-galactosamine, acetominophen, carbon tetrachloride and concanavalin A[9]. TAA is a hepatotoxin frequently used in experimental studies due to its efficacy in inducing hepatic failure in rodents[10,11]. Following hepatic failure, TAA is able to induce HE[8,12]. Most studies using TAA were performed in rats[8,13]. The best dose of TAA to induce HE in mice is still not well established.

Experimental HE has been defined using only severe neurological signs, such as coma followed by death[13]. Some studies have evaluated a few cognitive domains using standardized tests[8,14,15]. However, these parameters may be too restrictive as the HE encompasses a wide range of clinical manifestations. In this context, it is important to use a validated battery of tests that can evaluate several behavioral functions.

In the present study, we aimed to standardize an animal model of HE induced by TAA in C57BL/6 mice evaluating a large spectrum of behavioral symptoms as assessed by the SHIRPA battery, glutamate release from brain cortical isolated nerve terminals and liver damage.

## METHOD

### Animals

All experiments were approved by the Animal Ethics Committee of the Federal University of Minas Gerais (UFMG). Sixty Male C57BL/6 mice (20-25g), aged 8-12 weeks, were obtained from Animal Care Facilities of the Institute of Biological Sciences, UFMG. Thirty six mice were used to induce HE and twenty four were used as controls.

### Induction of hepatic encephalopathy

HE was induced by an intraperitoneal single dose of TAA (Sigma Chemical Co., St. Louis, MO, USA). We tested the following doses: 200 mg/kg, 600 mg/kg and 1,200 mg/kg of TAA dissolved in saline (NaCl 0.9%; 0.3 mL). Controls received the same volume of saline. Survival curve and weight of animals using these three doses of TAA were evaluated daily until 8$^{th}$ and 6$^{th}$ day post-induction (p.i), respectively.

### Histological analysis

Livers were removed immediately from mice after cervical dislocation at 48h p.i. and fixed in 10% buffered formalin. The sections (5.0 μm) were stained with hematoxylin and eosin (H&E) and analyzed in an Olympus BX51 microscope. Six liver sections from each animal were used for this analysis. Liver damage was also evaluated by transaminase activity, glutamic oxalacetic (GOT) and glutamic pyruvic (GPT) in sera from mice using standard kits (Bioclin, Belo Horizonte, Brazil).

### SHIRPA screen

Behavioral and functional parameters were evaluated using a screening battery called SHIRPA until 3$^{rd}$ day p.i. The SHIRPA screen was conceived as a multi-test behavioral battery used for longitudinal studies with standardized guidelines and materials[16]. This battery encompasses a wide range of tests, organized in five functional categories: neuropsychiatric state, motor behavior, reflex and sensory function, autonomous function and muscle tone and strength[17]. Animals were allowed to accommodate to their new environment for 2 days before behavioral assessment.

### Glutamate release and measurement

Synaptosomes were prepared as previously described[18]. Mice were decapitated and had their cortices removed and homogenized in 1:10 (w/v) 0.32M sucrose solution containing dithiothreitol (0.25 mM) and EDTA (1 mM). Homogenates were then submitted to low-speed centrifugation (1000g / 10 min) and isolated nerve terminals (synaptosomes) were purified from the supernatant by discontinuous Percoll-density gradient centrifugation[19], The synaptosomes were resuspended in Krebs-Ringer-HEPES solution (KRH, 124 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 25 mM HEPES, pH 7.4) with no added $CaCl_2$, to a concentration of approximately 10 mg/mL, divided into aliquots of 200 μL and kept on ice for posterior measurement of glutamate release. The

glutamate release assay was performed using a RF5301-PC spectrofluorimeter (Shimadzu, Kyoto, Japan) following the increase in the fluorescence due to the production of $NADPH^+$ in the presence of glutamate dehydrogenase and $NADP^+$[20,21].

### Statistical analysis

One-way analysis of variance (ANOVA) with Dunnett's multiple comparison post-test was used to analyze behavioral and functional categories of SHIRPA and the serum levels of GOT and GPT. To analyze the survival rate, the Logrank test was used. The glutamate release was analyzed using the unpaired t-student test. All analyses were performed using Prism 4 software (GraphPad, La Jolla, CA, USA).

## RESULTS

First, we evaluated survival rate using three different doses of TAA. We found that 200 mg/kg and 600 mg/kg doses lead to a higher survival rate ($p \leq 0.01$) in comparison with 1,200 mg/kg (Fig 1A). All animals exhibited a decrease in body weight after TAA injection and start recovering at day 3 p.i. (Fig 1B). Due to the high mortality rate associated with the dose of 1,200 mg/kg, behavioral and functional parameters were assessed with the other two doses.

The neuropsychiatric state ($p=0.02$), motor behavior ($p=0.03$), reflex and sensory function ($p=0.03$) from the SHIRPA battery were significantly altered in the group receiving 600mg/kg of TAA in comparison with animals receiving saline (Fig 2). No difference was found between the group receiving 200 mg/kg of TAA and controls. Thus, the dose of 600 mg/kg seemed to be the most appropriate to induce HE in mice.

To confirm liver damage induced by 600 mg/kg of TAA, we performed histological analyses at 48h p.i. An extensive hemorrhagic necrotic area was found in TAA injected mice compared to controls (Figs 3A, 3B). Serum levels of GOT and GPT were significantly increased after 24h and 48h ($p \leq 0.01$) (Fig 3C, 3D), confirming the extensive liver damage.

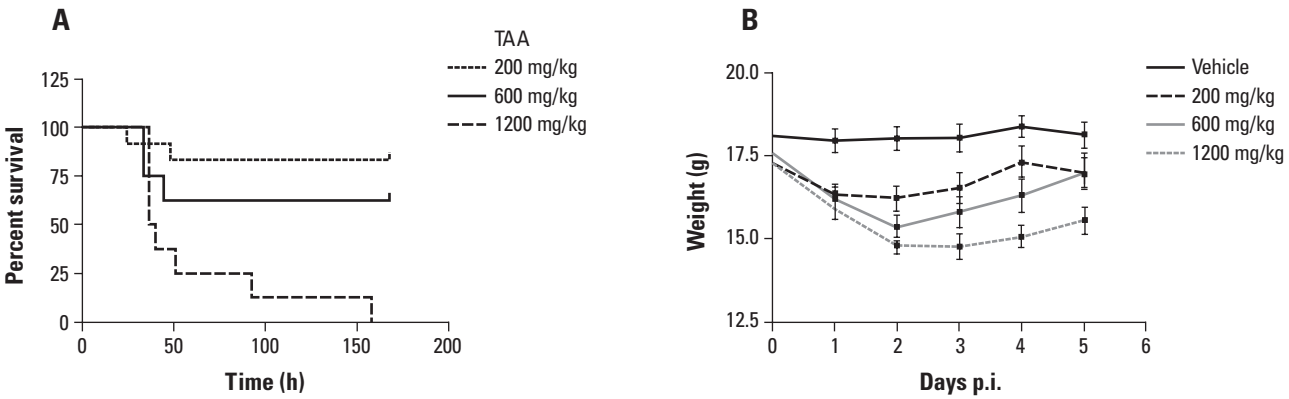


**Fig 1.** Survival curve [A] and weight [B] were evaluated daily until 8th and 6th day post-induction (p.i), respectively. Three different concentrations of thioacetamide were used to induce hepatic encephalopathy in the C57BL/6 wild type mice: 200 mg/kg (n=12), 600 mg/kg (n=12) and 1200 mg/kg (n=12).

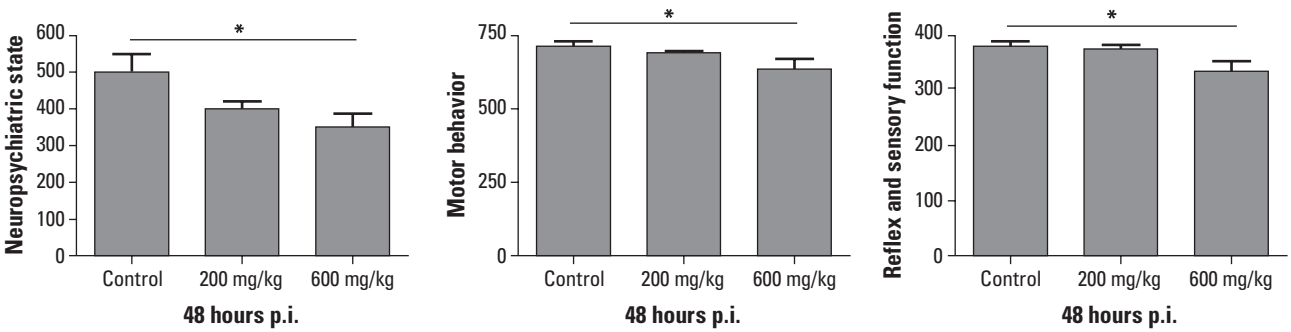


**Fig 2.** Performance of C57BL/6 mice hepatic encephalopathy induced with 200 mg/kg (n=10) and 600 mg/kg (n=6) of thioacetamide at 48 hours post-induction and of control group (n=6) in the functional categories of the SHIRPA battery. Data are presented as mean±SEM. *p≤0.05; **p≤0.01.

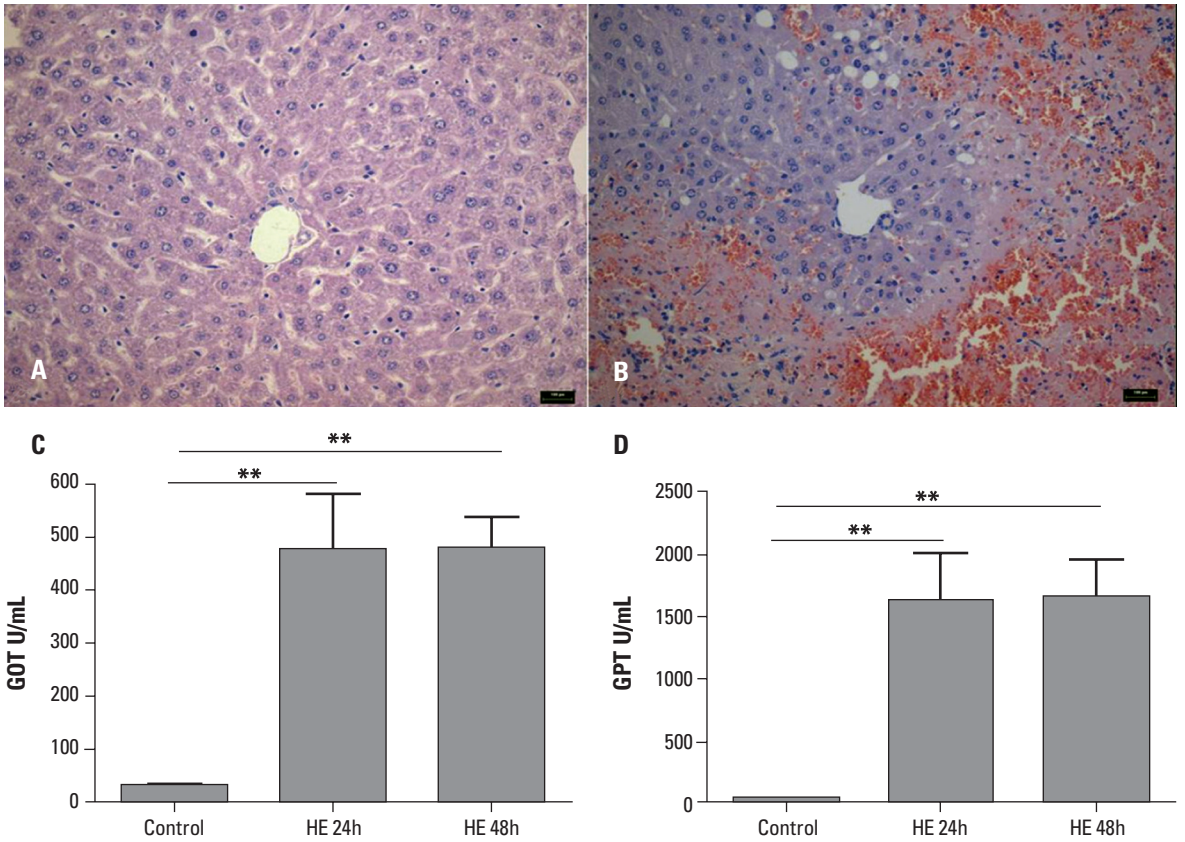


**Fig 3.** Liver damage following injection of 600 mg/kg of thioacetamide. Histological section (5 µm) of liver from control mice (n=3) with normal portal triad and hepatocytes [A]. The liver from hepatic encephalopathy mice (n=5) induced with 600 mg/kg of thioacetamide exhibits intense periacinar hemorrhagic necrosis with preservation of periportal zone [B]. The serum levels of GOT [C] and GPT [D] were assessed in hepatic encephalopathy group and in control group (n=11) at 24h (n=6) and 48h (n=10) post-induction. Data are presented as mean±SEM. **p≤0.01.

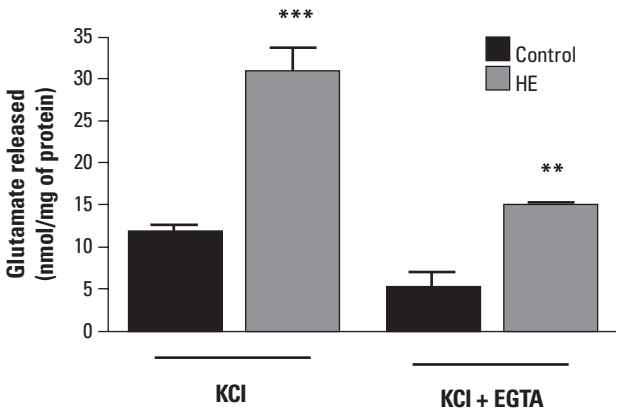


**Fig 4.** HE induces glutamate release from brain synaptosomes. First bar: 33 mM KCl-evoked glutamate release from C57BL/6 mice; Second bar: 33 mM KCl-evoked glutamate release from C57BL/6 mice with hepatic encephalopathy induced with 600 mg/kg of thioacetamide at 72 hours post-induction; Third bar: Calcium-independent glutamate release evoked by 33 mM KCl from C57BL/6 mice synaptosomes. Forth bar: Calcium- independent glutamate release evoked by 33 mM KCl from C57BL/6 mice with hepatic encephalopathy induced with 600 mg/kg of thioacetamide at 72 hours post-induction. The results are mean±SEM of at least three independent experiments for each experimental condition. **p≤0.01; ***p≤0.001.

All neurological functions altered in HE seem to be the final result of changes in neurotransmission. Considering that abnormal glutamatergic neurotransmission was previously reported in HE, we measured glutamate release from brain cortical isolated nerve terminals (synaptosomes) in our HE model. We observed that glutamate release was significantly increased in the group receiving 600 mg/kg of TAA in comparison with animals receiving saline (Fig 4). Synaptosomes from control animals were exposed to 33 mM KCl to depolarize their membranes and induce glutamate release (Fig 4). KCl-evoked glutamate release from synaptosomes obtained from HE animals was approximately three fold higher than control animals (Fig 4; **p≤0.01).

When synaptosomes were depolarized with KCl, the release of glutamate is the sum of two components: one that is extracellular calcium dependent and inhibited by the calcium chelator EGTA, and a second which is extracellular calcium independent and not sensitive to EGTA. We therefore measured KCl-evoked glutamate release in control and HE mice synaptosomes in the presence of EGTA which reflects the calcium-independent pool. In

both conditions (control and HE mice), KCl-evoked glutamate release was reduced in the presence of EGTA (Fig 4; ***p≤0.001).

## DISCUSSION

In this study, the administration of 600 mg/kg of TAA was capable of inducing severe liver damage evidenced by histopathological analysis and increased serum levels of hepatic enzymes. These hepatic alterations were accompanied by neuropsychiatry, motor, reflex and sensory changes. Moreover, increased glutamate release was observed in the cerebral cortex of HE animals.

HE has been largely studied in rat models[12,14,22]. In mice, most studies using TAA have been performed to evaluate acute or chronic liver failure and not changes in the central nervous system[10,23,24]. Indeed, histopathological changes observed in the liver were similar to those described in previous studies. To the best of our knowledge, only few studies have used mice as an experimental model of HE. Avraham et al. investigated neurological functions in a HE model using a dose of 200 mg/kg of TAA in the Sabra mouse strain[8]. We could not find significant neuropsychiatric and motor changes using this dose of TAA, indicating that different mice strains may present distinct susceptibility to the drug.

In the present study, using C57BL/6 mice we found significant changes in the reflex and sensory function, motor and neuropsychiatric state using SHIRPA battery. Other studies have also evaluated some behavioral changes of HE using different neurological and cognitive protocols. Similar to our study, they found significant alterations in motor behavior, reflex and cognitive function[8,15,25]. However no previous study used a broad behavioral battery such as SHIRPA to evaluate the clinical manifestations of HE. The present results and previous data with SHIRPA in other neurological conditions, such as cerebral malaria[17], suggest this battery seem to be an efficient tool to identify subtle behavioral changes in HE.

Neurotransmitters, such as glutamate, have been implicated in the pathogenesis of HE[26-28]. In our study, we found a significant increase of glutamate release in the cortex of HE mice, corroborating the view that glutamate release plays a role in the pathogenesis of HE. Indeed, previous studies in rodents showed that glutamate could mediate neurological symptoms of HE. For instance, Moroni et al. have demonstrated an increase in the synthesis and release of glutamate in rats using two different models of HE (ammonium acetate and portacaval anastomosis)[26]. Also, McArdle et al., using a TAA-induced HE model in rats, found increased extracellular levels of glutamate in hippocampus[27]. We also observed that KCl-evoked glutamate release from synaptosomes obtained from HE mice was partially inhibited by EGTA (approximately 50% inhibition compared to control, Fig 4). Previous work performed by Minelli et al. showed that alkalinization increases $Ca^{2+}$ release through intracellular stores and induces plasmalemmal calcium influx in microglia culture[29]. We could therefore suggest that the increase in KCl-evoked glutamate release observed in our experiments with HE mice synaptosomes might be due to a direct effect of ammonia or other mediator.

In conclusion, we standardized a HE model using 600 mg/kg of TAA in C57BL/6 mice. Due to the low mortality and clear neuropsychiatric and motor dysfunctions, this could be a useful model to study the pathogenesis and clinical disorders of HE. It is worth mentioning that the use of the C57BL/6 strain has several technical advantages. One is the high availability of knock out mice, which could contribute to the study of the mechanisms underlying this condition. Another advantage is that C57BL/6 is an inbred strain. Thus, HE model using C57BL/6 mice may account for less variability in experimental studies.

## REFERENCES

1. Ferenci P, Lockwood A, Mullen K, Tarter R, Weissenborn K, Blei AT. Hepatic encephalopathy: definition, nomenclature, diagnosis and quantification. Final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998. Hepatology 2002;35:716-721.
2. Raghavan M, Marik PE. Therapy of intracranial hypertension in patients with fulminant hepatic failure. Neurocrit Care 2006;4:179-189.
3. Rose C. Increased extracellular brain glutamate in acute liverfailure: decreased uptake or increased release? Metab Brain Dis 2002;17:251-261.
4. Butterworth RF. Pathophysiology of hepatic encephalopathy: a new look at ammonia. Metab Brain Dis 2002;17:221-227.
5. Shawcross D, Jalan R. The pathophysiologic basis of hepatic encephalopathy: central role for ammonia and inflammation. Cell Mol Life Sci 2005;62:2295-2304.
6. Palomero-Gallagher N, Bidmon HJ, Cremer M, et al. Neurotransmitter receptor imbalances in motor cortex and basal ganglia in hepatic encephalopathy. Cell Physiol Biochem 2009;24:291-306.
7. Bélanger M, Côté J, Butterworth RF. Neurobiological characterization of an azoxymethane mouse model of acute liver failure. Neurochem Int 2006;48:434-440.
8. Avraham Y, Israeli E, Gabbay E, et al. Endocannabinoids affect neurological and cognitive function in thioacetamide-induced hepatic encephalopathy in mice. Neurobiol Dis 2006;21:237-245.
9. Rahman TM, Hodgson HJ. Animal models of acute hepatic failure. Int J Exp Pathol 2000;81:145-157.
10. Wang CH, Jawan B, Lee TH, et al. Single injection of naked plasmid encoding alpha-melanocyte-stimulating hormone protects against thioacetamide-induced acute liver failure in mice. Biochem Biophys Res Commun 2004;322:153-161.
11. Reddy PV, Murthy ChR, Reddanna P. Fulminant hepatic failure induced oxidative stress in nonsynaptic mitochondria of cerebral cortex in rats. Neurosci Lett 2004;368:15-20.
12. Chu CJ, Chen CT, Wang SS, et al. Hepatic encephalopathy in rats with thioacetamide induced fulminant hepatic failure: role of endotoxin and tumor necrosis factor-α. Chin Med J 2001;64:321-330.
13. Dagon Y, Avraham Y, Ilan Y, Mechoulam R, Berry EM. Cannabinoids ameliorate cerebral dysfunction following liver failure via AMP-activated protein kinase. FASEB J 2007;21:2431-2441.
14. Bémeur C, Qu H, Desjardins P, Butterworth RF. IL-1 or TNF receptor gene deletion delays onset of encephalopathy and attenuates brain edema in experimental acute liver failure. Neurochem Int 2010;56:213-215.
15. Méndez M, Méndez-López M, López L, Aller MA, Arias J, Arias JL. Associative learning deficit in two experimental models of hepatic encephalopathy. Behav Brain Res 2009;198:346-351.

16. Sarhan S, Knödgen B, Grauffel C, Seiler N. Effects of inhibition of ornithine aminotransferase on thioacetamide-induced hepatogenic encephalopathy. Neurochem Res 1993;18:539-549.
17. Rogers DC, Fisher EM, Brown SD, Peters J, Hunter AJ, Martin JE. Behavioral and functional analysis of mouse phenotype: SHIRPA, a proposed protocol for comprehensive phenotype assessment. Mamm Genome 1997;8:711-713.
18. Lackner P, Beer R, Heussler V, et al. Behavioural and histopathological alterations in mice with cerebral malaria. Neuropathol Appl Neurobiol 2006;32:177-188.
19. Romano-Silva MA, Ribeiro-Santos R, Ribeiro AM, et al. Rat cortical synaptosomes have more than one mechanism for calcium entry linked to rapid glutamate release: studies using Phoneutria nigriventer toxin PhTX2 and potassium depolarization. Biochem J 1993;296:313-319.
20. Dunkley PR, Heath JW, Harrison SM, Jarvie PE, Glenfield PJ, Rostas JA. A rapid Percoll gradient procedure for isolation of synaptosomes directly from S1 fraction: homogeneity and morphology of subcellular fractions. Brain Res 1988;441:59-71.
21. Nicholls DG, Sihra TS, Sanchez-Prieto J. Calcium dependent and independent release of glutamate from synaptosomes monitored by continuous fluorometry. J Neurochem 1987;49:50-57.
22. Prado MAM, Guatimosim C, Gomez MV, Diniz CR, Cordeiro MN, Romano-Silva MA. A novel tool for the investigation of glutamate release from rat cerebrocortical synaptosomes: the toxin Tx3-3 from the venom of the spider Phoneutria nigriventer. Biochem J 1996;314:145-150.
23. Zarros A, Theocharis S, Skandali N, Tsakiris S. Effects of fulminant hepatic encephalopathy on the adult rat brain antioxidant status and the activities of acetylcholinesterase, $(Na^+,K^+)$- and $Mg^{2+}$-ATPase: comparison of the enzymes' response to in vitro treatment with ammonia. Metab Brain Dis 2008; 23:255-264.
24. Fernández-Martínez A, Callejas NA, Casado M, Boscá L, Martín-Sanz P. Thioacetamide-induced liver regeneration involves the expression of cyclooxygenase 2 and nitric oxide synthase 2 in hepatocytes. J Hepatol 2004;40: 963-970.
25. Okuyama H, Nakamura H, Shimahara Y, Araya S, Kawada N, Yamaoka Y, Yodoi J. Overexpression of thioredoxin prevents acute hepatitis caused by thioacetamide or lipopolysaccharide in mice. Hepatology 2003;37:1015-1025.
26. Moroni F, Lombardi G, Moneti G, Cortesini C. The release and neosynthesis of glutamic acid are increased in experimental models of hepatic encephalopathy. J Neurochem 1983;40:850-854.
27. McArdle P, Penning DH, Dexter F, Reynolds JD. Flumazenil does not affect the increase in rat hippocampal extracellular glutamate concentration produced during thioacetamide-induced hepatic encephalopathy. Metab Brain Dis 1996;11:329-342.
28. Albrecht J, Hilgier W, Zielinska M, Januszewski S, Hesselink M, Quack G. Extracellular concentrations of taurine, glutamate, and aspartate in the cerebral cortex of rats at the asymptomatic stage of thioacetamide-induced hepatic failure- modulation by ketamine anesthesia. Neurochem Res 2000;25:1497-1502.
29. Minelli A, Lyons S, Nolte C, Verkhratsky A, Kettenmann H. Ammonium triggers calcium elevation in cultured mouse microglial cells by initiating $Ca^{2+}$ release from thapsigargin-sensitive intracellular stores. Pflugers Arch 2000; 439:370-377.