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Original Article

Manifestation of psychiatric behaviors in a mouse model of griseofulvin-induced hepatic porphyria

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ABSTRACT — Most patients with hepatic porphyria exhibit neuropsychiatric symptoms, including abdominal pain, peripheral neuropathy, confusion, insomnia and mental disturbances such as anxiety and depression. Although heme deficiency and accumulation of heme precursors are thought to be responsible for neuropsychiatric manifestations in patients with acute porphyria, the pathogenetic mechanisms remain poorly understood. In the present study, we observed psychiatric behaviors in mice with hepatic porphyria induced by the ingestion of a griseofulvin (GF)-containing diet over a period of 12 weeks. GF ingestion by the mice caused an accumulation of porphyrins in the feces and a decrease in heme in the liver; these effects were observed throughout the entire duration of the experiment, with maximum levels observed after circa 1 week of ingestion of this diet. In addition, the mice developed enlargement of the liver, hepatocyte injury, and cholestasis. Mice with hepatic porphyria manifested an anxiety-like behavior by the long-term treatment (over 5 weeks) in a GF-dose and duration dependent manner. The hepatic porphyria mice also manifested depression-like behaviors by the short-term treatment (3 weeks) of GF2.0, which was reversed by administration of anti-depressant, imipramine. In conclusion, this study for the first time demonstrated psychiatric manifestations in GF-induced hepatic porphyria mice. The present results suggest that model animals could be useful for elucidating the mechanisms underlying psychiatric manifestations in syndromes such as hepatic porphyria and hepatic encephalopathy that are associated with the impairment of hepatic function.

Key words: Porphyria, Heme, Griseofulvin, Cholestasis, Anxiety, Depression

INTRODUCTION

Porphyria is a group of disorders that accompany abnormal heme biosynthesis due to functional impairment of enzymes corresponding to each step in heme biosynthesis (Anderson *et al*., 2005; Downey, 1999) by genetic defects and/or chemical modifications. Porphyrias are classified as either hepatic or erythrocytic porphyria, according to the origin of the dysfunction, and cases are also classified as acute or cutaneous in accord with the clinical manifestations. Cutaneous porphyria is associated with skin inflammation due to the accumulation of heme precursors (e.g., protoporphyrin IX (PPIX)) that act as photosensitizers in blood and skin tissue. On the other hand, acute porphyria clinically manifests with neuropsychiatric symptoms, including abdominal pain, peripheral neuropathy, confusion, seizures, loss of consciousness, insomnia, and mental disturbances such as anxiety and depression (Anderson *et al*., 2005; Kochar *et al*., 2007; Bonkowsky and Schady, 1982; Millward *et al*., 2005). Delaying the effective treatment of acute porphyric attacks can be fatal, or can cause long-term or permanent neurological damage (Thunell, 2006; Seth *et al*., 2007); therefore, it is crucial to treat porphyria symptoms effectively in order to prevent the development and progression of the syndrome.

All types of acute porphyria are hepatic in origin. Although porphyria patients carry genetic defects and mutations, the manifestation of neuropsychiatric symptoms in hepatic porphyria patients is triggered by precipitating factors, including various drugs, hormones, fasting, and other triggers (Anderson *et al*., 2005; Bonkowsky and Schady, 1982). All precipitating factors can increase the demand for hepatic heme and induce the production

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of δ-aminolevulinic acid synthase (Alas), which is the rate-limiting enzyme at the first step in the heme biosynthetic pathway (May *et al*., 1990). Depletion of free heme and the accumulation of heme precursors such as δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) are plausibly responsible for the neuropsychiatric manifestations seen in acute porphyria patients (Anderson *et al*., 2005). In addition, the activity of tryptophan 2,3-dioxygenase (TDO), an enzyme for Trp metabolism, is strictly regulated by free heme levels (Ren and Correia, 2000). It is estimated that inactivation of TDO in porphyria patients may elevate Trp contents in the brain, and subsequently activate serotonergic neurons (Litman and Correia, 1983). Moreover, porphyrin derivatives (e.g., PPIX, *N*-methyl protoporphyrin (NMPP), and hemin) may act as potential agonists for central benzodiazepine receptors (CBR), a component of $GABA_A$ receptor (Ruscito and Harrison, 2003). The porphyrins also bind to peripheral benzodiazepine receptors (PBR) (Verma *et al*., 1987; Cantoni *et al*., 1992). PBR is recently renamed as the 18 kDa translocator protein (Papadopoulos *et al.*, 2006), which may play a role in biosynthesis of steroid, including neurosteroids. Neurosteroids produced from glial cells can modulate $GABA_A$ receptors in the membranes of neurons (Henderson, 2007; Shen *et al*., 2007). These indicate that porphyrin derivatives may be directly and indirectly able to modulate GABAergic neurons. The perturbation of serotonergic and GABAergic neurons is known to be closely related to mental disturbances such as anxiety and depression, yet the mechanisms underlying the manifestation of neuropsychiatric symptoms in acute porphyria remain poorly understood. Therefore, it is considered of value to elucidate the mechanisms underlying the manifestation of neurological and psychiatric disturbances in porphyria patients as well as animal models.

Several animal models for porphyria have been established by genetic engineering (Lindberg *et al*., 1996; Ged *et al*., 2006; Phillips *et al*., 2001; Davies *et al*., 2005) or the administration of various chemicals (De Matteis and Rimington, 1963; Fonia *et al*., 1996; Krijt *et al*., 2003; Billi de Catabbi *et al*., 2005). Mice with a deficiency of porphobilinogen deaminase (PBGD) have been shown to exhibit peripheral neuropathy accompanied by a severe loss of axons in the sciatic nerve and atrophic fibers, although over one-half of PBGD activity in the liver was maintained, and ALA accumulation in the urine was only observed upon treatment with phenobarbital, a P450 inducer (Lindberg *et al*, 1996). Protoporphyria model mice have a mutated ferrochelatase (FC) gene that encodes a mitochondrial enzyme to catalyze the insertion of a ferrous ion into PPIX at the last step of heme biosynthesis; these mice exhibit hemolytic anemia, splenic enlargement, and cholestasis, but the heme regulatory pool in the liver is still maintained (Davies *et al*., 2005). Long-term administration of griseofulvin (GF) to rodent produces a well-established animal model for hepatic porphyria (De Matteis and Rimington, 1963; Hawkins *et al*., 1986; Berenson *et al*., 1991; Inafuku *et al*., 1999). GF is metabolized by cytochrome P450 with the concomitant production of NMPP, an inhibitor of FC; NMPP subsequently prevents heme biosynthesis and allows for the accumulation of heme precursors, especially the immediately preceding precursor, PPIX. Mice with GF-induced porphyria exhibit disturbances in heme metabolism (De Matteis and Rimington, 1963), liver injury (Inafuku *et al*., 1999; Berenson *et al*., 1991; Choi *et al*., 1991), and skin inflammation (Hawkins *et al*., 1986); however, little is known about the neuropsychiatric symptoms in these mice. In the present study, we evaluated psychiatric behaviors such as mouse-model anxiety and depression, as well as other pathological states, in association with disturbances in heme metabolism following GF ingestion.

MATERIALS AND METHODS

Animals

Male mice (ICR, 5-weeks-old, 20-24 g) were maintained on a 12:12-hr light/dark cycle and at a constant temperature (24 ± 2°C) with food (standard chow pellet, MF; Oriental Yeast Co., Tokyo, Japan) and water made available *ad libitum*. Mice were acclimated to these environmental conditions for at least 7 days before start of GF ingestion. According to the GF content in the powder chow, the mice were divided to three groups: HC, GF0.5, and GF2.0, of which diet contained 0%, 0.5%, and 2.0% (w/w) GF, respectively; the respective diets were maintained for up to 12 weeks. All experimental procedures were approved by the Josai International University Animal Care and Use Committee and complied with *the Helsinki Guidelines for the Care and Use of Laboratory Animals.*

Preparation of biological samples

On the indicated day, fecal samples were obtained from three mice in each group isolated in metabolic cages during single dark/light cycle, and the samples were collected during the final 24 hr of examination. Samples were stored at −20°C until used for analysis. Serum and liver tissue were obtained on the indicated days from three mice in each group anesthetized by the i.p. administration of pentobarbital around the time lights turned off. Blood samples were removed from the postcaval vein,

and serum separation was carried out by centrifugation to determine enzymatic activities, as described below. The liver was refluxed with excess phosphate-buffered saline. The isolated liver was weighed, immediately frozen in liquid nitrogen, and stored at −80°C before use. For the purpose of histological analysis, a segment of the liver was fixed in formalin solution without freezing immediately after isolation.

Histology

Histological analysis was carried out in order to evaluate the pathophysiological states of liver, ear, and skeletal muscle of one to three mice in each group on days 21, 49, and 85, after the initiation of GF ingestion. The fixed tissue was processed according to standard methods and stained with hematoxylin and eosin (H&E). The myelinated structure of neurons in the skeletal muscle was also stained with Luxol Fast Blue.

Fecal porphyrins

Porphyrin levels in the feces were determined according to the method of Hift *et al*. (2004) with minor modifications (Li *et al*., 1987). In brief, the fecal samples (ca. 50 mg) were homogenized in 2 ml of 5% (w/v) cH_2SO_4 in MeOH, and incubated overnight at ambient temperature for the methylation of porphyrins. Diethyl ether was then added to the samples, and the organic layer was dried with Na_2SO_4. After the filtration and evaporation of the organic layer, the residues were dissolved in MeOH and were separated by HPLC with a 15 cm x 2.0 mm i.d. Gemini-ODS column (GL Sciences Inc., Tokyo, Japan). The eluent was 5% (v/v) 1 M ammonium acetate (pH 5.16) in MeOH. The column was equilibrated with the eluent prior to sample injection. The retention times of the porphyrin derivatives, i.e., the tetramethylester of coproporphyrin III (CPIII) and dimethylester of PPIX, were 5.3 and 10.5 min, respectively. The standards of the porphyrin derivatives were obtained from Wako Pure Chemical Co. (Osaka, Japan). The flow rate was 0.2 ml/min. The porphyrins were detected by a fluorescence detector (ex., 400 nm; em., 618 nm). Porphyrin contents were determined as free acids and corrected by feces weight.

Serum enzyme activity assay

Serum enzyme activity was evaluated to determine the extent of liver damage. Levels of aspartic aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (γGTP) were analyzed according to the standard methods described in the literature (Kamei, 1989a; Kamei, 1989b; Kamei, 1990; Sekiguchi, 1995; respectively).

Liver mRNA expression assay

Total RNA was extracted from the liver using a commercially available kit (RNeasy; Qiagen, Valencia, CA, USA), and the remaining DNA was completely removed by RNase-free DNase treatment for the analysis of mRNA levels of Alas1 in the liver. Expression levels of mRNA were determined by RealTime-PCR with the following primers: Alas1, 5'-TCTTCCGCAAGGCCAGTCT-3' (forward) and 5'-TGGGCTTGAGCAGCCTCTT-3' (reverse); and GAPDH, 5'-GTGATGGGTGTGAACCACG-3' (forward) and 5'-GGATGCAGGGATGATGTTC-3' (reverse). The expression levels of Alas1 mRNA were normalized against those of GAPDH. Total RNA (10 ng) was reverse-transcribed with poly-d(T)12-18 as a first-strand primer using reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After the addition of gene-specific primers, the target genes were amplified by PCR using a thermal cycler (ABI Prism 7300; Applied Biosystems, Foster City, CA, USA) with SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The thermal-cycler protocol was as follows; 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec, 57°C for 30 sec, and 72°C for 1 min.

P450 contents in liver microsomes

According to the standard method described in the literature (Omura and Sato, 1964), liver microsomes were prepared and cytochrome P450 contents in the microsomes were determined by measuring the spectral difference following CO-binding ($\Delta\varepsilon_{450\text{-}490}$ = 91,000 $M^{-1}cm^{-1}$). Proteins in the microsomes were determined using a BCA assay kit (Sigma-Aldrich, St. Louis, MO, USA) and bovine serum albumin as the standard.

Light/dark box test

For the light/dark box test, the method of Costall *et al*. (1987) was used, with minor modifications. The acrylic test box consisted of a light (145 x 180 x 145 mm^3) and a dark (145 x 180 x 145 mm^3) compartment connected by an opening (30 x 35 mm) located in the center of the partition at the level of the floor. The light compartment had a transparent, covered top and white walls, and was brightly illuminated by a light source (ca. 800 lux at the floor level), whereas the dark compartment had an opaque, covered top and black, opaque walls. Mice were individually placed in the center of the light compartment facing the opening and were allowed to explore the test box freely for 5 min. The total period of time spent by each mouse in each compartment, and the times the mouse crossed the

interface between the two compartments were recorded.

Forced swimming test

For the forced swimming test, the method of Porsolt *et al*. (1978) was used, with minor modifications. In brief, the apparatus consisted of a plastic cylinder (25 cm high, 27 cm in diameter) containing 10 cm of water at 23-25°C. Mice were placed in the water, and the total duration in which the mice were immobile (immobility period) during the last 4 min of a 6-min test session was recorded. After each session, the previously used water was exchanged for fresh water. A mouse was considered to be immobile when it floated in an upright position and moved only slightly to keep its head above water. Imipramine (Wako Pure Chemical Co., Osaka, Japan) dissolved in saline was intraperitoneally injected 30 min prior to the test.

Locomotor activity

Mice were individually housed in a cage with a running wheel (Muromachi-Kikai, Tokyo, Japan). The locomotor activity of the mice was analyzed using a software package (CompAct 3.0, Muromachi-Kikai, Tokyo, Japan) and activity was determined according to the times the mice turned the wheel during a 24-hr period; results are shown as the relative rate, i.e., the number of turns after GF treatment compared to that observed prior to GF treatment. Foot print analysis was performed by measuring stride of left hind limb of the mice which had been painted with a black ink.

Statistical analysis

The significance of differences among two groups was determined by unpaired *t*-test. The significance of differences among more than two groups was determined by Kruskal Wallis H-test and Mann-Whitney U-test with Bonferroni correction in the case that the sample number in each group was less than 4, and in other case, by non-repeated measures ANOVA with Dunnett's test (nrANOVA with Dunnett's). Only significant statistical tests are reported in the text, with the significance established at a p-value < 0.05.

RESULTS

As shown in Fig. 1, GF2.0 mice retarded to gain the body weight for 6 weeks, compared to that of the controls, whereas the pattern of weight gain among GF0.5 mice did not differ from that of the controls. After 6 weeks of treatment, the body weight of GF2.0 mice became congruent with those of other groups.

Heme precursors such as PPIX and CPIII in the feces were extremely elevated, dose-dependently, and transiently in excess of 50-100 fold, by GF treatment (Fig. 2). Accumulation peaked on day 7, and then the heme precursor levels remained stable at 5- to 10-fold above that of the controls throughout the GF ingestion period. The color of the feces of GF-treated mice turned reddish-brown within one week, and then changed to yellow or brown with chronic GF treatment. Moreover, GF2.0 mice exhibited ear swelling and erythema, whereas no significant changes were observed in either the controls or GF0.5 mice (Fig. 3).

Fig. 4 shows the mRNA expression levels of Alas1 in the mouse livers. Marked induction of Alas1 was observed with GF ingestion. The peak Alas1 level was observed 3 days after the onset of GF ingestion, and this peak occurred in a dose-dependent manner. Alas1 mRNA levels in the livers of GF2.0 mice were restored to the control levels after day 21, while the Alas1 mRNA levels in the livers of GF0.5 mice remained higher than those of the controls even after day 21. Cytochrome P450 levels in the liver microsomes of GF2.0 mice declined by ca. 40% after 1 week of GF ingestion, and then returned to control levels after 5 weeks of GF ingestion (Fig. 5). No significant decrease in P450 contents in the liver microsomes of GF0.5 mice was observed.

Isolated liver samples from GF-treated mice were abnormally dark, and the wet weight had increased in a dose- and duration-dependent manner (Fig. 6). In the case of GF0.5, change of the liver weight was slight within 3 weeks, and then the weight of the liver elevated after

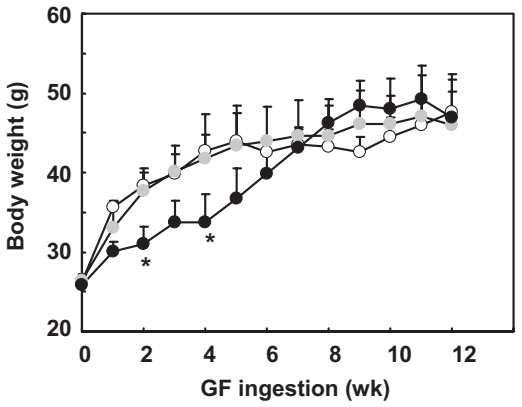

Fig. 1. Body weight changes in mice. The body weight of the mice was measured weekly. Data represent the mean ± S.D. (n = 4-5). Open circles, HC; gray circles, GF0.5; closed circles, GF2.0. Significant differences from HC are indicated by asterisks as follows: *p < 0.05 (nrANOVA with Dunnett's).

5 weeks of GF ingestion. In addition to the enlargement of the liver, the pathophysiological state of the liver in the experimental mice was analyzed by serum enzyme activity tests and tissue staining. Enzyme activity in the serum of GF2.0 mice (i.e., AST, ALT, ALP, and γ-GTP) increased, and peaked at around 3 weeks of GF treatment (Fig. 7); levels then declined, eventually to be reincreased. Enzyme levels in GF0.5 mouse serum, especially those of ALP, a marker for cholestasis, were elevated after 5 weeks of GF treatment. As shown in Fig. 8, H&E staining of liver sections revealed centrolobular hypertrophy of hepatocytes and intracellular vacuoles in the livers of GF0.5 mice, and hyperplasia of the bile ducts and cell infiltration around the periportal region in livers of GF2.0 were observed on day 21. Deposition of brown pigment around hepatocytes and hyperplasia around the portal veins were prominent in the livers of GF-treated mice on day 49. The severity of hepatocyte injury was GF-dose dependent. On day 85 after the onset of GF treatment, isolated livers from GF2.0 mice were found to be very stiff and hard to homogenize, indicating the development of fibrosis (data not shown).

Using the GF-induced mouse model of hepatic porphyria described above, the anxiety and depression status of the mice were examined. Fig. 9 presents the results of the light/dark box tests. The number of times the mice traversed between two compartments was significantly reduced in GF2.0 mice after 5 weeks of GF ingestion.

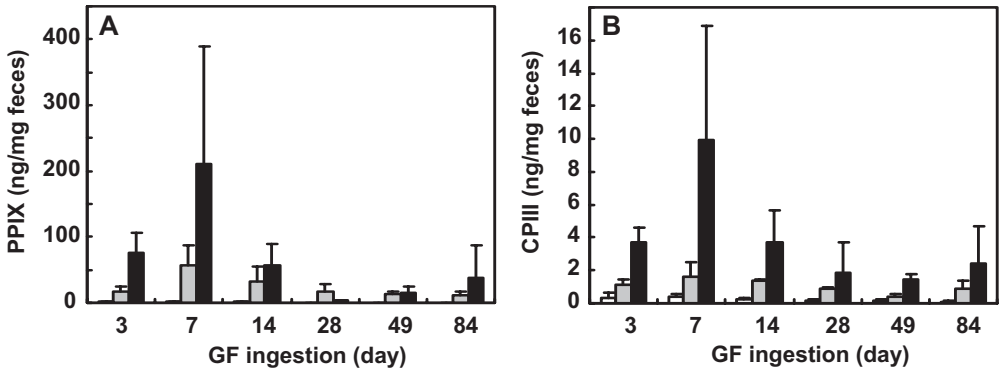

Fig. 2. Porphyrins in mouse feces. A, PPIX; B, CPIII. Data represent the mean ± S.D. (n = 3). Open bars, HC; gray bars, GF0.5; black bars, GF2.0.

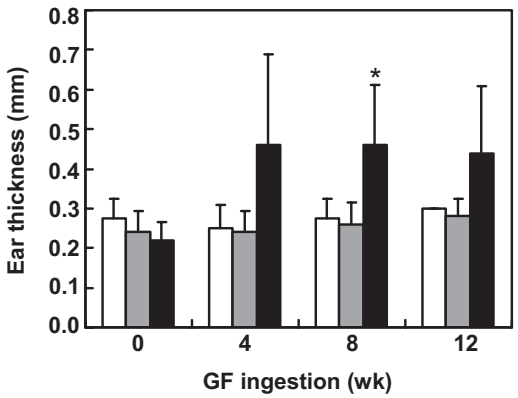

Fig. 3. Inflammation of the ear in mice. Inflammation was determined by ear thickness. Data represent the mean ± S.D. (n = 4-5). Open bars, HC; gray bars, GF0.5; black bars, GF2.0. Significant differences from HC are indicated by asterisks as follows: *p < 0.05 (nrANOVA with Dunnett's).

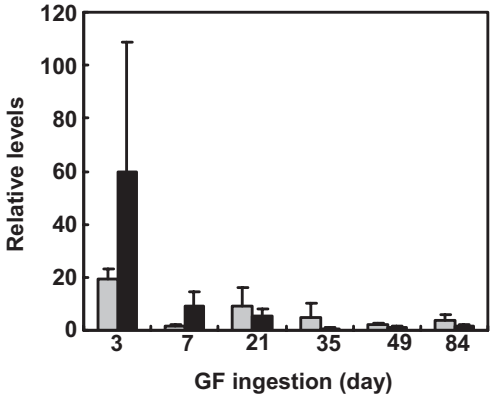

Fig. 4. Induction of Alas1 mRNA expression in the liver of mice by GF ingestion. Data are shown as relative levels compared with those of controls, and represent the mean ± S.D. (n = 3). Gray bars, GF0.5; black bars, GF2.0.

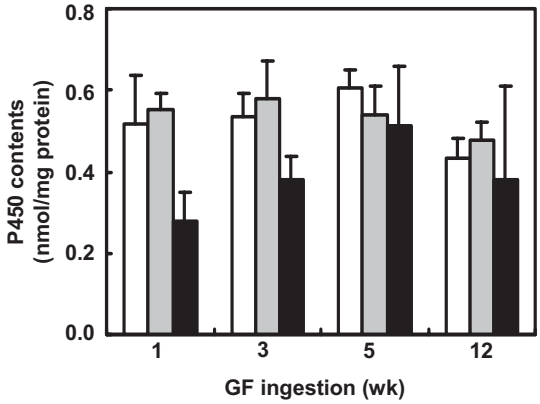

Fig. 5. P450 contents in liver microsomes of mice. Data represent the mean ± S.D. (n = 3). Open bars, HC; gray bars, GF0.5; black bars, GF2.0.

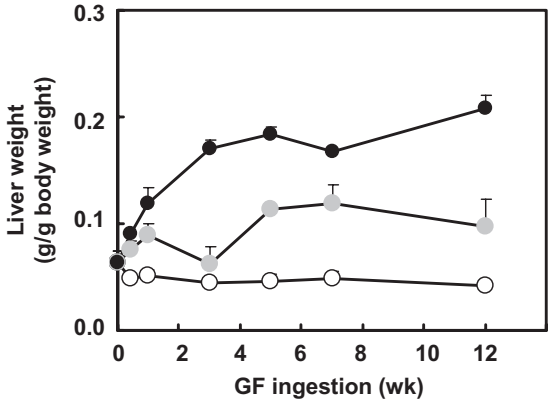

Fig. 6. Liver weight changes in mice. Data represent the mean ± S.D. (n = 3). Open circles, HC; gray circles, GF0.5; closed circles, GF2.0.

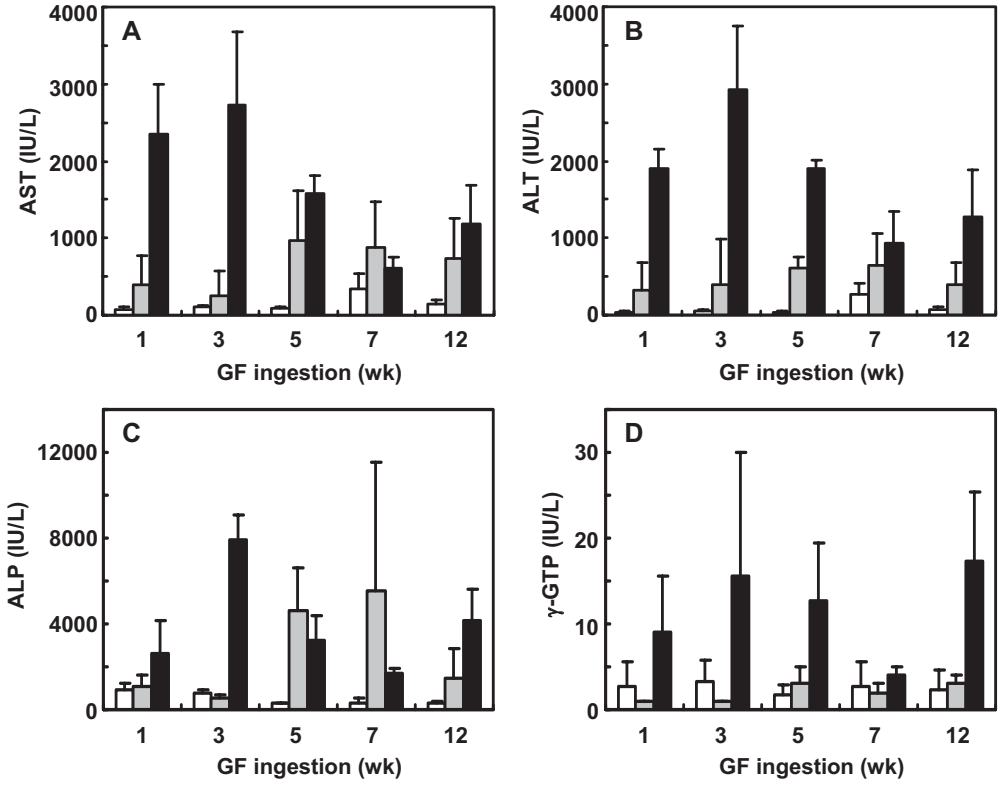

Fig. 7. Enzymatic activity in the serum of mice with or without GF ingestion. A, AST; B, ALT; C, ALP; D, γ-GTP. Data represent the mean ± S.D. (n = 3). Open bars, HC; gray bars, GF0.5; black bars, GF2.0.

The total duration spent by mice in the light area was significantly shortened in GF2.0 mice in 5th week or later of the experiment. Ten-week ingestion of GF0.5 significantly reduced the duration the mice spent in the light compartment. These effects of GF were dose and duration dependent.

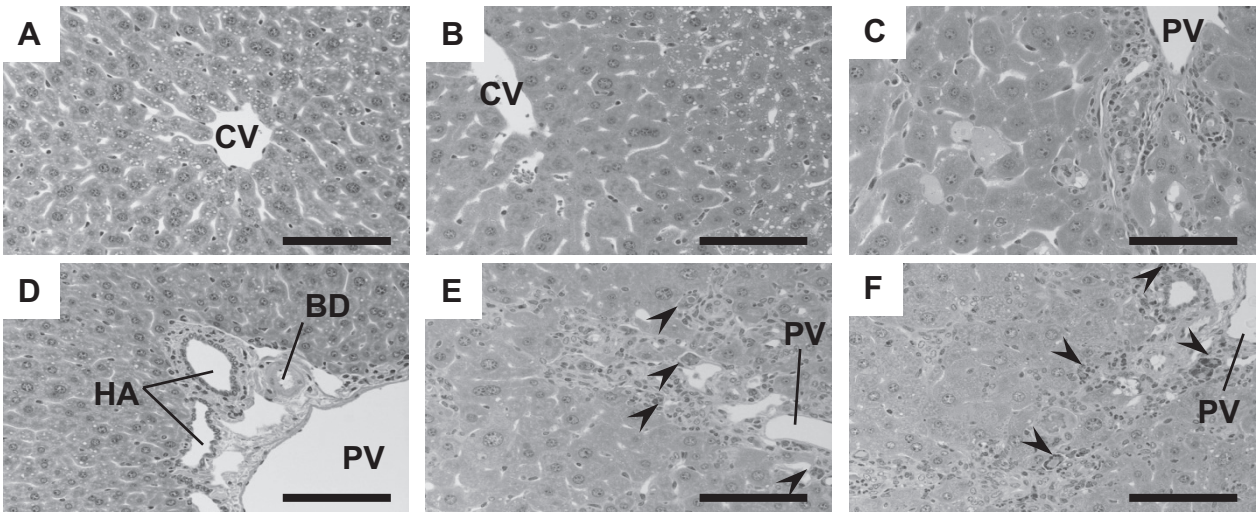

Fig. 8. Histological analysis of the liver in mice as determined by H&E staining. A-C, 21 days; D-F, 49 days. A and D, HC; B and E, GF0.5; C and F, GF2.0. Arrowheads indicate brown pigmentation. CV, central vein; PV, portal vein; HA, hepatic artery; BD, bile ducts. Bars indicate 100 μm.

Fig. 10 shows the results of forced swimming tests. The immobility period of GF2.0 mice was significantly longer than those of the controls by 3 weeks of treatment (Fig. 10A). The immobilized periods observed in control and GF2.0 mice were shortened by intraperitoneal administration of anti-depressant, imipramine, in a dose-dependent manner (Fig. 10B). Higher dose of imipramine was required in GF2.0 mice to reduce the immobility period, comparing to the control mice.

Locomotor activity of GF2.0 mice was mostly comparable levels to that of the control mice during the course of experiment, although the activity showed large individual variation (Fig. 11). Footprint analysis was performed to determine peripheral motor neuropathy. However, we failed to observe significant difference of stride between control and GF-treated mice (data not shown). Histological analysis of the muscles of the hind limb indicated no significant signs of peripheral neuropathy such as the destruction of muscle or nerve cells, and no demyelination was observed, even in GF2.0 mice (data not shown).

DISCUSSION

In the present study, we observed GF-induced hepatic porphyria mice exhibited shortening of the time spent in the light area by light/dark box test, and elongation of imipramine-inhibitable immobility period on the surface of water by forced swimming test. These observations are suggestive of anxiety and depressed states in GF-ingested mice, especially with high dose treatment. Since gain of body weight in GF2.0 mice delayed during several weeks and both behaviors described above are based on decrease of mobility, there maybe a possibility to reflect general toxicity due to the treatment of GF. However, it is unlikely that the anxiety- and depression-like behaviors in GF-ingested mice might result from general toxicity by the treatment of GF, since locomotor activity and foot print analysis revealed no significant difference between HC- and GF-ingested mice. Moreover, although peripheral neuropathy is a typical symptom in acute porphyria patients (Anderson *et al*., 2005), and a hepatic porphyria model with PBGD deficiency has shown peripheral motor neuropathy (Lindberg *et al*., 1996), histological analysis revealed no destruction of muscle and peripheral nerve cells in the hind limbs of the mice, even after 12 weeks of ingestion of GF2.0. Therefore, it is unlikely that peripheral motor neuropathy is manifested in the mouse model of hepatic porphyria induced by GF. In addition, there has been no report to show neuropsychiatric behaviors in animal models of chemical-induced hepatic porphyria, so far. In general, it became evident that GF ingestion by mice leads to a manifestation of mental disturbances in addition to the disturbance of heme biosynthesis.

Cytochrome P450 is a major heme protein in the liver and it utilizes heme as a cofactor. Alas1 mRNA is also an indicator of a reduction in the heme pool, since expression levels are negatively regulated by free heme (May *et al*., 1990). Reduction of P450 contents in liver microsomes

and elevation of Alas1 mRNA expression levels in the liver may reflect the status of the heme pool in the liver, that is, it is likely that hepatic free heme pools are severely decreased by GF ingestion within several days. Heme precursors in the feces were transiently elevated, but this occurred after a peak in Alas1 mRNA expression. Restoration of Alas1 mRNA levels then led to a decrease in heme precursor accumulation in the feces. The P450 contents in GF2.0 mice remained lowered levels than those in control mice during 3 weeks. In sum, heme biosynthesis was highly affected by the short-term treatment of GF2.0 until 3 weeks of treatment; however, it is likely that the mice adapted to these homeostatic changes and reached a metastable state of heme biosynthesis during long-term (i.e., more than 5 weeks) treatment with GF2.0.

In GF-induced hepatic porphyria mice, it has been ascertained that levels of PPIX and CPIII in feces are increased, and Alas1 mRNA are elevated and P450 contents in liver microsomes are decreased. Under these conditions, it is suggested that heme biosynthesis is clearly disturbed and the free heme levels could be reduced. The porphyrin derivatives, including heme, are plausible endogenous benzodiazepine-like agonists (Verma *et al*., 1987; Cantoni *et al*., 1992; Ruscito and Harrison,

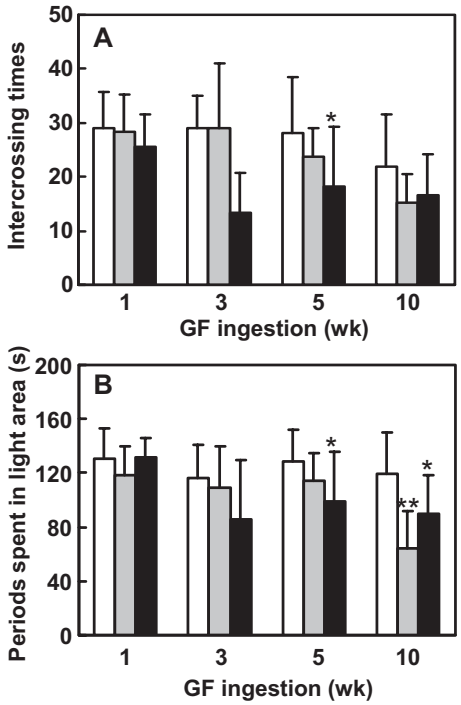

Fig. 9. Anxiety-like behaviors in mice. Anxiety-like behavior was evaluated by the number of crossings over the border between the two compartments (dark and light), and the total duration mice spent in the light compartment using a light/dark box test. A, The number of times mice traversed the border between light and dark compartments; B, Period mice spent in the light compartment. Data represent the mean ± S.D. (n = 8-11). Open bars, HC; gray bars, GF0.5; black bars, GF2.0. Significant differences from HC are indicated by asterisks as follows: *p < 0.05, **p < 0.01 (nrANOVA with Dunnett's).

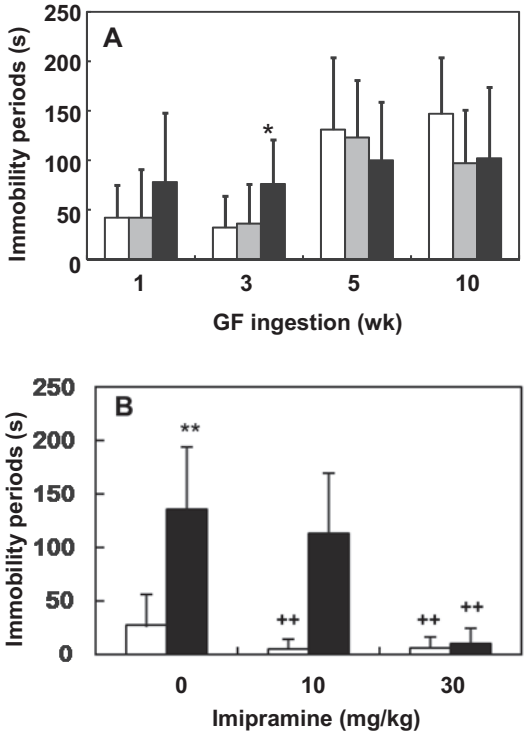

Fig. 10. Swimming-test evaluation of depression-like behaviors in mice. Depression was defined as periods of immobilization on the surface of the water. A, Total immobilized periods of mice as a function of duration of GF-ingestion; Data represent the mean ± S.D. (n = 8-11). Open bars, HC; gray bars, GF0.5; black bars, GF2.0. Significant differences from HC are indicated by asterisks as follows: *p < 0.05 (nrANOVA with Dunnett's). B, Effect of imipramine on the immobility period of mice with GF ingestion for 3 weeks; Data represent the mean ± S.D. (n = 6-8). Open bars, HC; black bars, GF2.0. Significant differences from HC are indicated by asterisks as follows: **p < 0.01 (unpaired t-test). Significant differences from vehicle in each group are also indicated by plus as follows: ++p < 0.01 (nrANOVA with Dunnett's).

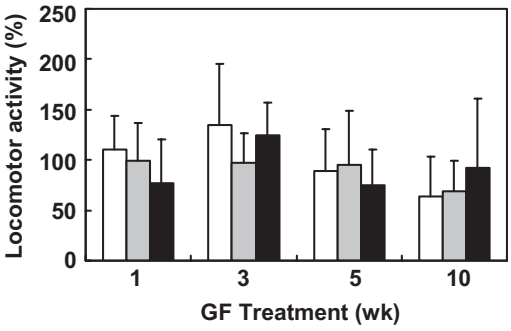

Fig. 11. Autonomic locomotor activities in mice. Data are shown as relative rates, compared to control levels, and represent the mean ± S.D. (n = 8-11). Open bars, HC; gray bars, GF0.5; black bars, GF2.0.

2003), which directly and indirectly lead to the activation of GABAergic neurons (Ruscito and Harrison, 2003; Verma *et al*., 1987; Shen *et al*., 2007). Benzodiazepines have sedative, anti-depressant, and anxiolytic effects (Kalueff and Nutt, 2007), which are mediated by functional activation of $GABA_A$ receptor. It is considered that a reduction in the heme pool and the accumulation of porphyrins could modulate GABAergic neural function. The roles played by hemin, PPIX, and NMPP as endogenous mediators remain obscure under physiological and pathological conditions; however, the psychiatric behaviors observed in the short-term treatment (3 weeks) of GF may be due to interactions between porphyrin derivatives and $GABA_A$ receptors. However, this speculation cannot rule out the possibility of involvement of other neuronal mediators in the manifestation of neuropsychiatric behaviors.

The porphyria-model mice exhibited chronic liver injury during the entire experimental period. After long-term ingestion of GF, the mice showed hepatomegaly, degenerative rearrangement, deposition of porphyrins, and cholestasis, all of which are consistent with the findings of previous reports (Inafuku *et al*., 1999; Berenson *et al*., 1991; Choi *et al*., 1991). Chronic hepatic injury may cause neuropsychiatric symptoms such as aggressiveness and loss of consciousness, as observed in hepatic encephalopathy (Ostapowicz *et al*., 2002). Therefore, it is likely that the manifestation of mental disturbances in mice with long term GF ingestion (over 5 weeks) maybe due to the chronic impairment of liver function rather than a reduction in the heme pool.

In conclusion, we observed anxiety-like and depression-like behaviors in a mouse with GF-induced hepatic porphyria. This mouse model may be a useful tool for analyzing behavioral symptoms and mechanisms underlying the psychiatric manifestations of liver diseases such as hepatic porphyria and hepatic encephalopathy.

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REFERENCES

Anderson, K.E., Bloomer, J.R., Bonkovsky, H.L., Kushner, J.P., Pierach, C.A., Pimstone, N.R. and Desnick, R.J. (2005): Recommendations for the diagnosis and treatment of the acute porphyrias. Ann. Intern. Med., **142**, 439-450.

Berenson, M.M., Welch, V. and Garcia-Marin, J.J. (1991): Importance of bile acid structure in amelioration of griseofulvin-induced murine protoporphyric hepatopathy. J. Lab. Clin. Med., **118**, 89-98.

Billi de Catabbi, S.C., Faletti, A., Fuentes, F., San Martín de Viale, L.C. and Cochón, A.C. (2005): Hepatic arachidonic acid metabolism is disrupted after hexachlorobenzene treatment. Toxicol. Appl. Pharmacol., **204**, 187-195.

Bonkowsky, H.L. and Schady, W. (1982): Neurologic manifestations of acute porphyria. Semin. Liver Dis., **2**, 108-124.

Cantoni, L., Rizzardini, M., Skorupska, M., Cagnotto, A., Codegoni, A., Pecora, N., Frigo, L., Ferrarese, C. and Mennini, T. (1992): Hepatic protoporphyria is associated with a decrease in ligand binding for the mitochondrial benzodiazepine receptors in the liver. Biochem. Pharmacol., **44**, 1159-1164.

Choi, S.W., Han, J.H., Lim, K.T., Cho, H.M., Chung, K.W., Sun, H.S., Park, D.H., Kim, B.S. and Seo, E.J. (1991): Effect of ursodeoxycholic acid on experimental hepatic porphyria induced by griseofulvin. J. Korean Med. Sci., **6**, 146-156.

Costall, B., Hendrie, C.A., Kelly, M.E. and Naylor, R.J. (1987): Actions of sulpiride and tiapride in a simple model of anxiety in mice. Neuropharmacology, **26**, 195-200.

Davies, R., Schuurman, A., Barker, C.R., Clothier, B., Chernova, T., Higginson, F.M., Judah, D.J., Dinsdale, D., Edwards, R.E., Greaves, P., Gant, T.W. and Smith, A.G. (2005): Hepatic gene expression in protoporphyic FECH mice is associated with cholestatic injury but not a marked depletion of the heme regulatory pool. Am. J. Pathol., **166**, 1041-1053.

De Matteis, F. and Rimington, C. (1963): Disturbance of porphyrin metabolism caused by griseofulvin in mice. Br. J. Dermatol., **75**, 91-104.

Downey, D.C. (1999): Porphyria and chemicals. Med. Hypotheses, **53**, 166-171.

Fonia, O., Weizman, R., Coleman, R., Kaganovskaya, E. and Gavish, M. (1996): PK11195 aggravates 3,5-diethoxycarbonyl-1,4-dihydrocollidine-induced hepatic porphyria in rats. Hepatology, **24**, 697-701.

Ged, C., Mendez, M., Robert, E., Lalanne, M., Lamrissi-Garcia, I., Costet, P., Daniel, J.Y., Dubus, P., Mazurier, F., Moreau-Gaudry, F. and de Verneuil, H. (2006): A knock-in mouse model of con-

genital erythropoietic porphyria. Genomics, **87**, 84-92.
Hawkins, C.W., Bickers, D.R., Mukhtar, H. and Elmets, C.A. (1986): Cutaneous porphyrin photosensitization: Murine ear swelling as a marker of the acute response. J. Invest. Dermatol., **86**, 638-642.
Henderson, L.P. (2007): Steroid modulation of $GABA_A$ receptor-mediated transmission in the hypothalamus: effects on reproductive function. Neuropharmacology, **52**, 1439-1453.
Hift, R.J., Davidson, B.P., van der Hooft, C., Meissner, D.M. and Meissner, P.N. (2004): Plasma fluorescence scanning and fecal porphyrin analysis for the diagnosis of variegate porphyria: Precise determination of sensitivity and specificity with detection of protoporphyrinogen oxidase mutations as a reference standard. Clin. Chem., **50**, 915-923.
Inafuku, K., Takamiyagi, A., Oshiro, M., Kinjo, T., Nakashima, Y. and Nonaka, S. (1999): Alteration of mRNA levels of d-aminolevulinic acid synthase, ferrochelatase and heme oxygenase-1 in griseofulvin induced protoporphyria mice. J. Dermatol. Sci., **19**, 189-198.
Kalueff, A.V. and Nutt, D.J. (2007): Role of GABA in anxiety and depression. Depress. Anxiety, **24**, 495-517.
Kamei, Y. (1989a): Recommendation method for measurement of enzyme activity in human serum. Aspartate aminotransferase. Jpn. J. Clin. Chem., **18**, 226-230.
Kamei, Y. (1989b): Recommendation method for measurement of enzyme activity in human serum. Alanine aminotransferase. Jpn. J. Clin. Chem., **18**, 250-254.
Kamei, Y. (1990): Recommendation method for measurement of enzyme activity in human serum. Alkaline phosphatase. Jpn. J. Clin. Chem., **19**, 209-212.
Kochar, D.K., Pal, M., Kochar, S.K., Vyas, A., Kochar, A., Bindal, D. and Agrawal, R.P. (2007): Acute intermittent porphyria presenting with neurological emergency: Review of six cases. Neurology India, **55**, 413-415.
Krijt, J., Pšenák, O., Vokurka, M., Chlumská, A. and Fakan, F. (2003): Experimental hepatic uroporphyria induced by the diphenyl-ether herbicide fomesafen in male DBA/2 mice. Toxicol. Appl. Pharmacol., **189**, 28-38.
Li, F., Lim, C.K. and Peters, T.J. (1987): An h.p.l.c. assay for protoporphyrinogen oxidase activity in rat liver. Biochem. J., **243**, 863-866.
Lindberg, R.L.P., Porcher, C., Grandchamp, B., Ledermann, B., Bűrki, K., Brandner, S., Aguzzi, A. and Meyer, U.A. (1996): Porphobilinogen deaminase deficiency in mice causes a neuropathy resembling that of human hepatic porphyria. Nat. Genet., **12**, 195-199.
Litman, D.A. and Correia, M.A. (1983): L-Tryptophan: A common denominator of biochemical and neurological events of acute hepatic porphyria? Science, **222**, 1031-1033.
May, B.K., Ramana Bhasker, C., Bawden, M.J. and Cox, T.C. (1990): Molecular regulation of 5-aminolevulinate synthase. Diseases related to heme biosynthesis. Mol. Biol. Med., **7**, 405-421.
Millward, L.M., Kelly, P., King, A. and Peters, T.J. (2005): Anxiety and depression in the acute porphyrias. J. Inherit. Metab. Dis., **28**, 1099-1107.
Omura, T. and Sato, R. (1964): The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. J. Biol. Chem., **239**, 2370-2378.
Ostapowicz, G., Fontana, R.J., Schiødt, F.V., Larson, A., Davern, T.J., Han, S.H., McCashland, T.M., Shakil, A.O., Hay, J.E., Hynan, L., Crippin, J.S., Blei, A.T., Samuel, G., Reisch, J. and Lee, W.M. (2002): Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann. Intern. Med., **137**, 947-954.
Papadopoulos, V., Baraldi, M., Guilarte, T.R., Knudsen, T.B., Lacapère, J.-J., Lindemann, P., Norenberg, M.D., Nutt, D,. Weizman, A., Zhang, M.-R. and Gavish, M. (2006): Translocator protein (18kDa): new nomenclature for the peripheral-type benzodiazepine receptor based on its structure and molecular function. Trends Pharmacol. Sci., **27**, 402-409.
Phillips, J.D., Jackson, L.K., Bunting, M., Franklin, M.R., Thomas, K.R., Levy, J.E., Andrews, N.C. and Kushner, J.P. (2001): A mouse model of familial porphyria cutanea tarda. PNAS, **98**, 259-264.
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.
Ren, S. and Correia, A. (2000): Heme: A regulator of rat hepatic tryptophan 2,3-dioxygenase? Arch. Biochem. Biophys., **377**, 195-203.
Ruscito, B.J. and Harrison, N.L. (2003): Hemoglobin metabolites mimic benzodiazepines and are possible mediators of hepatic encephalopathy. Blood, **102**, 1525-1528.
Sekiguchi, M. (1995): Recommendation method for measurement of enzyme activity in human serum. γ-Glutamyltranspeptidase. Jpn. J. Clin. Chem., **24**, 106-121.
Seth, A.K., Badminton, M.N., Mirza, D., Russell, S. and Elias, E. (2007): Liver transplantation for porphyria: Who, when, and how? Liver Transpl., **13**, 1219-1227.
Shen, H., Gong, Q.H., Aoki, C., Yuan, M., Ruderman, Y., Dattilo, M., Williams, K. and Smith, S.S. (2007): Reversal of neurosteroid effects at alpha4beta2delta $GABA_A$ receptors triggers anxiety at puberty. Nat. Neurosci., **10**, 469-477.
Thunell, S. (2006): Genomic approach to acute porphyria. Physiol. Res., **55**, S43-S66.
Verma, A., Nye, J.S. and Snyder, S.H. (1987): Porphyrins are endogenous ligands for the mitochondrial (peripheral-type) benzodiazepine receptor. PNAS, **84**, 2256-2260.