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Author(s)	Furugen, Ayako; Kanno, Yuki; Ohyama, Nanami; Kurosawa, Yuko; Jinno, Naoko; Narumi, Katsuya; Iseki, Ken; Kobayashi, Masaki
Citation	Drug metabolism and pharmacokinetics, 40, 100409 https://doi.org/10.1016/j.dmpk.2021.100409
Issue Date	2021-10
Doc URL	http://hdl.handle.net/2115/87117
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Type	article (author version)
File Information	HUSCAP.pdf

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Effects of valproate, an HDAC inhibitor, on the expression of folate carriers and folate metabolism-related genes in the placenta of rats

Ayako Furugen*, Yuki Kanno, Nanami Ohyama, Yuko Kurosawa, Naoko Jinno, Katsuya Narumi, Ken Iseki[a], Masaki Kobayashi*

Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University

[a] Present affiliation: Health Sciences University of Hokkaido.

*Corresponding authors

Masaki Kobayashi, Ph.D. Phone/Fax: +81-11-706-3772/3770; E-mail: masaki@pharm.hokudai.ac.jp

Ayako Furugen, Ph.D. Phone/Fax: +81-11-706-3235/3235; E-mail: afurugen@pharm.hokudai.ac.jp

Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12-jo, Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan

Abbreviations

CBE, cystathionine b-synthase; CSE, cystathionine gamma-lyase; DHFR, dihydrofolate reductase; FRα, folate receptor alpha; GD, gestational day; HDAC, histone deacetylase; MS, methionine synthase; MSR, methionine synthase reductase; MTHFR, methylenetetrahydrofolate reductase; PCFT, proton-coupled folate transporter; RFC, reduced folate carrier; VPA, valproate

Abstract

Valproate (VPA), an antiepileptic drug, is known to inhibit histone deacetylases (HDACs). Exposure to VPA during pregnancy increases several fetal risks. The maintenance of folate level during pregnancy is essential for adequate fetal development, and the placenta plays a critical role in supplying nutrients to the fetus. The aim of this study was to elucidate the effects of VPA on the gene expression of folate carriers and metabolizing enzymes in the rat placenta at both mid and late gestation periods. Pregnant rats were orally administered VPA on a single day or 4 days (repeated administration). Gene expression of folate carriers (*Folr1*, *Slc19a1*, *Slc46a1*) and metabolizing enzymes (*Cth*, *Mtr*, *Mtrr*, *Mthfr*, *Dhfr*) was assessed in the placenta on gestational day (GD) 13 or GD20. In the control rats, the expression of *Folr1*, *Slc46a1*, *Cth*, and *Mthfr* tended to be upregulated, whereas that of *Mtrr* and *Dhfr* was downregulated during gestation; the expression of *Slc19a1* and *Mtr* did not change. Repeated VPA administration reduced the placental expression of *Folr1*and *Mtr* on GD20 and increased the expression of *Dhfr* on GD13 compared with the control. These findings indicate that administration of VPA alters the placental gene expression of folate carriers and metabolism-related enzymes.

Keywords: folate; transporter; enzyme; placenta; rat; valproate; histone deacetylase

1. Introduction

Maternal folate sustention during pregnancy is critical for adequate fetal growth. Studies have indicated that folate deficiency during pregnancy is associated with several fetal risks [1-4]. It is well known that folic acid supplementation during pregnancy prevents the incidence of neural tube defects [1] and reduces the risk of congenital heart defects in the fetus [2]. Furthermore, folic acid supplementation during pregnancy may prevent gestational hypertension and preeclampsia [3] and improve fetal growth [4].

The placenta is a crucial organ for normal fetal development; it has various functions, such as substance exchange, gas exchange, and hormone secretion. Folate carriers such as folate receptor-α (FRα/*FOLR1*), reduced folate carrier (RFC/*SLC19A1*), and proton-coupled folate transporter (PCFT/ *SLC46A1*) are expressed in the placenta and contribute to the transport of folates [5]. Folates are involved in one-carbon metabolism and are important for the DNA methylation cycle and cell division. Besides folate carriers, studies have indicated the presence of folate metabolic enzymes in the placenta of humans [6–9] and rodents [10]. Dihydrofolate reductase (DHFR/*DHFR*) is an enzyme that converts dihydrofolate to tetrahydrofolate. Methylenetetrahydrofolate reductase (MTHFR/*MTHFR*) converts 5,10-metylenetrahydrofolate to 5-methyltetrahydrofolate. 5-Methyltetrahydrofolate provides the methyl group for the remethylation of homocysteine to methionine, and the process is catalyzed by methionine synthase (MS/*MTR*). Methionine synthase

reductase (MSR/*MTRR*) regulates the activity of MS. Cystathionine b-synthase (CBS/*CBS*) and cystathionine gamma-lyase (CSE/*CTH*) contribute to the conversion of homocysteine to cysteine.

The expression and function of folate carriers can be altered by maternal conditions, such as exposure to some compounds (e.g., alcohol and pharmacotherapies) [11,12] and pregnancy complications (e.g., preeclampsia, diabetes, and preterm birth) [13–15]. Besides folate carriers, the expression of genes involved in folate metabolism can also be altered by maternal conditions (e.g., preeclampsia and neural tube defects) [8,9]. Therefore, information on the effects of maternal conditions, including the use of medications, on folate dynamics in the placenta is necessary to predict fetal risks.

Valproate (VPA), a widely prescribed antiepileptic drug, is used to treat bipolar disorder. However, caution should be exerted when administering VPA to women with epilepsy at childbearing age. Exposure to VPA during the periconception period increases fetal malformation risk [16]. In addition, VPA decreases the IQ score and increases neurodevelopmental disorder risk in children [17,18]. Mechanisms underlying the toxicological effects of VPA in the reproductive tissues have not been elucidated. VPA reduces the serum folate level and elevates the homocysteine level [19], and the disruption of folate level has been hypothesized as one of the action mechanisms of VPA [20]. VPA can affect the mitochondria by interfering with mitochondrial pathways, functions, or structures [21]. Recently, VPA has garnered attention as an inhibitor of histone deacetylases (HDACs). The HDAC isoforms in mammals are classified into four classes based on their structure:

class I (HDAC1- HDAC3 and HDAC8), class II (class IIa: HDAC4, HDAC5, HDAC7 and HDAC9, class IIb: HDAC6 and HDAC10), class III (SIRT1- SIRT7), and class IV (HDAC11) [22]. VPA is known to act on class I and IIa HDACs [22].

Studies have investigated the influence of VPA on the functions and expression of folate carriers in various models, including trophoblastic cell lines, cell culture models, and *ex vivo* placenta [23–25]. As each model has advantages and limitations, it is necessary to comprehensively employ different models to better understand the effects of VPA on the fetus. Because the expression of placental genes changes throughout gestation, evaluation at each gestational stage is important. Furthermore, gestational changes in several genes, such as those encoding folate-metabolizing enzymes and HDACs in the rat placenta, have not been fully characterized. We previously reported that VPA alters the expression of several transporters in the rat placenta and that the sensitivity to VPA differs among gestational stages [26]. In this study, we used pregnant rats as *an in vivo* animal model to investigate the effects of VPA administration on the expression of folate carriers, folate metabolism-related genes, and HDACs in the placenta.

2. Material and Methods

2.1. Chemicals

Valproate (valproic acid sodium salt) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals, drug administration, and tissue collection

Animal experimental protocols in this study were approved by the Hokkaido University Animal Care Committee (Approval No. 17–0005) and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Detailed protocols, including housing conditions, administration schedules, and placental sample collection have been described previously [26]. The present study was associated with a previous report, which reported the expression of placental drug transporters after administration of VPA (400 mg/kg) [26]. Placental samples for gene expression analyses used in this study were the same as those in the previous study. Briefly, VPA (400 mg/kg/day) was orally administered to pregnant female Wistar rats. Control rats were administered an equivalent volume of water. To investigate the effects of a single administration of VPA, rats were orally administered VPA on gestation day (GD)12 or GD19. To investigate the effects of repeated administrations of VPA, rats were orally administered VPA for 4 successive days at mid-gestation (GD9–GD12) or late gestation (GD16–GD19). To assess dose-dependent effects, rats were orally administered VPA (200, 400, or 600 mg/kg/day) for 4 successive days during late gestation (GD16–GD19). After 24 h of the last administration of VPA/water, the placentas were collected.

2.4. Real-time polymerase chain reaction

Real-time polymerase chain reaction (PCR) was conducted using the KAPA SYBR® Fast qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) as described previously [26], and the primers used are shown in Supplemental Table 1. Folate carrier genes were amplified through 40 PCR cycles at 95°C for 30 s, 52°C (*Folr1*) or 60 °C (*Slc19a1* and *Slc46a1*) for 30 s, and 72°C for 15 s using the Mx3000™ real-time PCR system (StrataGene) or 40 PCR cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 1 s using the LightCycler® 480 System II (Roche, Basel, Switzerland). *Hdac9* was amplified through 50 PCR cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 1 s using the LightCycler® 480 System II. Other targets were amplified through 40 PCR cycles at 95°C for 10 s, 55°C (*Ugt1a6*, *Mtr*, *Mthfr*, *Dhfr*, *Hdac1-Hdac5*, and *Hdac7–Hdac8*) or 60°C (*Cse* and *Mtrr*) for 20 s, and 72°C for 1 s using the LightCycler® 480 System II. β-Actin (*Actb*) was used as the housekeeping gene for the normalization of target gene expression. The reference gene was not changed by gestational age or VPA administration [26]. Three to four placentas per dam were used in the real-time PCR analysis. The placenta samples of each litter were individually analyzed, and the results were averaged for each dam.

2.6. Western blotting

Western blotting was conducted as described previously [26]. The placental tissue on GD20 was minced and homogenized in ice-cold lysis buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 4.5 M urea). The lysis buffer was supplemented with cOmplete™ Mini protease

inhibitor cocktail tablets (Millipore Sigma, Burlington, MA) and 1 mM phenylmethylsulfonyl fluoride. The sample was subjected to SDS-PAGE (12.5% acrylamide gel for FRα and 15% acrylamide gel for acetyl-histone H3). Ten micrograms of protein was loaded per well to detect FRα; 100 μg of protein was loaded per well to detect acetyl-Histone H3 (Lys9/Lys14). The primary antibodies used were rabbit anti-FRα monoclonal antibody (ab221543; Abcam, Cambridge, UK), rabbit anti-acetyl-Histone H3 (Lys9/Lys14) polyclonal antibody (#9677; Cell Signaling Technology, Beverly, MA), and mouse anti-actin monoclonal antibody (#517310; Merck Millipore, Burlington, MA). Two to four placentas per dam were used for western blotting. The placenta samples of each litter were individually analyzed, and the results were averaged for each dam.

2.7. Statistical analyses

Data are presented as mean ± standard deviation (S.D.). Student's *t*-test was used for comparisons between two groups. Tukey–Kramer test and Dunnett's test were used for multiple comparisons. Statistical analyses were conducted using JMP pro (SAS Institute, Cary, NC, USA). Statistical significance was defined at $p < 0.05$.

3. Results

3.1. Acetyl-histone H3 (Lys9/Lys14) expression in the placenta after VPA administration

In the present study, the effects of single-dose (single administration) and repeated-dose (repeated administration) administration of VPA on placental gene expression were assessed. The placental samples were collected 24 h after VPA administration on GD13 or GD20. Western blotting revealed that single and repeated administrations of VPA increased the placental level of acetyl-histone H3 (Lys9/Lys14) compared with the control on GD20 (Figure 1). In the repeated-administration group, the acetyl-histone H3 level was significantly increased by 2.5-fold compared with that in the control (Figure 1B).

3.2. Effect of VPA on the expression of folate carrier genes in the placenta

First, we evaluated the effect of single administration of VPA on the expression of folate carrier genes, namely, FRα (*Folr1*), RFC (*Slc19a1*), and PCFT (*Slc46a1*). There was no significant change in the gene expression of folate carriers after single VPA administration in both gestational stages (Figure 2A). Next, the effect of repeated administrations of VPA was evaluated. The multiple comparison analyses showed that the expression of FRα on GD20 was significantly reduced by VPA to 64% compared with that of the control. Although the PCFT mRNA expression tended to decrease to 72% compared with that of the control on GD20, the decrease was not statistically significant. With regard to the changes with gestational stage, the expression of FRα and PCFT was considerably

increased on GD20 compared with that on GD13 (FRα: approximately 8–9-fold; PCFT: approximately 3–4-fold). The expression of RFC did not show significant changes with gestational stage.

3.3. Effect of VPA on the expression of folate metabolism-related genes in the placenta

We analyzed the effect of VPA on the expression of folate metabolism-related genes, namely, CSE (*Cth*), MSR (*Mtrr*), MS (*Mtr*), MTHFR (*Mthfr*), and DHFR (*Dhfr*) in the placentas. There was no significant change in the expression of these genes after single VPA administration at both gestational stages (Figure 3A). Repeated administrations of VPA decreased the gene expression of MS on GD20 by 35% (Figure 3B) compared with the control. The DHFR mRNA level increased to 188% compared with that of the control on GD13. Although the CSE mRNA expression tended to increase to 191% compared with that of the control on GD20, the increase was not statistically significant. With respect to the changes associated with gestational stage, the gene expression of MSR and DHFR on GD13 was higher than that on GD20 (1.5–1.8- and 2.7–3.3-fold, respectively), whereas the gene expression of MTHFR on GD20 was approximately 2-fold higher than that on GD13. CSE gene expression tended to increase and MS gene expression did not change with the gestational stage.

3.4. mRNA expression of class I and IIa HDACs in the placenta

VPA has been shown to inhibit class I and IIa HDACs [22]. In addition, some reports showed that HDAC inhibitors can alter the expression of HDAC [27, 28]. Although placental HDACs can be targets of VPA, the isoforms of HDACs expressed in the rat placenta and gestational changes have not yet been fully elucidated. Here, we evaluated the placental expression of HDACs at two gestation stages and the effects of VPA on it.

Single VPA administration did not affect the mRNA expression of class I HDACs on GD13 or GD20 (Figure 4A top). Repeated administrations of VPA decreased the expression of HDAC3 mRNA by 24% compared with the control on GD20 (Figure 5B top). The mRNA expression of HDAC2 and HDAC8 on GD13 was higher than that on GD20 (1.6–1.8- and 1.9–2.4-fold, respectively), whereas that of HDAC1 and HDAC3 was similar on GD13 and GD20.

With regard to class IIa isoforms, single administration of VPA decreased the HDAC5 mRNA level by 22% compared with the control on GD20 (Figure 4B bottom). Repeated administrations of VPA decreased the mRNA expression of HDAC5 by 35% compared with the control (Figure 5B bottom), although the decrease was not statistically significant ($p = 0.053$). Although HDAC4 mRNA expression tended to decrease by 35% on GD13 and GD20, the change was not statistically significant. With regard to alterations with gestational stage, HDAC5 mRNA expression on GD20 was higher than that on GD13 (3.1–3.5-fold), whereas HDAC9 mRNA

expression tended to decrease with gestational. HDAC4 mRNA expression on GD13 and GD20 was similar. There was no significant change in HDAC7 expression with the gestational stage.

3.5. Changes in the placental level of FRα on GD20 after repeated administrations of VPA

As FRα mRNA was considerably reduced by repeated administrations of VPA in the placenta on GD20, the protein expression of FRα was investigated. Western blotting showed that FRα was significantly decreased by approximately 35% after repeated VPA administrations (Figure 5). This tendency was consistent with mRNA level.

4. Discussion

Nutrient requirements, including folate, are elevated during pregnancy owing to increased maternal demand and fetal cell development [5]. Folates are involved in one-carbon metabolism and are important for the DNA methylation cycle and cell division. Folate level influences homocysteine level, which can be a risk factor for pregnancy complications such as preeclampsia, intrauterine growth restriction, placenta separation, and recurrent miscarriages [5]. The present study was performed to elucidate the effects of VPA administration on the expression of folate carriers and folate metabolism-related enzymes in the rat placenta.

In this study, we selected the dose to observe rat fetal effects without leading to maternal and fetal death after prolong administration of VPA, based on the findings of a previous study [29]. Furthermore, previous studies that investigated the effects of prenatal VPA exposure on rat pups

selected an administration dose ranging from 300 to 800 mg/kg [30]. In a previous study, we confirmed that the administration regimen reached a clinical concentration range [26]. VPA upregulates the UDP glucuronosyltransferase family 1 member A6 (UGT1A6/*Ugt1a6*) mRNA level in male rat liver [31]. As a positive control, we analyzed UGT1A6 mRNA levels in the liver of GD20 rats after repeated administration of VPA. VPA administration increased UGT1A6 levels in the liver by 152% compared with that in the control (Supplemental Fig. 1). Although the experimental conditions were not completely identical to those of the previous study, the tendency in the study was consistent with previously reported results.

It has been reported that FRα, RFC, and PCFT contribute to the influx of folate in the placenta [5]. In the human placenta, FRα and PCFT are localized to the microvillous plasma membrane, and RFC exists in both apical and basal membranes [6]. In the present study, the expression of FRα (*Folr1*) and PCFT (*Slc46a1*) was considerably increased during gestation, whereas the expression of RFC (*Slc19a1*) did not show a significant change (Figure 2). In a previous study, the expression of FRα and PCFT was considerably increased at GD20 compared with that at GD14 (FRα: 9-fold; PCFT: 6-fold), whereas the increase in the level of RFC was still at twofold [32]. These results suggest that the increase in the expression of rat FRα and PCFT during gestation was more drastic than that of RFC. The accumulation of [^{3}H]-folic acid after intravenous injection increased with the progress of gestation in the rat placenta and fetus [32]. These results suggest that

the expression levels of folate carriers play an important role in the response to the need for folate in the rat placenta and fetus during development.

Repeated administrations of VPA tended to decrease the levels of FRα and PCFT mRNA in GD20 placenta, whereas in the level of RFC mRNA did not show significant changes between the two gestation stages (Figure 2). Furthermore, the FRα level was significantly decreased, consistent with its mRNA level (Figure 5). FRα is a high-affinity folate carrier that transports folate at a neutral to mildly acidic pH. Because FRα is highly expressed in the rat placenta, this alteration may decrease folate transport to the fetus. Fetal dysgenesis has been reported in mice fed a folic acid-deficient diet [33]. Furthermore, we previously reported that rat placental weight decreased after repeated administration of VPA [26]. These results indicate that the reduction of rat FRα expression after VPA administration may be associated with placental and fetal growth. Studies have investigated the effects of VPA on the expression and function of folate carriers in various models [23-25]. Fathe *et al*. showed that VPA at high concentration can inhibit folate receptors such as FRα in HEK293T cells [24]. In contrast, we previously reported that VPA at clinical concentrations did not inhibit the uptake of ^{3}H-folic acid in human placental choriocarcinoma cell lines [23]. However, 24-h treatment of the cells with VPA induced the mRNA expression of FRα and PCFT. An *ex vivo* study reported that VPA exposure for 180 min significantly reduced the folate concentration by 25%–35% and altered the mRNA level of FRα (downregulation) and RFC (upregulation) (perfusion of term human placentas) [25]. These results suggest that VPA can alter the folate level in placental cells

277 accompanied by the changes in gene expression. As each model has advantages and limitations, it is
278 necessary to comprehensively employ different model [35]. In this study, we utilized pregnant rats as
279 an *in vivo* animal model; the results indicated that FRα expression on GD20 was reduced by repeated
280 VPA administrations. These tendencies were inconsistent with the findings of our previous *in vitro*
281 study in human choriocarcinoma cell lines [23], although the altered expression of genes was
282 consistent. The discordance can be attributed to the differences in the characteristics of models, such
283 as species differences, normal cells, and cancerous cells. Furthermore, in the present *in vivo* study,
284 entire placentas containing several types of cells, including trophoblasts, were utilized for
285 assessment, whereas the cell lines have characteristics of trophoblasts. Future studies should
286 investigate the regulation mechanisms of VPA using human primary trophoblasts to better
287 understand the effects of VPA on folate dynamics in the placenta.

288 Although the relevant data are limited, several studies have indicated the presence of folate-
289 metabolizing enzymes in the placenta. Solanky *et al*. reported that MS and MTHFR mRNAs were
290 highly expressed in the human placenta in both first trimester and term, at equivalent levels [6]. In
291 contrast, the expression of CBS mRNA was lower than that in the liver during gestation. They
292 concluded that the remethylation of homocysteine from 5-methyltetrahydrofolate might be the
293 underlying pathway. Shin *et al*., based on their immunohistochemistry analysis, reported the
294 expression of MS in the cytoplasm of villous syncytiotrophoblasts and MTHFR in extravillous
295 trophoblasts in human placenta [7]. Seremak-Mrozikiewicz *et al*. showed the mRNA expression of

296 MTHFR, MS, MSR, and CSE in human placenta [8]. Recently, Mohanraj *et al*. reported the mRNA
297 expression of CSE, MS, MSR, and MTHFR in human placental villous tissues during gestation [9].
298 They reported that the expression of MTHFR and CBS decreased, whereas that of MS increased with
299 gestation. In the mouse placenta, MTHFR and MS are expressed in labyrinth trophoblast cells [10].
300 In the present study, the DHFR (*Dhfr*) and MTHFR (*Mthfr*) mRNAs were detected in the rat
301 placenta, suggesting that the placenta can convert folate to its active form, 5-methytetrahydrofolate.
302 Furthermore, the expression of CSE (*Cth*), MS (*Mtr*), and MSR (*Mtrr*) indicates homocysteine
303 metabolism in the placenta. Considering the expression patterns of MSR and CSE during gestation,
304 the remethylation of homocysteine from 5-methyltetrahydrofolate might be an important pathway at
305 GD13, whereas conversion from homocysteine to cysteine might be more important at GD20.
306 Although both MTHFR and DHFR are involved in reduced reactions to generate the activated form
307 of folate, the expression patterns during gestation were reversed. MTHFR plays a central role in
308 folate metabolism as it regulates the availability of 5,10-methyltetrahydrofolate in cells [5]. The
309 expression pattern of MTHFR during gestation was similar to that of FRα and PCFT. FRα has a
310 higher affinity for the oxidized form of folate than for the reduced for [34]. PCFT transports both the
311 oxidized and reduced forms of folate. Conversely, RFC transports the reduced form of folate into
312 cells. These results indicate that these carriers and MTHFR may contribute to the utilization of
313 oxidized forms of folate at late gestation to meet the demand for folate. However, the expression
314 patterns in the rat placenta shown in the present study were not fully consistent with those in

315 previous reports in the human placenta [9]. The possibility of species differences between human and
316 rat placentas should be considered when interpreting the results. In the present study, we found that
317 the expression of MSR and DHFR on GD13 was higher than that on GD20, whereas that of MTHFR
318 on GD20 was higher than that on GD13. In contrast, CSE expression tended to increase and MS
319 expression did not change with the gestational stage. "Repeated administration of VPA decreased the
320 mRNA expression of MS in the GD20 placenta; MS catalyzes 5-methyltetrahydrofolate, which
321 donates the methyl group, during the remethylation of homocysteine to methionine, indicating that
322 homocysteine levels in the placenta may be altered by VPA in the late gestational stage. VPA has
323 been reported to reduce serum folate levels and elevate homocysteine levels [19]. Disruption of
324 maternal folate and homocysteine may be involved in the alteration of folate metabolism-related
325 genes. Elevated homocysteine is known to be involved in the formation of free radicals, leading to
326 increased oxidative stress [36]. Higher oxidative stress is associated with increased apoptotic markers
327 in the placenta [36]. Future studies are required to investigate MS function and homocysteine levels
328 in serum and placenta after administration of VPA and their effects on the fetus. In addition, repeated
329 administrations of VPA increased the DHFR mRNA level in GD13 placenta, suggesting that VPA
330 increased the ability of conversion to tetrahydrofolate from dihydrofolate in the early gestational
331 stage. DHFR expression at GD13 was high in the rat placenta, and VPA is generally known to reduce
332 the serum folate level [19]. These results suggest that the function of DHFR at an earlier gestational
333 period plays a role in folate requirement, and the elevated DHFR in the placenta after VPA

administration is probably the result of a compensatory mechanism of folate deficiency. In a rat model, exposure to VPA at embryonic stage E12 has been reported to alter prenatal behavior (autism model), whereas exposure at E9.5 induces the highest teratogenic effect [30]. Because exposure during the early gestational period is critical for the onset of toxic effects of VPA and folates are involved in one-carbon metabolism and cell division, disruption of folate metabolism-related genes may be associated with the risks of VPA. However, in this study, we did not investigate the serum folate levels after VPA administration. In addition, we did not reveal changes at the protein levels. It is essential to evaluate the protein levels to precisely justify their function. Future studies are required to better understand the alteration of DHFR by VPA at GD13.

In the present study, we determined the mRNA levels of class I and IIa HDACs in the rat placenta. The mRNA expression of several isoforms showed differences with the gestational stage; the HDAC2 and HDAC8 mRNA levels on GD13 were higher than those on GD20, whereas the HDAC5 mRNA level on GD13 was lower that on GD20. Furthermore, acetyl-histone H3 expression was increased after VPA administration (Figure 1), suggesting that VPA has inhibitory effects on HDACs in the placenta. However, information on the regulation of folate carriers and metabolizing enzymes by HDACs is still not available. Future studies should assess detailed regulation mechanisms, using isoform-specific inhibitors and gene knockdown.

Although VPA administration during pregnancy has risks to the fetus, such as malformations, cognitive defects, and autism spectrum disorders [16–18], the mechanisms underlying the adverse

353 effects of VPA have not been fully elucidated. As mentioned in section 1, Introduction, VPA reduces
354 the serum folate level and elevates homocysteine level [19]. The supplementation of folic acid to the
355 mother in the periconceptional period prevents neural tube defects in the fetus [1], and its
356 supplementation is also recommended for women on antiepileptic drugs [37]. Furthermore, there are
357 reports that folic acid supplementation reduces the risks of cognitive defects and autism in children
358 exposed to antiepileptic drugs *in utero* [38,39]. However, in the present study, we did not directly
359 evaluate the folate dynamics in the placenta and relationships between the changes in genes and
360 adverse effects of VPA. The risks of VPA administration, such as malformations and reduced
361 cognitive abilities, in the fetus are dose dependent [16, 40]. In the present study, we investigated the
362 effects of VPA on FRα (*Folr1*) at different doses. Placental expression of FRα after repeated
363 administration of VPA (200, 400, and 600 mg/kg) was assessed (Supplemental Figure 2). Although
364 we investigated only three dosage selections, the effects of VPA on FRα mRNA tended to be dose
365 dependent. In our preliminary study, repeated administration of a dose of 800 mg/kg led to maternal
366 oversedation and fetal death. The results suggest that the reducing effects of VPA on FRα mRNA in
367 rat placenta were approximately 40% at a maximum. One limitation of the present study is that we
368 did not analyze the folate transport function in the placenta after administration of VPA. FRα is a
369 high-affinity folate carrier that transports folate at a neutral to mildly acidic pH. Because FRα is
370 highly expressed in the rat placenta, its alteration can change folate transport to the fetus. However,
371 as described above, the placenta expresses several folate carriers, such as RFC and PCFT, in addition

to FRα. Therefore, it is essential to evaluate the transport function to precisely justify the effects of VPA. Future studies should investigate whether VPA changes the transport function of folates in the rat placenta by analyzing folate levels in the placenta and fetus or analyzing the placental and fetal profiles after injection of isotope-labeled folic acid.

5. Conclusions

In this study, we comprehensively evaluated the effects of VPA on the gene expression of folate carriers and metabolizing enzymes in the rat placenta. Repeated VPA administrations reduced the expression of FRα in GD20 placenta. As for folate metabolism-related genes, repeated VPA administrations reduced the expression of MS on GD20, but increased the expression of DHFR on GD13 compared with the control. These results suggest that VPA may alter folate uptake and metabolism in the placenta, and thereby alter folate and homocysteine levels in the placenta. Future research on folate dynamics in the placenta and gene regulation mechanisms are required to better understand the adverse effects of VPA on the fetus and to develop strategies to reduce the associated risks.

Authorship contributions

Participated in research design: Ayako Furugen, Yuko Kurosawa, Naoko Jinno, Masaki Kobayashi.

Performed the experiments: Ayako Furugen, Yuki Kanno, Nanami Ohyama, Yuko Kurosawa, Naoko Jinno.

Analyzed the data: Ayako Furugen, Yuki Kanno, Nanami Ohyama, Yuko Kurosawa, Naoko Jinno.

Contributed to the writing of the manuscript: Ayako Furugen, Katsuya Narumi, Ken Iseki, Masaki Kobayashi. All authors read and approved the final manuscript.

Acknowledgment

Funding

This work was supported by the OTC Self-Medication Promotion Foundation (grant number, 31-1A-010) (provided to A.F.).

Conflicts of Interest

The authors declare no conflicts of interest.

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576 **Figure captions**

577 **Figure 1** Acetyl-histone H3 (Lys9/Lys14) level in the placenta of pregnant rats treated with
578 valproate (VPA). (A) Pregnant rats on GD19 were orally administered VPA (400 mg/kg) or water
579 (control). (B) Pregnant rats on GD16–GD19 were orally administered VPA (400 mg/kg) or water
580 (control) for 4 successive days. The placenta samples were collected from the rats (on GD20) 24 h
581 after the last administration of VPA/water. Total proteins were assessed by western blotting. Actin
582 was used as the loading control. Each column represents the mean with S.D. (n = 3 dams).

583

584 **Figure 2** Effect of valproate (VPA) on the expression of folate carriers in the placenta of rats. (A)
585 Pregnant rats on GD12 and GD19 were orally administered VPA (400 mg/kg) or water (control). (B)
586 Pregnant rats on GD9–GD12 and GD16–GD19 were orally administered VPA (400 mg/kg) or water
587 (control) for 4 successive days. The placenta samples were collected from the rats (GD13 and GD20)
588 24 h after the last administration of VPA/water. Gene expression of FRα (*Folr1*), RFC (*Slc19a1*),
589 and PCFT (*Slc46a1*) was analyzed by real-time PCR. Each column represents the mean with S.D. of
590 three dams. **: significantly different from the control at $p < 0.01$, respectively. †, ††: significantly
591 different between the GD13 and GD20 controls at $p < 0.05$ and $p < 0.01$, respectively.

592

593 **Figure 3** Effect of valproate (VPA) on folate metabolism-related genes in the placenta of rats. (A)
594 Pregnant rats on GD12 and GD19 were orally administered VPA (400 mg/kg) or water (control). (B)

595 Pregnant rats on GD9–GD12 and GD16-19 were orally administered VPA (400 mg/kg) or water
596 (control) for 4 successive days. The placenta samples were collected from the rats (GD13 and GD20)
597 24 h after the last administration of VPA/water. Gene expression of CSE (*Cth*), MSR (*Mtrr*), MS
598 (*Mtr*), MTHFR (*Mthfr*), and DHFR (*Dhfr*) was assessed by real-time PCR. Each column represents
599 the mean with S.D. of three dams. *, **: significantly different from the control at $p < 0.05$ and $p <$
600 0.01, respectively. ††; significantly different between the GD13 and GD20 controls at $p < 0.01$.
601
602 **Figure 4** Effect of valproate (VPA) on the mRNA expression of HDACs in the placenta of rats. (A)
603 Pregnant rats on GD12 and GD19 were orally administered VPA (400 mg/kg) or water (control). (B)
604 Pregnant rats on GD9–GD12 and GD16–GD19 were orally administered VPA (400 mg/kg) or water
605 (control) for 4 successive days. The placenta samples were collected from the rats (GD13 and GD20)
606 24 h after the last administration of VPA/water. Gene expression of class I (HDAC1, HDAC2,
607 HDAC3, HDAC8/*Hdac1*, *Hdac2*, *Hdac3*, *Hdac8*) and class IIa (HDAC4, HDAC5, HDAC7,
608 HDAC9/*Hdac4*, *Hdac5*, *Hdac7*, *Hdac9*) HDACs was assessed by real-time PCR. Each column
609 represents the mean with S.D. of three dams. *, significantly different from the control at $p < 0.05$. †,
610 ††; significantly different between the GD13 and GD20 controls at $p < 0.05$ and $p < 0.01$,
611 respectively.
612

Figure 5 Effect of repeated administrations of valproate (VPA) on FRα expression in placenta f rats on GD20. Pregnant rats on GD16–GD19 were orally administered VPA (400 mg/kg) or water (control) for 4 successive days. The placenta samples were collected from the rats 24 h after the last administration of VPA/water. Total proteins were assessed by western blotting. Actin was used as the loading control. Each column represents the mean with S.D. of three dams. *: significantly different from the control at $p < 0.05$.

Supplemental Figure 1. Effect of valproate (VPA) on the expression of UGT1A6 in the rat liver. Pregnant rats on GD16–GD19 were orally administered VPA (400 mg/kg) or water (control) for 4 successive days. Liver samples were collected from GD20 rats 24 h after the last administration of VPA/water. The expression of UGT1A6 (*Ugt1a6*) was analyzed by real-time PCR. Each column represents the mean ± S.D. of five to six dams. *: significantly different from the control at $p < 0.05$.

Supplemental Figure 2. Dose-dependent analysis of the effect of valproate (VPA) on the expression of FRα in the rat placentaPregnant rats on GD16–GD19 were orally administered VPA (200, 400, or 600 mg/kg) or water (control) for 4 successive days. The samples were collected from GD20 rats 24 h after the last administration of VPA/water. Expression of FRα (*Folr1*) was analyzed by real-time PCR. Each column represents the mean ± S.D. of three to six dams. Dunnett's test was used for statistical analysis. **: significantly different from the control at $p < 0.01$.

Supplemental Table 1 Primer sequences for real-time PCR.

Name		Primer sequence	Product size (bp)
FRα (*Folr1*)	Forward	5'-GCCCAGAGGACAAGTTACA-3'	116
	Reverse	5'-CCAGTTGAATCGGTACAG-3'	
RFC (*Slc19a1*)	Forward	5'-CATGCTAAGCGAACTGGTGA-3'	122
	Reverse	5'-TTTTCCACAGGACATGGACA-3'	
PCFT (*Slc46a1*)	Forward	5'-CCTTCTGGGAGATTTCAACG-3'	184
	Reverse	5'-CCAGAAAGGGTTGGCATAAC-3'	
CSE (*Cth*)	Forward	5'-CAGTGATGTTGTCATGGGCTTAGTG-3'	148
	Reverse	5'-CATCCGGATCTGCAGTGTCTTC-3'	
MSR (*Mtrr*)	Forward	5'-CAAAGTATGTGCAAGACAACCTCCA-3'	138
	Reverse	5'-TGATTTCTACAAGGGCGTCGTG-3'	
MS (*Mtr*)	Forward	5'-ACTTGCGCAAACTCCGCTATG-3'	140
	Reverse	5'-TGCCAAGGATTCTGTCAACCTG-3'	
MTHFR (*Mthfr*)	Forward	5'-TATGCCACAGACCTGGTGAA-3'	117
	Reverse	5'-CTTCAGGTCATCCTCGAAGC-3'	
DHFR (*Dhfr*)	Forward	5'-ACCAGGAAGCCATGAATCAG-3'	228
	Reverse	5'-AGCAGTAGGACTTGGGAGCA-3'	
HDAC1 (*Hdac1*)	Forward	5'-TCTGACAAACGCATTGCCTG-3'	258
	Reverse	5'-AGGGACTTGGAGAGAAGATGGA-3'	
HDAC2 (*Hdac2*)	Forward	5'-AATCCAAGGACAATAGTGGTGAG-3'	147
	Reverse	5'-ACTTCCTCAAACAGCGAAGG-3'	
HDAC3 (*Hdac3*)	Forward	5'-CCAGATTTCACGCTCCATC-3'	126
	Reverse	5'-GACACTGGGTGCATGGTTC-3'	
HDAC8 (*Hdac8*)	Forward	5'-ATCGAATCCAGCAAATCCTC-3'	143
	Reverse	5'-TCACAAATCCCACAAACTGC-3'	
HDAC4 (*Hdac4*)	Forward	5'-GGGCACTCTCTGATTGAGG-3'	149
	Reverse	5'-AGCTTCGGCTACAGTGGTG-3'	
HDAC5 (*Hdac5*)	Forward	5'-GCCACACTAGAGAAAGTCATCG-3'	126
	Reverse	5'-CACAGTCTCGGCCTCCTC-3'	
HDAC7 (*Hdac7*)	Forward	5'-GAGCTGATGCAGAAGTGGAG-3'	118
	Reverse	5'-CCCTAGAGGTTCATGGGTTC-3'	
HDAC9 (*Hdac9*)	Forward	5'-TCTGAACATCACTCACTACT-3'	156
	Reverse	5'-GTGCAGCTCATTCCAAA-3'	
UGT1A6 (*Ugt1a6*)	Forward	5'-ACTCAAAGTATGAGATCCTTGC-3'	190
	Reverse	5'-TCAAATTCCTGAGACAGGTTC-3'	
β-Actin (*Actb*)	Forward	5'-CTATCGGCAATGAGCGGTTC-3'	134
	Reverse	5'-GAGGTCTTTACGGATGTCAACG-3'	

636

Figure

Figure 1

Click here to access/download;Figure;Figure (revise).pptx

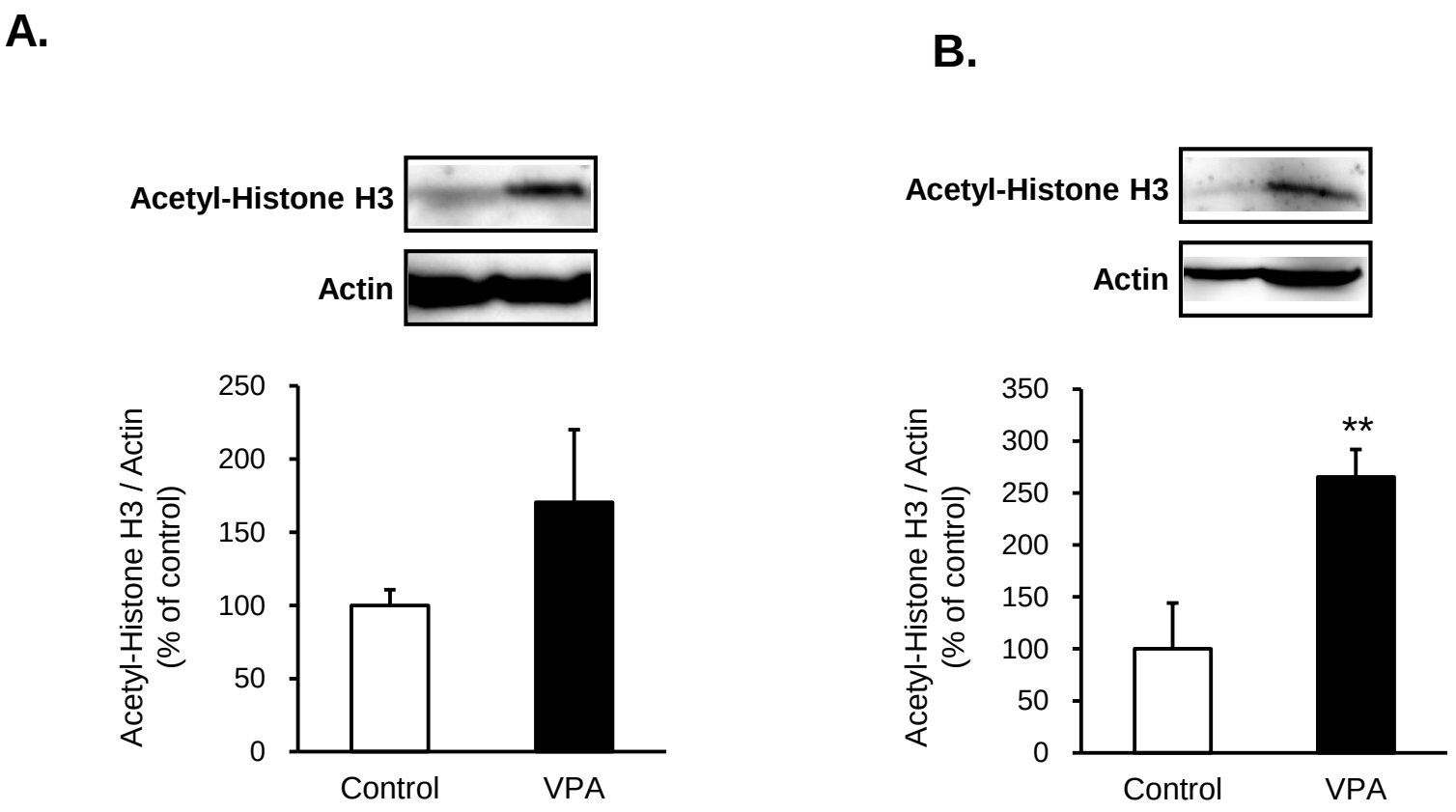
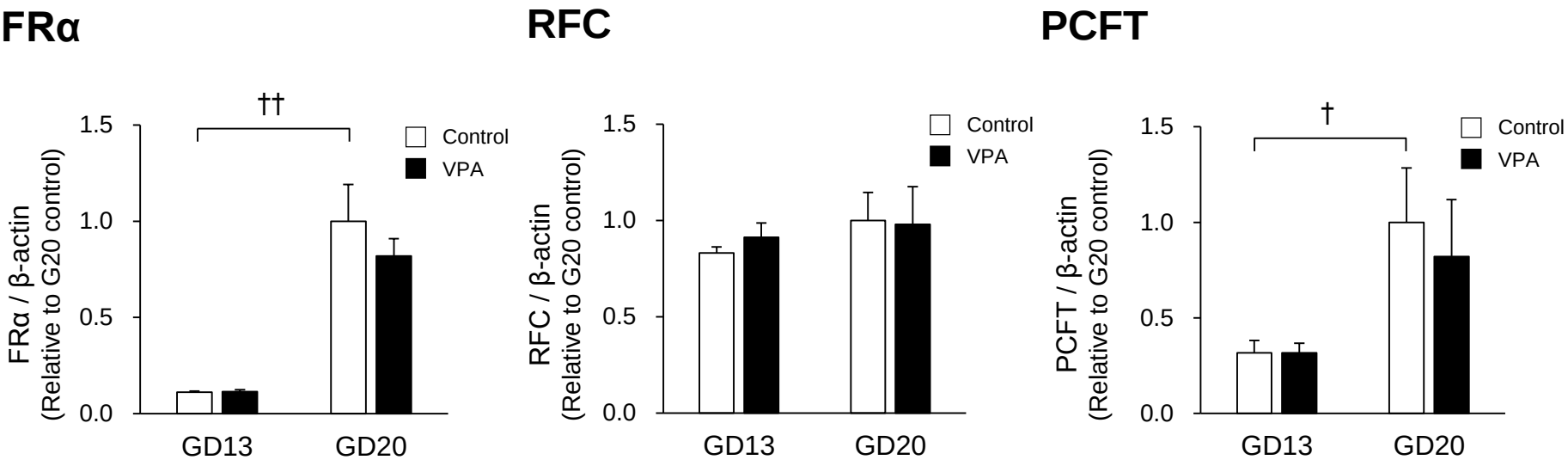


Figure 2

A.


FRα
RFC
PCFT
††
†
Control
VPA
FRα / β-actin (Relative to G20 control)
RFC / β-actin (Relative to G20 control)
PCFT / β-actin (Relative to G20 control)
1.5
1.0
0.5
0.0
GD13
GD20

B.

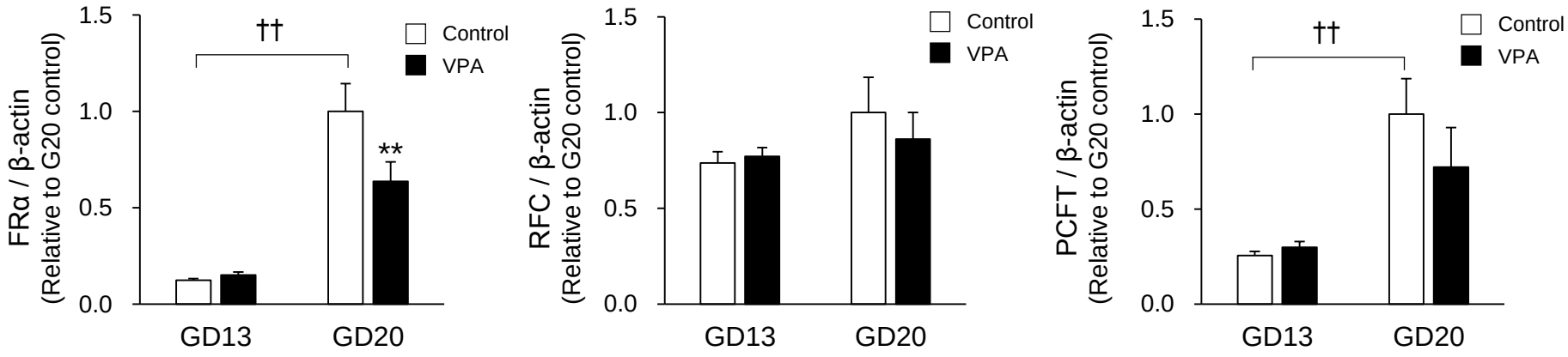
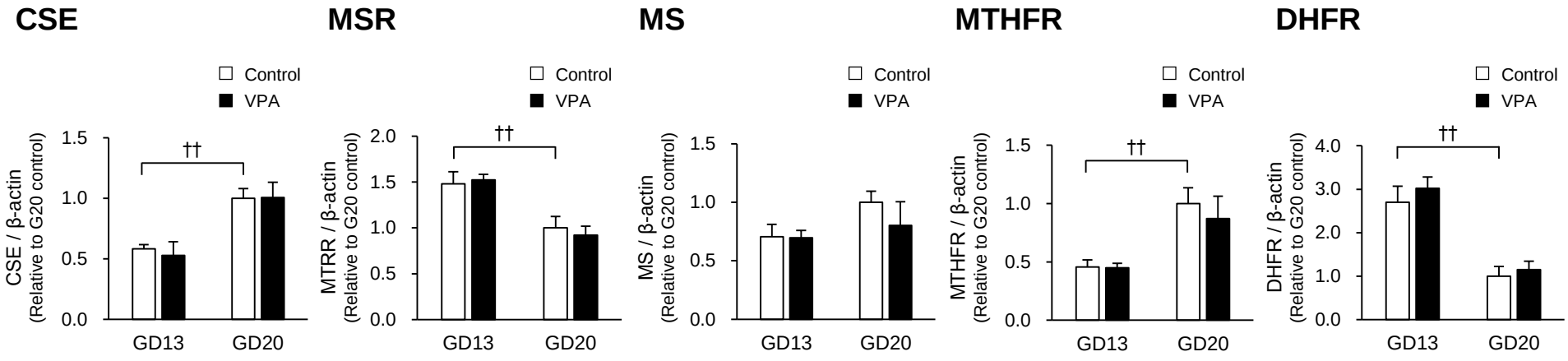

FRα
RFC
PCFT
††
**
Control
VPA
FRα / β-actin (Relative to G20 control)
RFC / β-actin (Relative to G20 control)
PCFT / β-actin (Relative to G20 control)
1.5
1.0
0.5
0.0
GD13
GD20

Figure 3

A.


CSE
MSR
MS
MTHFR
DHFR
Control
VPA
CSE / β-actin (Relative to G20 control)
MTRR / β-actin (Relative to G20 control)
MS / β-actin (Relative to G20 control)
MTHFR / β-actin (Relative to G20 control)
DHFR / β-actin (Relative to G20 control)
††
GD13
GD20

B.

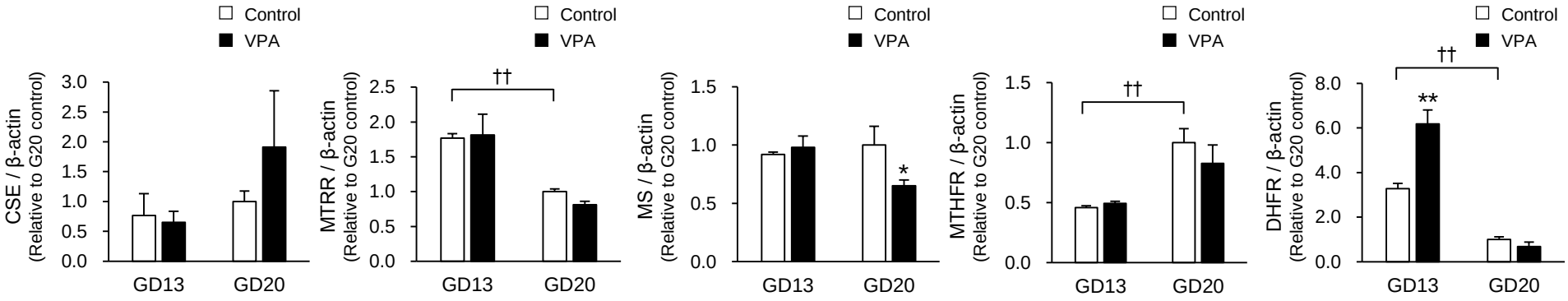

CSE
MSR
MS
MTHFR
DHFR
Control
VPA
CSE / β-actin (Relative to G20 control)
MTRR / β-actin (Relative to G20 control)
MS / β-actin (Relative to G20 control)
MTHFR / β-actin (Relative to G20 control)
DHFR / β-actin (Relative to G20 control)
††
*
**
GD13
GD20

Figure 4

A.

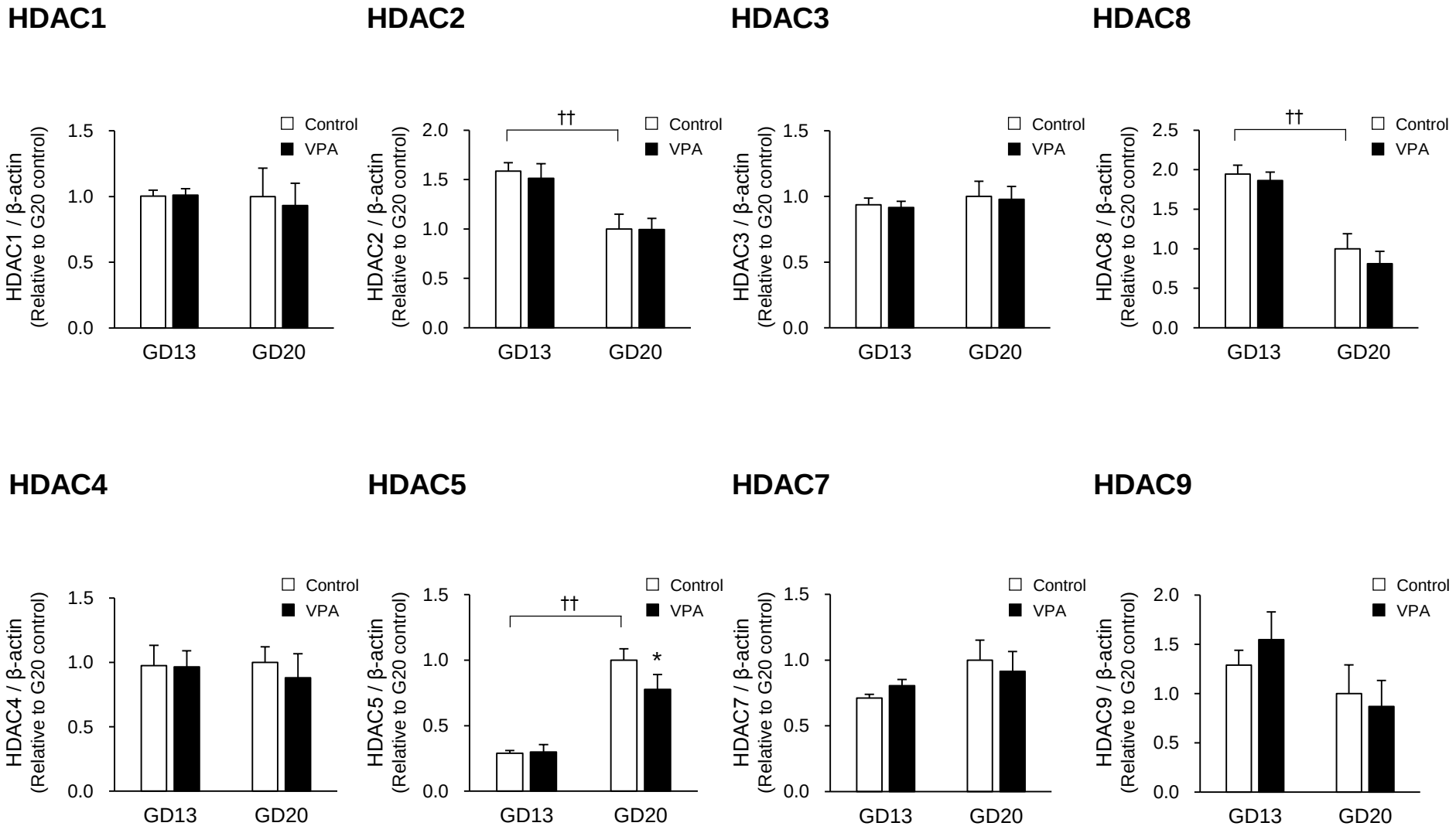

HDAC1
HDAC2
HDAC3
HDAC8
HDAC4
HDAC5
HDAC7
HDAC9
HDAC1 / β-actin (Relative to G20 control)
HDAC2 / β-actin (Relative to G20 control)
HDAC3 / β-actin (Relative to G20 control)
HDAC8 / β-actin (Relative to G20 control)
HDAC4 / β-actin (Relative to G20 control)
HDAC5 / β-actin (Relative to G20 control)
HDAC7 / β-actin (Relative to G20 control)
HDAC9 / β-actin (Relative to G20 control)
Control
VPA
GD13
GD20
††
*

Figure 4

B.

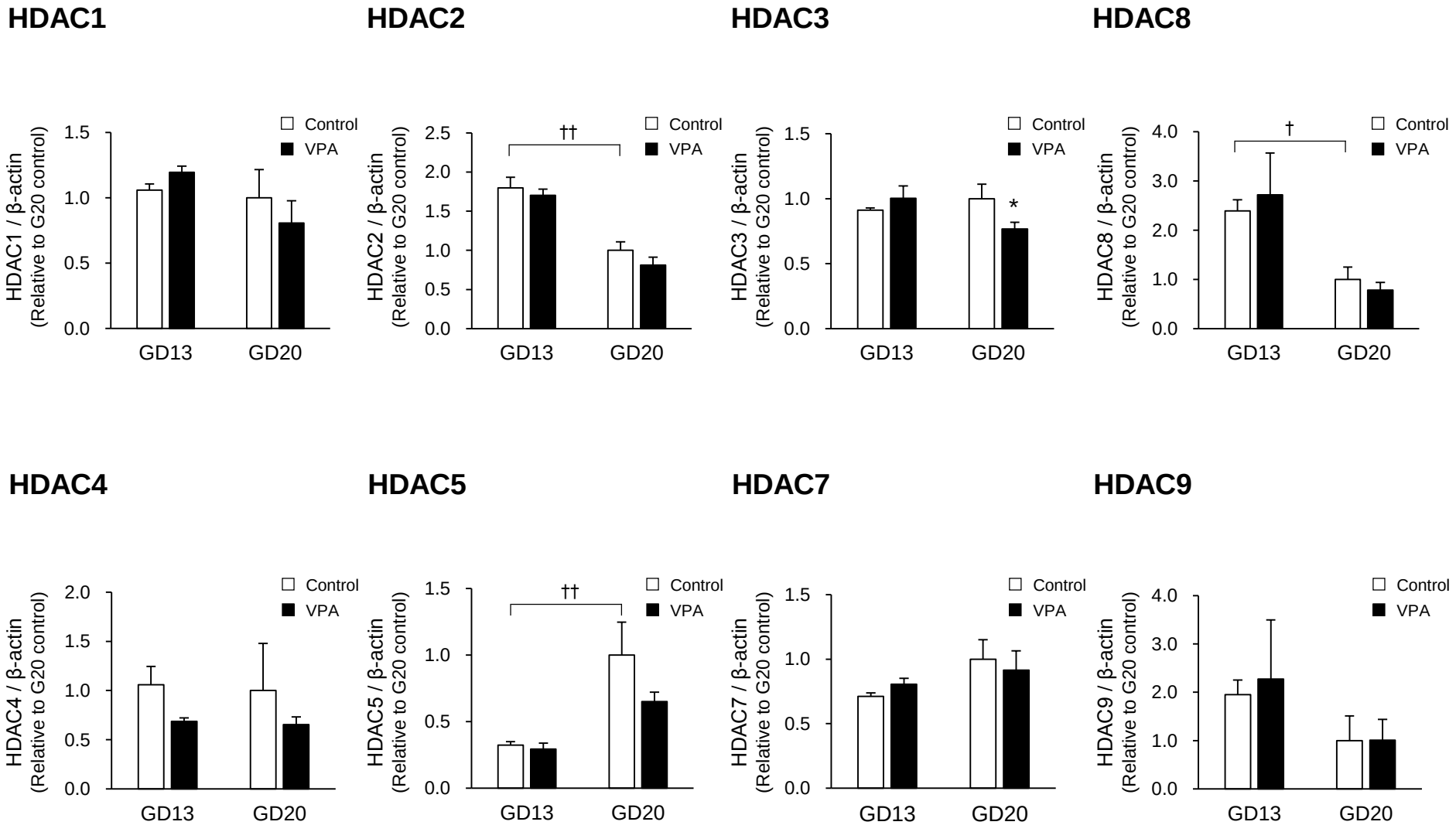
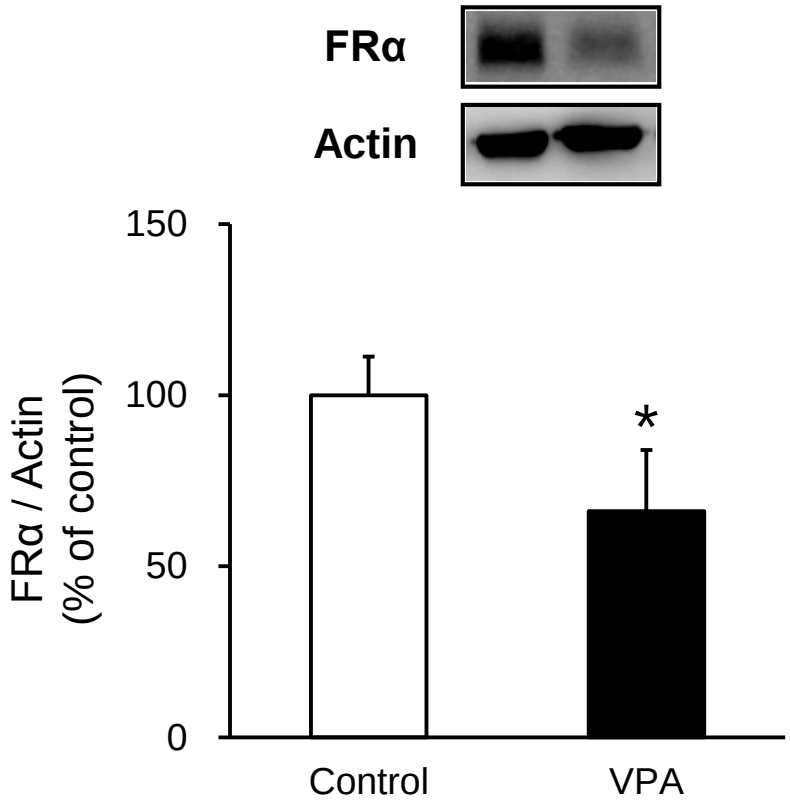
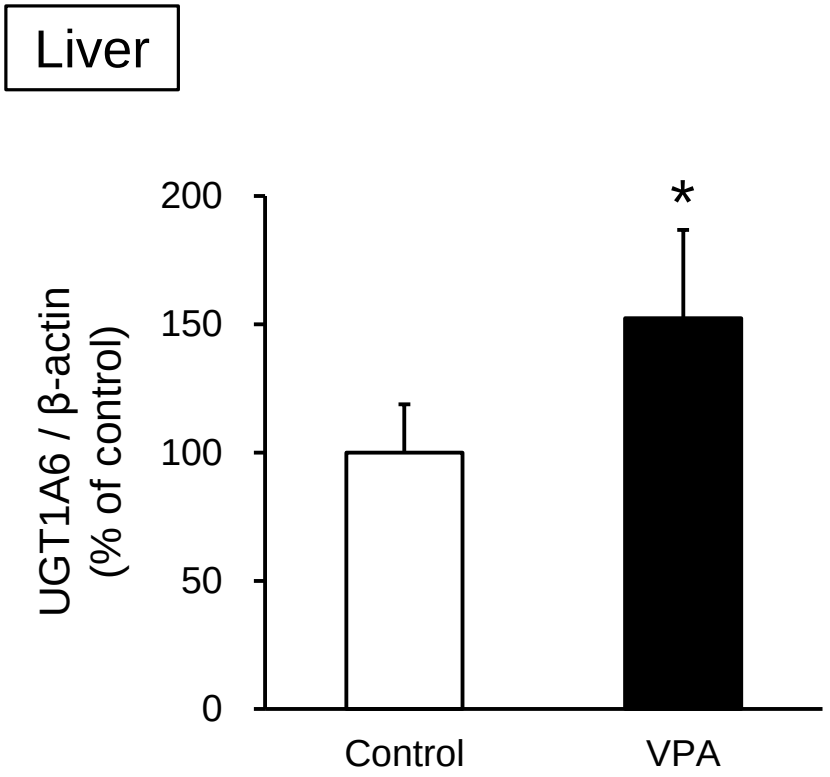

HDAC1
HDAC2
HDAC3
HDAC8
HDAC4
HDAC5
HDAC7
HDAC9
HDAC1 / β-actin (Relative to G20 control)
HDAC2 / β-actin (Relative to G20 control)
HDAC3 / β-actin (Relative to G20 control)
HDAC8 / β-actin (Relative to G20 control)
HDAC4 / β-actin (Relative to G20 control)
HDAC5 / β-actin (Relative to G20 control)
HDAC7 / β-actin (Relative to G20 control)
HDAC9 / β-actin (Relative to G20 control)
Control
VPA
GD13
GD20
††
†
*

Figure 5



Supplemental Figure 1



Supplemental Figure 2