



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

3

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20 **Abbreviations**

21 CBE, cystathionine b-synthase; CSE, cystathionine gamma-lyase; DHFR, dihydrofolate reductase;

22 FR α , folate receptor alpha; GD, gestational day; HDAC, histone deacetylase; MS, methionine

23 synthase; MSR, methionine synthase reductase; MTHFR, methylenetetrahydrofolate reductase;

24 PCFT, proton-coupled folate transporter; RFC, reduced folate carrier; VPA, valproate

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38 **Abstract**

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

53

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

55 **1. Introduction**

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

62 The placenta is a crucial organ for normal fetal development; it has various functions, such as
63 substance exchange, gas exchange, and hormone secretion. Folate carriers such as folate receptor- α
64 ($FR\alpha/FOLR1$), reduced folate carrier ($RFC/SLC19A1$), and proton-coupled folate transporter ($PCFT/$
65 $SLC46A1$) are expressed in the placenta and contribute to the transport of folates [5]. Folates are
66 involved in one-carbon metabolism and are important for the DNA methylation cycle and cell
67 division. Besides folate carriers, studies have indicated the presence of folate metabolic enzymes in
68 the placenta of humans [6-9] and rodents [10]. Dihydrofolate reductase ($DHFR/DHFR$) is an enzyme
69 that converts dihydrofolate to tetrahydrofolate. Metylenetetrahydrofolate reductase
70 ($MTHFR/MTHFR$) converts 5,10-metylenetrahydrofolate to 5-methyltetrahydrofolate. 5-
71 Methyltetrahydrofolate provides the methyl group for the remethylation of homocysteine to
72 methionine, and the process is catalyzed by methionine synthase (MS/MTR). Methionine synthase

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

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

82 Valproate (VPA), a widely prescribed antiepileptic drug, is used to treat bipolar disorder.
83 However, caution should be exerted when administering VPA to women with epilepsy at
84 childbearing age. Exposure to VPA during the periconception period increases fetal malformation
85 risk [16]. In addition, VPA decreases the IQ score and increases neurodevelopmental disorder risk in
86 children [17,18]. Mechanisms underlying the toxicological effects of VPA in the reproductive tissues
87 have not been elucidated. VPA reduces the serum folate level and elevates the homocysteine level
88 [19], and the disruption of folate level has been hypothesized as one of the action mechanisms of
89 VPA [20]. VPA can affect the mitochondria by interfering with mitochondrial pathways, functions,
90 or structures [21]. Recently, VPA has garnered attention as an inhibitor of histone deacetylases
91 (HDACs). The HDAC isoforms in mammals are classified into four classes based on their structure:

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

95 Studies have investigated the influence of VPA on the functions and expression of folate
96 carriers in various models, including trophoblastic cell lines, cell culture models, and *ex vivo* placenta
97 [23–25]. As each model has advantages and limitations, it is necessary to comprehensively employ
98 different models to better understand the effects of VPA on the fetus. Because the expression of
99 placental genes changes throughout gestation, evaluation at each gestational stage is important.
100 Furthermore, gestational changes in several genes, such as those encoding folate-metabolizing
101 enzymes and HDACs in the rat placenta, have not been fully characterized. We previously reported
102 that VPA alters the expression of several transporters in the rat placenta and that the sensitivity to
103 VPA differs among gestational stages [26]. In this study, we used pregnant rats as *an in vivo* animal
104 model to investigate the effects of VPA administration on the expression of folate carriers, folate
105 metabolism-related genes, and HDACs in the placenta.

106

107 **2. Material and Methods**

108 **2.1. Chemicals**

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

111

112 **2.2. Animals, drug administration, and tissue collection**

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

128

129 **2.4. Real-time polymerase chain reaction**

130 Real-time polymerase chain reaction (PCR) was conducted using the KAPA SYBR[®] Fast
131 qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) as described previously [26], and the primers
132 used are shown in Supplemental Table 1. Folate carrier genes were amplified through 40 PCR cycles
133 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
134 Mx3000[™] real-time PCR system (StrataGene) or 40 PCR cycles at 95°C for 10 s, 55°C for 20 s, and
135 72°C for 1 s using the LightCycler[®] 480 System II (Roche, Basel, Switzerland). *Hdac9* was
136 amplified through 50 PCR cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 1 s using the
137 LightCycler[®] 480 System II. Other targets were amplified through 40 PCR cycles at 95°C for 10 s,
138 55°C (*Ugt1a6*, *Mtr*, *Mthfr*, *Dhfr*, *Hdac1-Hdac5*, and *Hdac7-Hdac8*) or 60°C (*Cse* and *Mtrr*) for 20 s,
139 and 72°C for 1 s using the LightCycler[®] 480 System II. β -Actin (*Actb*) was used as the housekeeping
140 gene for the normalization of target gene expression. The reference gene was not changed by
141 gestational age or VPA administration [26]. Three to four placentas per dam were used in the real-
142 time PCR analysis. The placenta samples of each litter were individually analyzed, and the results
143 were averaged for each dam.

144

145 **2.6. Western blotting**

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

149 inhibitor cocktail tablets (Millipore Sigma, Burlington, MA) and 1 mM phenylmethylsulfonyl
150 fluoride. The sample was subjected to SDS-PAGE (12.5% acrylamide gel for FR α and 15%
151 acrylamide gel for acetyl-histone H3). Ten micrograms of protein was loaded per well to detect FR α ;
152 100 μ g of protein was loaded per well to detect acetyl-Histone H3 (Lys9/Lys14). The primary
153 antibodies used were rabbit anti-FR α monoclonal antibody (ab221543; Abcam, Cambridge, UK),
154 rabbit anti-acetyl-Histone H3 (Lys9/Lys14) polyclonal antibody (#9677; Cell Signaling Technology,
155 Beverly, MA), and mouse anti-actin monoclonal antibody (#517310; Merck Millipore, Burlington,
156 MA). Two to four placentas per dam were used for western blotting. The placenta samples of each
157 litter were individually analyzed, and the results were averaged for each dam.

158

159 **2.7. Statistical analyses**

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

164

165 **3. Results**

166 **3.1. Acetyl-histone H3 (Lys9/Lys14) expression in the placenta after VPA administration**

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

174

175 **3.2. Effect of VPA on the expression of folate carrier genes in the placenta**

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

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

187

188 **3.3. Effect of VPA on the expression of folate metabolism-related genes in the placenta**

189 We analyzed the effect of VPA on the expression of folate metabolism-related genes, namely,
190 CSE (*Cth*), MSR (*Mtrr*), MS (*Mtr*), MTHFR (*Mthfr*), and DHFR (*Dhfr*) in the placentas. There was
191 no significant change in the expression of these genes after single VPA administration at both
192 gestational stages (Figure 3A). Repeated administrations of VPA decreased the gene expression of
193 MS on GD20 by 35% (Figure 3B) compared with the control. The DHFR mRNA level increased to
194 188% compared with that of the control on GD13. Although the CSE mRNA expression tended to
195 increase to 191% compared with that of the control on GD20, the increase was not statistically
196 significant. With respect to the changes associated with gestational stage, the gene expression of
197 MSR and DHFR on GD13 was higher than that on GD20 (1.5–1.8- and 2.7–3.3-fold, respectively),
198 whereas the gene expression of MTHFR on GD20 was approximately 2-fold higher than that on
199 GD13. CSE gene expression tended to increase and MS gene expression did not change with the
200 gestational stage.

201

202 **3.4. mRNA expression of class I and IIa HDACs in the placenta**

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

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

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

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

222

223 **3.5. Changes in the placental level of FR α on GD20 after repeated administrations of VPA**

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

228

229 **4. Discussion**

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

237 In this study, we selected the dose to observe rat fetal effects without leading to maternal and
238 fetal death after prolong administration of VPA, based on the findings of a previous study [29].

239 Furthermore, previous studies that investigated the effects of prenatal VPA exposure on rat pups

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

248 It has been reported that FR α , RFC, and PCFT contribute to the influx of folate in the
249 placenta [5]. In the human placenta, FR α and PCFT are localized to the microvillous plasma
250 membrane, and RFC exists in both apical and basal membranes [6]. In the present study, the
251 expression of FR α (*Folr1*) and PCFT (*Slc46a1*) was considerably increased during gestation,
252 whereas the expression of RFC (*Slc19a1*) did not show a significant change (Figure 2). In a previous
253 study, the expression of FR α and PCFT was considerably increased at GD20 compared with that
254 GD14 (FR α : 9-fold; PCFT: 6-fold), whereas the increase in the level of RFC was still at twofold
255 [32]. These results suggest that the increase in the expression of rat FR α and PCFT during gestation
256 was more drastic than that of RFC. The accumulation of [³H]-folic acid after intravenous injection
257 increased with the progress of gestation in the rat placenta and fetus [32]. These results suggest that

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

260 Repeated administrations of VPA tended to decrease the levels of FR α and PCFT mRNA in
261 GD20 placenta, whereas in the level of RFC mRNA did not show significant changes between the
262 two gestation stages (Figure 2). Furthermore, the FR α level was significantly decreased, consistent
263 with its mRNA level (Figure 5). FR α is a high-affinity folate carrier that transports folate at a neutral
264 to mildly acidic pH. Because FR α is highly expressed in the rat placenta, this alteration may decrease
265 folate transport to the fetus. Fetal dysgenesis has been reported in mice fed a folic acid-deficient diet
266 [33]. Furthermore, we previously reported that rat placental weight decreased after repeated
267 administration of VPA [26]. These results indicate that the reduction of rat FR α expression after
268 VPA administration may be associated with placental and fetal growth. Studies have investigated the
269 effects of VPA on the expression and function of folate carriers in various models [23-25]. Fathe *et*
270 *al.* showed that VPA at high concentration can inhibit folate receptors such as FR α in HEK293T
271 cells [24]. In contrast, we previously reported that VPA at clinical concentrations did not inhibit the
272 uptake of ³H-folic acid in human placental choriocarcinoma cell lines [23]. However, 24-h treatment
273 of the cells with VPA induced the mRNA expression of FR α and PCFT. An *ex vivo* study reported
274 that VPA exposure for 180 min significantly reduced the folate concentration by 25%–35% and
275 altered the mRNA level of FR α (downregulation) and RFC (upregulation) (perfusion of term human
276 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 FR α 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-methyltetrahydrofolate.
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 FR α and PCFT. FR α has a
310 higher affinity for the oxidized form of folate than for the reduced form [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

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

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

351 Although VPA administration during pregnancy has risks to the fetus, such as malformations,
352 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 periconceptual 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 FR α /*Folr1* at different doses. Placental expression of FR α 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 FR α 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 FR α 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. FR α is a
369 high-affinity folate carrier that transports folate at a neutral to mildly acidic pH. Because FR α 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

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

376

377 **5. Conclusions**

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

387

388 **Authorship contributions**

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

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

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

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

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400

401 **Conflicts of Interest**

402 The authors declare no conflicts of interest.

403

404 **References**

405 [1] De-Regil LM, Peña-Rosas JP, Fernández-Gaxiola AC, Rayco-Solon P. Effects and safety of
406 periconceptional oral folate supplementation for preventing birth defects. *Cochrane Database Syst*
407 *Rev.* 2015;(12):CD007950. <https://doi.org/10.1002/14651858.CD007950.pub3>.

408

409 [2] Feng Y, Wang S, Chen R, Tong X, Wu Z, Mo X. Maternal folic acid supplementation and the risk

410 of congenital heart defects in offspring: a meta-analysis of epidemiological observational studies. *Sci*
411 *Rep.* 2015;5:8506. <https://doi.org/10.1038/srep08506>.

412

413 [3] Hua X, Zhang J, Guo Y, Shen M, Gaudet L, Janoudi G, Walker M, Wen SW. Effect of folic acid
414 supplementation during pregnancy on gestational hypertension/preeclampsia: A systematic review
415 and meta-analysis. *Hypertens Pregnancy.* 2016;35:447-460.
416 <https://doi.org/10.1080/10641955.2016.1183673>.

417

418 [4] Zhang Q, Wang Y, Xin X, Zhang Y, Liu D, Peng Z, He Y, Xu J, Ma X. Effect of folic acid supplementation
419 on preterm delivery and small for gestational age births: A systematic review and meta-analysis. *Reprod*
420 *Toxicol.* 2017;67:35-41. <https://doi.org/10.1016/j.reprotox.2016.11.012>.

421

422 [5] Radziejewska A, Chmurzynska A. Folate and choline absorption and uptake: Their role in fetal
423 development. *Biochimie.* 2019;158:10-19. <https://doi.org/10.1016/j.biochi.2018.12.002>.

424

425 [6] Solanky N, Requena Jimenez A, D'Souza SW, Sibley CP, Glazier JD. Expression of folate
426 transporters in human placenta and implications for homocysteine metabolism. *Placenta.*
427 2010;31:134-43. <https://doi.org/10.1016/j.placenta.2009.11.017>.

428

- 429 [7] Shin JA, Kim YJ, Park H, Kim HK, Lee HY. Localization of folate metabolic enzymes,
430 methionine synthase and 5,10-methylenetetrahydrofolate reductase in human placenta. *Gynecol*
431 *Obstet Invest.* 2014;78:259-65. <https://doi.org/10.1159/000364866>.
- 432
- 433 [8] Seremak-Mrozikiewicz A, Bogacz A, Bartkowiak-Wieczorek J, Wolski H, Czerny B, Gorska-
434 Paukszta M, Drews K. The importance of MTHFR, MTR, MTRR and CSE expression levels in
435 Caucasian women with preeclampsia. *Eur J Obstet Gynecol Reprod Biol.* 2015;188:113-7.
436 <https://doi.org/10.1016/j.ejogrb.2015.03.009>.
- 437
- 438 [9] Mohanraj PS, Rahat B, Mahajan A, Bagga R, Kaur J. Temporal expression of genes involved in
439 folate metabolism and transport during placental development, preeclampsia and neural tube defects.
440 *Mol Biol Rep.* 2019;46:3193-3201. <https://doi.org/10.1007/s11033-019-04776-w>.
- 441
- 442 [10] Cherukad J, Wainwright V, Watson ED. Spatial and temporal expression of folate-related
443 transporters and metabolic enzymes during mouse placental development. *Placenta.* 2012;33:440-8.
444 <https://doi.org/j.placenta.2012.02.005>.

445

- 446 [11] Hutson JR, Stade B, Lehotay DC, Collier CP, Kapur BM. Folic acid transport to the human
447 fetus is decreased in pregnancies with chronic alcohol exposure. *PLoS One*. 2012;7:e38057.
448 <https://doi.org/10.1371/journal.pone.0038057>.
- 449
- 450 [12] Keating E, Gonçalves P, Campos I, Costa F, Martel F. Folic acid uptake by the human
451 syncytiotrophoblast: interference by pharmacotherapy, drugs of abuse and pathological conditions.
452 *Reprod Toxicol*. 2009;28:511-20. doi: 10.1016/j.reprotox.2009.07.001.
- 453
- 454 [13] Williams PJ, Mistry HD, Morgan L. Folate transporter expression decreases in the human
455 placenta throughout pregnancy and in pre-eclampsia. *Pregnancy Hypertens*. 2012;2:123-31.
456 <https://doi.org/10.1016/j.preghy.2011.12.001>.
- 457
- 458 [14] Araújo JR, Correia-Branco A, Moreira L, Ramalho C, Martel F, Keating E. Folic acid uptake by
459 the human syncytiotrophoblast is affected by gestational diabetes, hyperleptinemia, and TNF- α .
460 *Pediatr Res*. 2013;73:388-94. <https://doi.org/10.1038/pr.2013.14>.
- 461
- 462 [15] Castaño E, Caviedes L, Hirsch S, Llanos M, Iñiguez G, Ronco AM. Folate Transporters in
463 Placentas from Preterm Newborns and Their Relation to Cord Blood Folate and Vitamin B12 Levels.
464 *PLoS One*. 2017;12:e0170389. <https://doi.org/10.1371/journal.pone.0170389>.

465

466 [16] Tomson T, Battino D, Bonizzoni E, Craig J, Lindhout D, Sabers A, Perucca E, Vajda F; EURAP
467 study group. Dose-dependent risk of malformations with antiepileptic drugs: an analysis of data from
468 the EURAP epilepsy and pregnancy registry. *Lancet Neurol.* 2011;10:609-17. [https://doi.org/](https://doi.org/10.1016/S1474-4422(11)70107-7)
469 10.1016/S1474-4422(11)70107-7.

470

471 [17] Baker GA, Bromley RL, Briggs M, Cheyne CP, Cohen MJ, García-Fiñana M, Gummery A,
472 Kneen R, Loring DW, Mawer G, Meador KJ, Shallcross R, Clayton-Smith J; Liverpool and
473 Manchester Neurodevelopment Group. IQ at 6 years after in utero exposure to antiepileptic drugs: a
474 controlled cohort study. *Neurology.* 2015;84:382-90. [https://doi.org/](https://doi.org/10.1212/WNL.0000000000001182)
475 10.1212/WNL.0000000000001182.

476

477 [18] Christensen J, Grønberg TK, Sørensen MJ, Schendel D, Parner ET, Pedersen LH, Vestergaard
478 M. Prenatal valproate exposure and risk of autism spectrum disorders and childhood autism. *JAMA.*
479 2013;309:1696-703. <https://doi.org/10.1001/jama.2013.2270>.

480

481 [19] Karabiber H, Sonmezgoz E, Ozerol E, Yakinci C, Otlu B, Yologlu S. Effects of valproate and
482 carbamazepine on serum levels of homocysteine, vitamin B12, and folic acid. *Brain Dev.*
483 2003;25:113-5. [https://doi.org/10.1016/s0387-7604\(02\)00163-8](https://doi.org/10.1016/s0387-7604(02)00163-8).

484

485 [20] Reynolds EH, Green R. Valproate and folate: Congenital and developmental risks. *Epilepsy*
486 *Behav.* 2020 Jul;108:107068. <https://doi.org/10.1016/j.yebeh.2020.107068>.

487

488 [21] Silva MF, Aires CC, Luis PB, Ruiten JP, IJlst L, Duran M, Wanders RJ, Tavares de Almeida I.
489 Valproic acid metabolism and its effects on mitochondrial fatty acid oxidation: a review. *J Inherit*
490 *Metab Dis.* 2008;31:205-16. <https://doi.org/10.1007/s10545-008-0841-x>.

491

492 [22] Chuang DM, Leng Y, Marinova Z, Kim HJ, Chiu CT. Multiple roles of HDAC inhibition in
493 neurodegenerative conditions. *Trends Neurosci.* 2009;32:591-601.
494 <https://doi.org/10.1016/j.tins.2009.06.002>.

495

496 [23] Kurosawa Y, Furugen A, Nishimura A, Narumi K, Kobayashi M, Iseki K. Evaluation of the
497 effects of antiepileptic drugs on folic acid uptake by human placental choriocarcinoma cells. *Toxicol*
498 *In Vitro.* 2018;48:104-110. <https://doi.org/10.1016/j.tiv.2017.12.003>.

499

500 [24] Fathe K, Palacios A, Finnell RH. Brief report novel mechanism for valproate-induced
501 teratogenicity. *Birth Defects Res A Clin Mol Teratol.* 2014;100:592-7.
502 <https://doi.org/10.1002/bdra.23277>.

503

504 [25] Rubinchik-Stern M, Shmuel M, Bar J, Kovo M, Eyal S. Adverse placental effects of valproic
505 acid: Studies in perfused human placentas. *Epilepsia*. 2018;59:993-1003.
506 <https://doi.org/10.1111/epi.14078>.

507

508 [26] Jinno N, Furugen A, Kurosawa Y, Kanno Y, Narumi K, Kobayashi M, Iseki K. Effects of single
509 and repetitive valproic acid administration on the gene expression of placental transporters in
510 pregnant rats: An analysis by gestational period. *Reprod Toxicol*. 2020;96:47-56.
511 <https://doi.org/10.1016/j.reprotox.2020.04.077>.

512

513 [27] Duan H, Wang C, Zhou K, Wang T, Li Y, Qiu D, Li Q, Zhang Y, Hua Y. The effect of histone
514 deacetylase inhibition on the expression of P-glycoprotein in human placental trophoblast cell lines.
515 *Placenta*. 2017;49:37-47. <https://doi.org/10.1016/j.placenta.2016.11.011>.

516

517 [28] Talwadekar M, Fernandes S, Kale V, Limaye L. Valproic acid enhances the neural
518 differentiation of human placenta derived-mesenchymal stem cells in vitro. *J Tissue Eng Regen Med*.
519 2017;11:3111-3123. <https://doi.org/10.1002/term.2219>.

520

- 521 [29] Vorhees CV. Teratogenicity and developmental toxicity of valproic acid in rats. *Teratology*.
522 1987;35:195-202. <https://doi.org/10.1002/tera.1420350205>
- 523
- 524 [30] Rouillet FI, Lai JK, Foster JA. In utero exposure to valproic acid and autism-a current review of
525 clinical and animal studies. *Neurotoxicol Teratol*. 2013;36:47-56. [https://doi.org/](https://doi.org/10.1016/j.ntt.2013.01.004)
526 10.1016/j.ntt.2013.01.004
- 527
- 528 [31] Zhang L, Chu X, Wang H, Xie H, Guo C, Cao L, Zhou X, Wang G, Hao H. Dysregulations of
529 UDP-glucuronosyltransferases in rats with valproic acid and high fat diet induced fatty liver. *Eur J*
530 *Pharmacol*. 2013;721:277-285. <https://doi.org/10.1016/j.ejphar.2013.09.024>.
- 531
- 532 [32] Yasuda S, Hasui S, Yamamoto C, Yoshioka C, Kobayashi M, Itagaki S, Hirano T, Iseki K.
533 Placental folate transport during pregnancy. *Biosci Biotechnol Biochem*. 2008;72:2277-84.
534 <https://doi.org/10.1271/bbb.80112>.
- 535
- 536 [33] Rosario FJ, Nathanielsz PW, Powell TL, Jansson T. Maternal folate deficiency causes inhibition
537 of mTOR signaling, down-regulation of placental amino acid transporters, and fetal growth
538 restriction in mice. *Sci Rep*. 2017;7:3982. <https://doi.org/10.1038/s41598-017-03888-2>.
- 539

540 [34] Zhao R, Diop-Bove N, VisentinM, Goldman ID. Mechanisms of membrane transport of folates
541 into cells and across epithelia. *Annu Rev Nutr.* 2011;31:177-201. [https://doi.org/10.1146/annurev-](https://doi.org/10.1146/annurev-nutr-072610-145133)
542 [nutr-072610-145133](https://doi.org/10.1146/annurev-nutr-072610-145133).

543

544 [35] Myllynen P, Vähäkangas K. Placental transfer and metabolism: an overview of the experimental
545 models utilizing human placental tissue. *Toxicol In Vitro.* 2013;27:507-12.
546 <https://doi.org/10.1016/j.tiv.2012.08.027>.

547

548 [36] Kasture VV, Sundrani DP, Joshi SR. Maternal one carbon metabolism through increased
549 oxidative stress and disturbed angiogenesis can influence placental apoptosis in preeclampsia. *Life*
550 *Sci.* 2018;206:61-69. <https://doi.org/10.1016/j.lfs.2018.05.029>.

551

552 [37] Harden CL, Pennell PB, Koppel BS, Hovinga CA, Gidal B, Meador KJ, Hopp J, Ting TY,
553 Hauser WA, Thurman D, Kaplan PW, Robinson JN, French JA, Wiebe S, Wilner AN, Vazquez B,
554 Holmes L, Krumholz A, Finnell R, Shafer PO, Le Guen C; American Academy of Neurology;
555 American Epilepsy Society. Practice parameter update: management issues for women with
556 epilepsy--focus on pregnancy (an evidence-based review): vitamin K, folic acid, blood levels, and
557 breastfeeding: report of the Quality Standards Subcommittee and Therapeutics and Technology

558 Assessment Subcommittee of the American Academy of Neurology and American Epilepsy Society.
559 Neurology. 2009;73:142-9. <https://doi.org/10.1212/WNL.0b013e3181a6b325>.

560

561 [38] Meador KJ, Baker GA, Browning N, Cohen MJ, Clayton-Smith J, Kalayjian LA, Kanner A,
562 Liporace JD, Pennell PB, Privitera M, Loring DW; NEAD Study Group. Foetal antiepileptic drug
563 exposure and verbal versus non-verbal abilities at three years of age. Brain. 2011;134:396-404.
564 <https://doi.org/10.1093/brain/awq352>.

565

566 [39] Bjørk M, Riedel B, Spigset O, Veiby G, Kolstad E, Daltveit AK, Gilhus NE. Association of
567 Folic Acid Supplementation During Pregnancy With the Risk of Autistic Traits in Children Exposed
568 to Antiepileptic Drugs In Utero. JAMA Neurol. 2018;75:160-168.
569 <https://doi.org/10.1001/jamaneurol.2017.3897>.

570

571 [40] Meador KJ, Baker GA, Browning N, Cohen MJ, Bromley RL, Clayton-Smith J, Kalayjian LA,
572 Kanner A, Liporace JD, Pennell PB, Privitera M, Loring DW; NEAD Study Group. Fetal
573 antiepileptic drug exposure and cognitive outcomes at age 6 years (NEAD study): a prospective
574 observational study. Lancet Neurol. 2013;12:244-252. [https://doi.org/10.1016/S1474-](https://doi.org/10.1016/S1474-4422(12)70323-X)
575 [4422\(12\)70323-X](https://doi.org/10.1016/S1474-4422(12)70323-X).

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 FR α (*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

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

619

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

625

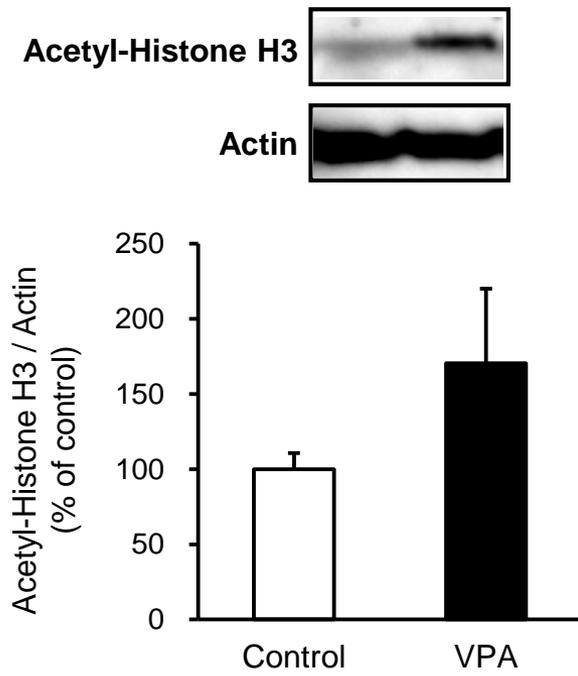
626 **Supplemental Figure 2.** Dose-dependent analysis of the effect of valproate (VPA) on the expression
627 of FR α in the rat placenta Pregnant rats on GD16–GD19 were orally administered VPA (200, 400, or
628 600 mg/kg) or water (control) for 4 successive days. The samples were collected from GD20 rats 24 h
629 after the last administration of VPA/water. Expression of FR α (*Folr1*) was analyzed by real-time PCR.
630 Each column represents the mean \pm S.D. of three to six dams. Dunnett's test was used for statistical
631 analysis. **: significantly different from the control at $p < 0.01$.

632

Supplemental Table 1 Primer sequences for real-time PCR.

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

A.



B.

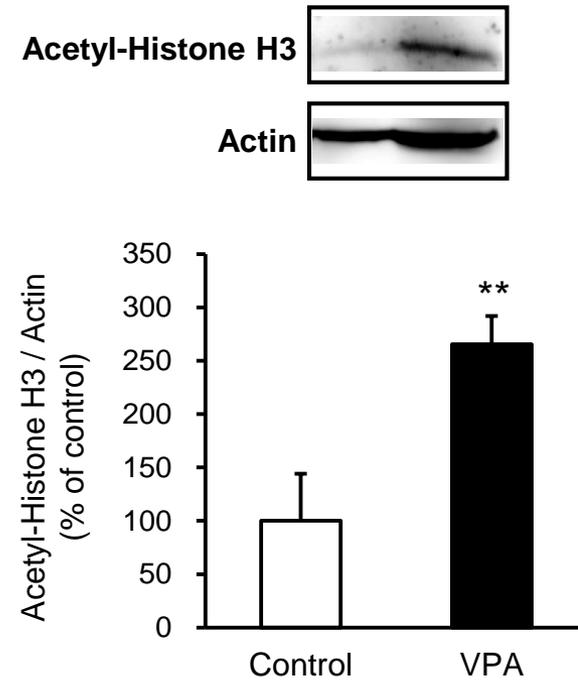
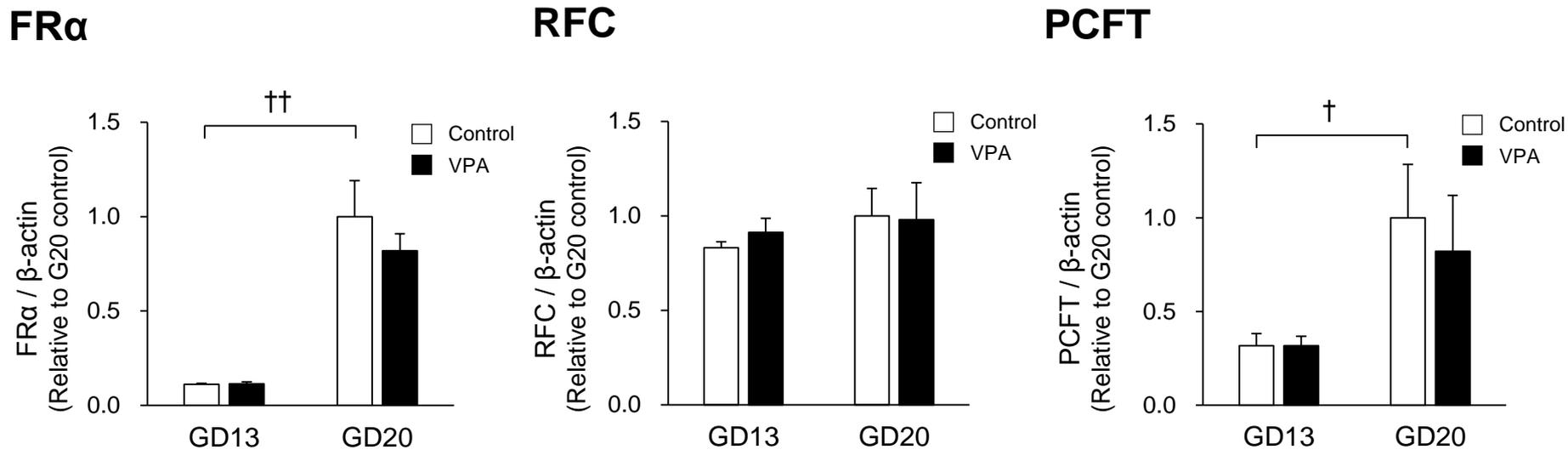


Figure 2

A.



B.

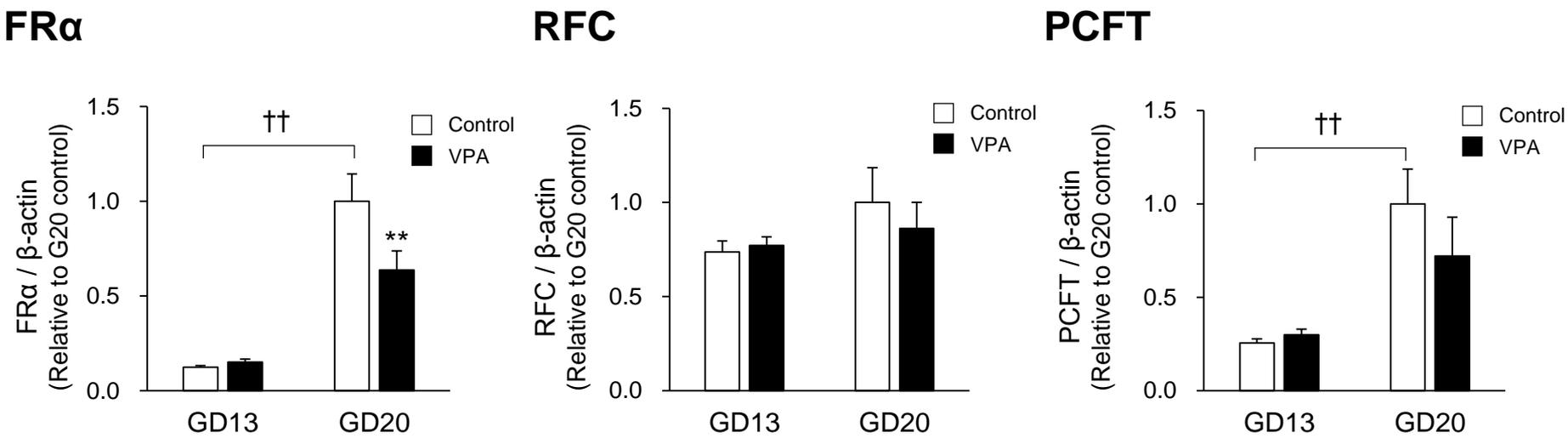
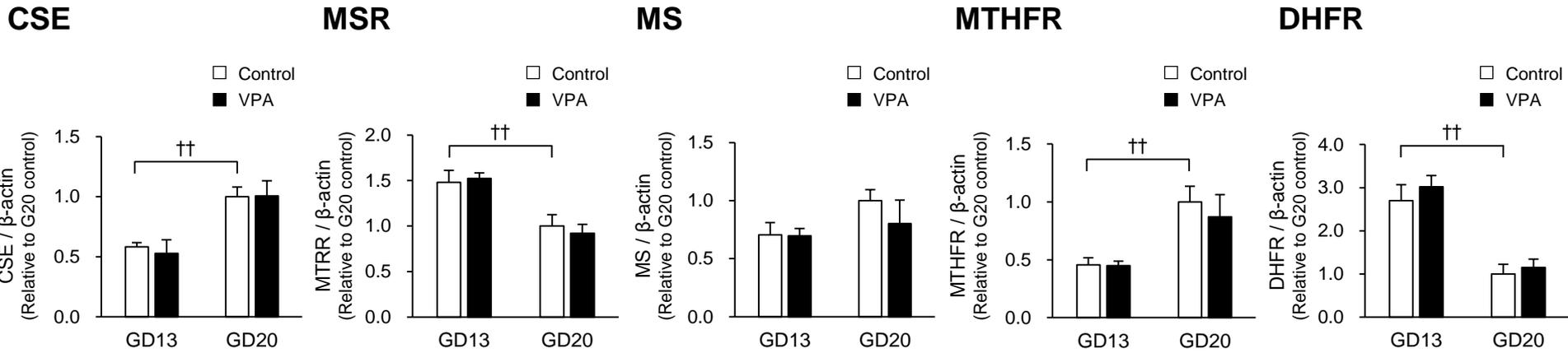


Figure 3

A.



B.

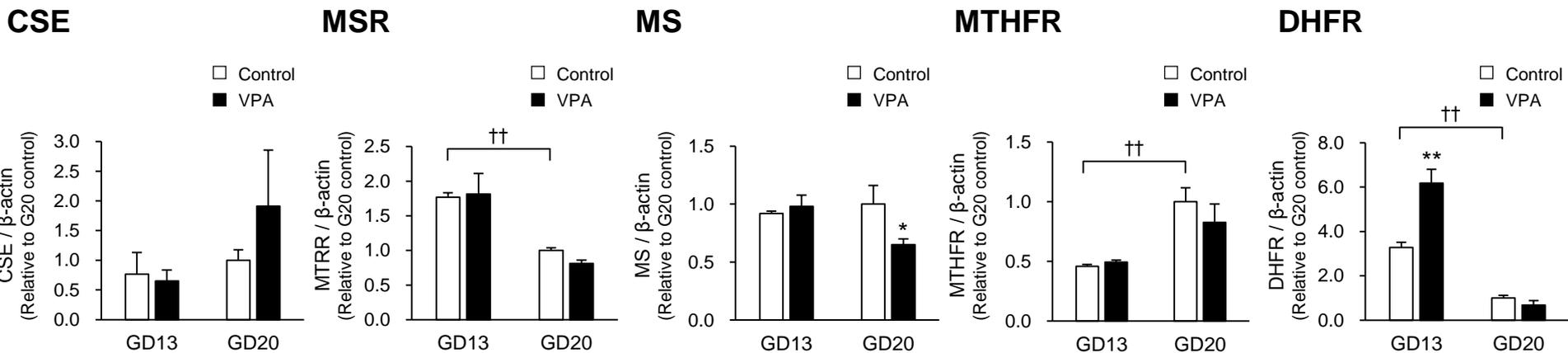


Figure 4

A.

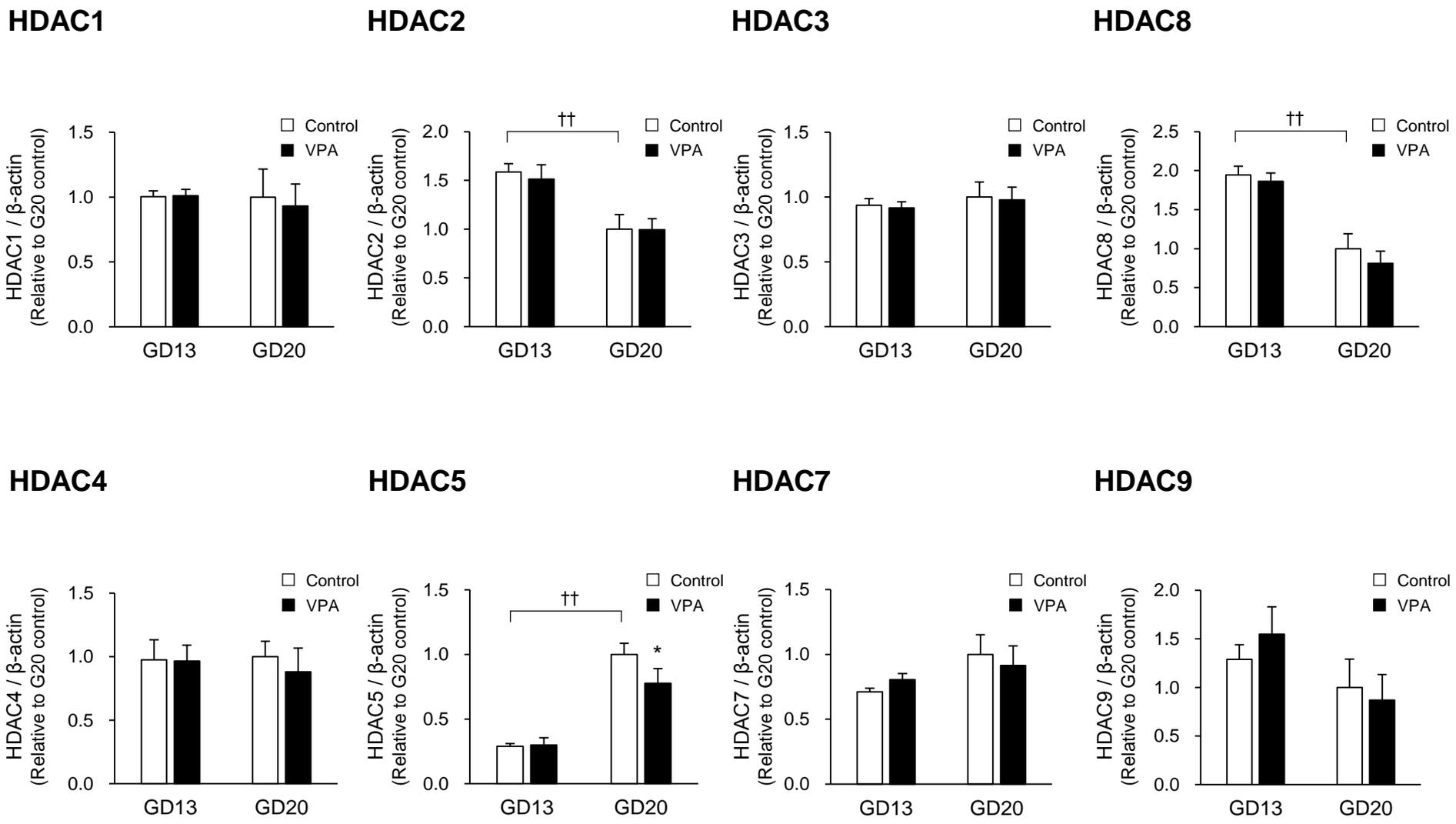


Figure 4

B.

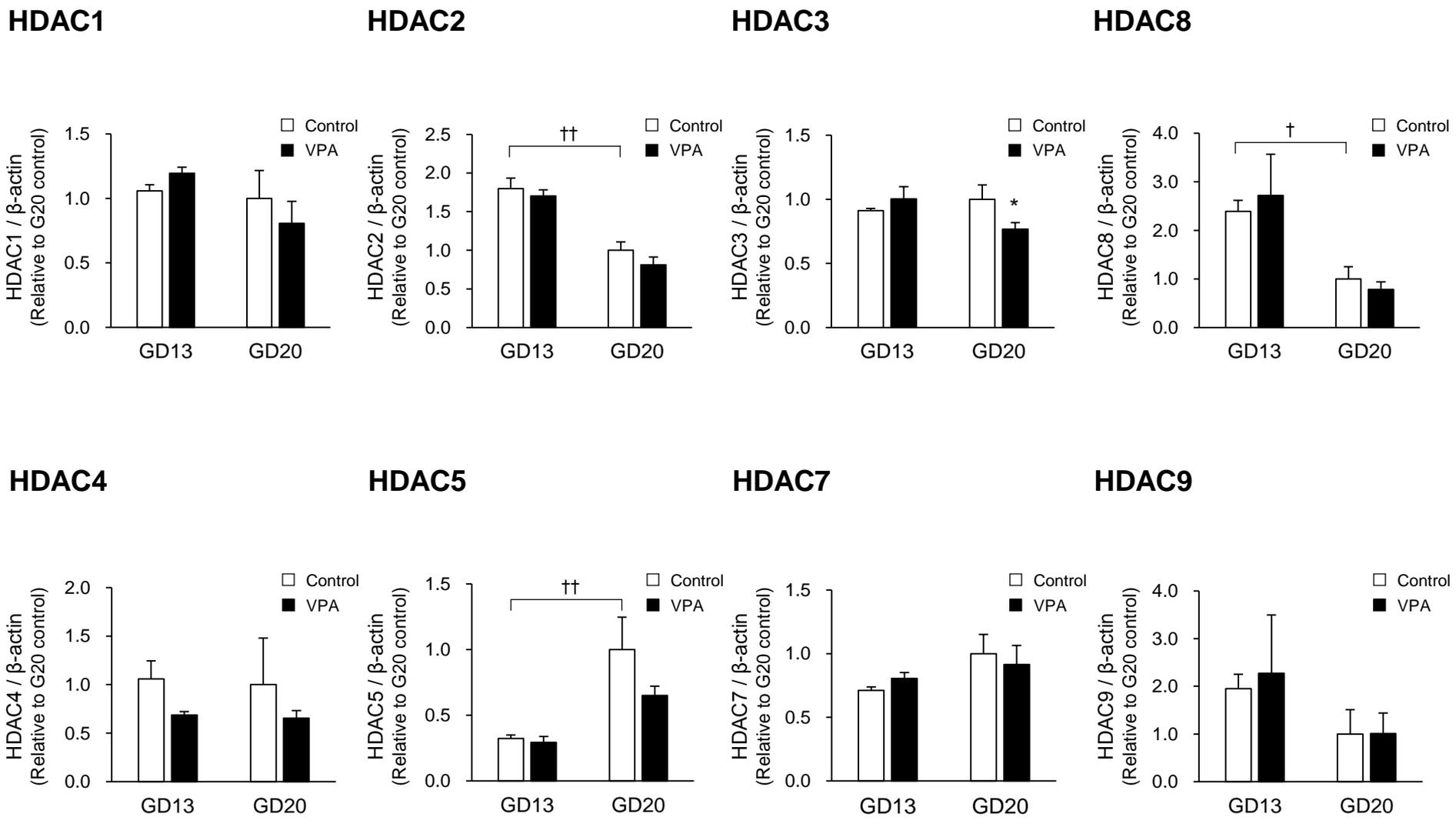
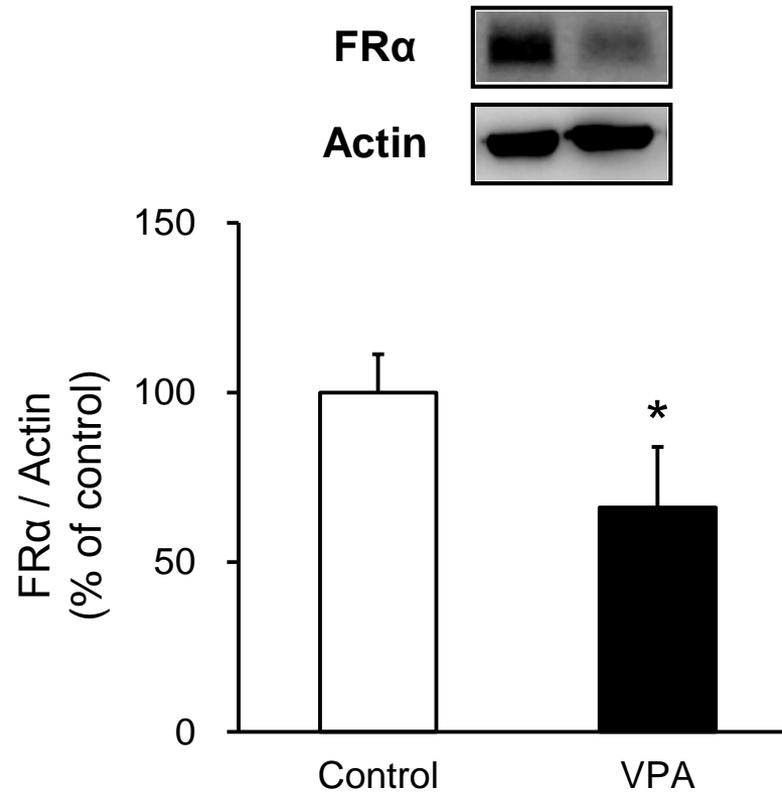
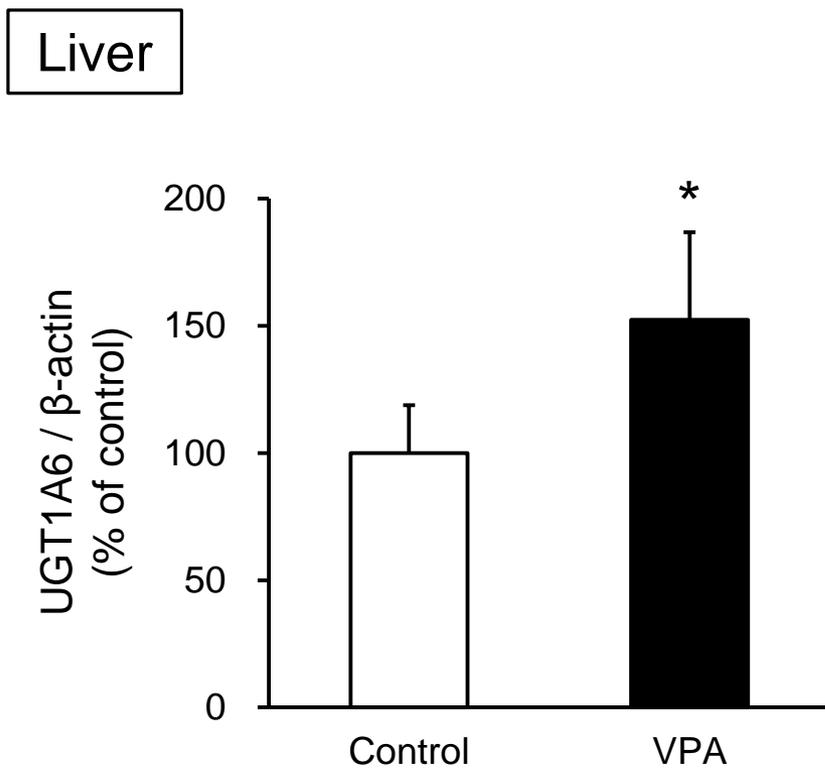


Figure 5



Supplemental Figure 1



Supplemental Figure 2

