Effects of single and repetitive valproic acid administration on the gene expression of placental transporters in pregnant rats: An analysis by gestational period.

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Abbreviations

ABC, ATP-binding cassette; ANOVA, analysis of variance; BCRP, breast cancer resistance protein; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; FRα, folate receptor alpha; G, gestational day; GLUT, glucose transporter; HDAC, histone deacetylase; IS, internal standard; LAT, L-type amino acid transporter; LC/MS/MS, liquid chromatography tandem mass spectrometry; MCT, monocarboxylate transporter; MDR, multiple drug resistant; MRP, multidrug resistance-associated protein; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter; PCR, polymerase chain reaction; SLC, solute carrier; VPA, valproic acid

Abstract

The use of valproic acid (VPA), an antiepileptic drug, during pregnancy, is known to increase various fetal risks. Since VPA has been known to inhibit histone deacetylases (HDACs); its administration could alter gene transcription levels. However, *in vivo* effects of VPA administration on placental transporters have not been fully elucidated. The purpose of the present study was to comprehensively evaluate the effects of single and repetitive VPA administration on the expression of placental transporters and analyze them by gestational day. We investigated 18 transporters (8 ATP-binding cassette (ABC) and 10 solute carrier (SLC) transporters) in the placentas of pregnant rats that were orally administered 400 mg/kg/day VPA for one or four days, during mid- or late gestation. In the control rats, 4 ABC transporter genes (*Abcb1a*, *1b*, *Abcc2*, *Abcc4*) were upregulated, 3 (*Abcc3*, *Abcc5*, *Abcg2*) downregulated through gestation, whereas 1 (*Abcc1*) was not changed. Regarding SLC transporters, 6 genes (*Slc7a5*, *Slc16a3*, *Slc22a3*, *Slc22a4*, *Slco2b1*, *Slco4a1*) were increased, 1 (*Slc29a1*) decreased through gestation, whereas 3 (*Slc7a8*, *Slc22a5*, *Slco2a1*) showed no significant change. Single VPA administration altered the expression of 9 transporters and repetitive administration, 13 transporters. In particular, VPA remarkably decreased *Abcc4* and *Slc22a4* in late gestation and increased *Abcc5* during mid-gestation. Our findings indicated that VPA administration changed transporter expression levels in rat placenta, and suggested that sensitivity to VPA differs across gestational stages.

Key words: Placenta; antiepileptic drug; valproic acid; ABC transporter; SLC transporter; gestational period; Rat

1. Introduction

About 0.3–0.7 % of pregnant women have epilepsy (Viinikainen et al., 2006). Seizure control needs to be maintained by continuous pharmacotherapy throughout pregnancy. Generally, medication use during pregnancy is a fetal risk factor, needing consideration in gestational pharmacotherapy.

Valproic acid (VPA) is a well-established and frequently used antiepileptic drug. However, it increases the risk of fetal malformations, autism spectrum disorders, and cognitive defects (Baker et al., 2015; Christensen et al., 2013; Jentink et al., 2010; Tomson et al., 2011). VPA treatment should be avoided in women of childbearing age; however, it is prescribed to pregnant women when required (Ishikawa et al., 2019; Yoshimura et al., 2018). Hence, the effect of VPA on the fetus and related organs needs to be evaluated.

The placenta is a crucial temporary organ, which is in contact with both maternal and fetal blood. ATP-binding cassette (ABC) and solute carrier (SLC) transporters play a role in nutrient, metabolic waste, and xenobiotic exchange between mother and fetus (Leazer and Klaassen, 2003; Staud et al., 2012). Hence, the expression levels of placental transporters are one of the factors affecting the intrauterine environment and fetal growth. Since the expression of placental transporters changes across gestation, their evaluation at each gestational stage is important. Additionally, transporters can be affected in a sex-specific manner, in some cases (Song et al., 2017).

VPA has been reported to inhibit histone deacetylases (HDACs), which remove acetyl groups from histones. Four classes of HDACs have been identified, and VPA acts predominantly on Class I HDACs, including HDAC 1–3 and 8 (Grabiec and Potempa, 2018; Gurvich et al., 2004). Therefore, VPA likely affects the expression of various genes. So far, there are several reports that have investigated the effects of VPA on placental transporters. An *in vitro* study indicated that VPA treatment altered the expression and function of breast cancer resistance protein (BCRP) (Rubinchik-Stern et al., 2015). Moreover, we previously showed that VPA exposure induces increased mRNA levels of folate receptor alpha (FRα) and proton-coupled folate transporter in BeWo and JEG-3 cell lines, derived from human choriocarcinoma (Kurosawa et al., 2018). Additionally, perfusion with VPA reportedly has reduced FRα and glucose transporter (GLUT) 1 in *ex vivo* human placentas (Rubinchik-Stern et al., 2018; Tetro et al., 2019). However, the perfusion time was short and variability among samples was high in the model. In particular, placental structure, function, and transporters are dramatically altered. Therefore, an *in vivo* study is well suited to sequentially investigate, throughout gestation, and assess the influence of fetal sex on VPA-mediated changes. Although a previous *in vivo* study indicated that the expression of L-type amino acid transporter (LAT) 1, organic anion transporting polypeptide (OATP) 4a1, and reduced folate carrier were lower in placentas from VPA-treated mid-pregnant mice (Meir et al., 2016), transporter expression level changes were not recorded.

This study aimed to comprehensively reveal the effects of both single and repetitive VPA treatment on placental transporters, using pregnant rats. We analyzed the expression of 8 ABC transporters, including multiple drug-resistant (MDR) 1a and 1b (*Abcb1a, 1b*), multidrug resistance-associated proteins (MRPs) 1–5 (*Abcc1–5*), and BCRP (*Abcg2*), as well as 10 SLC transporters, including LAT1 and 2 (*Slc7a5* and *Slc7a8*), monocarboxylate transporter (MCT) 4 (*Slc16a3*), organic cation transporter (OCT) 3 (*Slc22a3*), organic cation/carnitine transporter (OCTN) 1 and 2 (*Slc22a4*, *5*), equilibrative nucleoside transporter (ENT) 1 (*Slc29a1*), and OATP2a1, 2b1, and 4a1 (*Slco2a1*, *2b1*, *4a1*), by gestational age. Furthermore, the results were secondarily analyzed by fetal sex.

2. Material and Methods

2.1. Chemicals and reagents.

Valproic acid sodium salt (VPA Na) and valproic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterium-labeled valproic acid (VPA-d_6) and 2-propyl-4-pentenoic acid (4-ene-VPA) were purchased from Toronto Research Chemicals (Toronto, Canada).

2.2. Animals.

Pregnant female Wistar rats (12–13 weeks old) were provided by CLEA Japan (Tokyo, Japan). The existence of a vaginal plug indicated the first day of gestation (Gestational day 0; G0). G9–13 and G16–20 were considered as mid- and late gestation, respectively (Sun et al., 2015; Kalisch-Smith et al., 2017). The rats were housed at 23 ± 2°C and 60 ± 10 % relative humidity under a 12-h light/dark cycle, with *ad libitum* access to food and water. The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee, in accordance with the National Institutes of Health guide for the care and use of laboratory animals.

2.3. Drug administration and collection of plasma and placentas.

VPA Na was dissolved in distilled water (200 mg/mL), filter-sterilized, and then orally administered to pregnant rats at a daily dose of 400 mg/kg body weight, by gavage. We determined the appropriate experimental dose for observing fetal effects, which does not cause maternal death, based on the findings of a previous study (Vorhees, 1987). Control rats were administered the same

112 volume of distilled water. Rats of the single administration groups were treated on G12
113 (mid-gestation) or G19 (late gestation), and those in the repetitive groups were treated for 4 days on
114 G9–12 (mid-gestation) or G16–19 (late gestation). All pregnant rats were weighed before every
115 administration and euthanization.

116 Thirty minutes and 24 h post-VPA administration, blood was collected from the tail vein and
117 centrifuged at 800 *g* and 4℃ for 15 min, to obtain plasma. Twenty-four hours post the last
118 administration, G13 or G20 rats were anesthetized with sevoflurane and euthanized by decapitation;
119 their placentas were then immediately collected, washed briefly with ice-cold PBS, and weighed.
120 Three to four placentas per dam were used for real-time PCR, and the others were frozen in liquid
121 nitrogen and stored at -80℃ until further experiments.

122

123 **2.4. Real-time PCR.**

124 Total RNA was extracted from the placental homogenates using ISOGEN II (Nippon Gene,
125 Tokyo, Japan). RNA concentration was measured using Thermo Scientific™ NanoDrop 2000
126 (Thermo Fisher Scientific, Waltham, MA, USA). DNase treatment was performed using the
127 RNase-free DNase Set (QIAGEN, Venlo, Netherlands). Samples after DNase treatment were
128 reverse-transcribed using ReverTra Ace (Toyobo, Osaka, Japan) and iCycler™ (Bio-Rad
129 Laboratories, Hercules, CA), according to manufacturer's instructions. The A260/A280 and
130 A260/A230 ratios of isolated RNA were 1.95 ± 0.05 and 2.00 ± 0.10, respectively.

Real-time PCR was performed using KAPA SYBR® Fast qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) and LightCycler® 480 System II (Roche, Basel, Switzerland), through 40 cycles of 95℃ for 10 s, 55℃ or 60℃ for 20 s, and 72℃ for 1 s. Primers used for the real-time PCR are shown in Supplemental Table 1. Beta-actin was used as the reference gene. We determined that the reference gene in the rat placenta was not influenced by gestational age or VPA administration. Data were analyzed by the relative standard curve method. As a calibrator, cDNA from a G20 placenta (1 lot) was used. The Ct values of rat placenta samples are shown in Supplemental Table 2.

Fetal sex was determined by detecting the Y-chromosome-linked gene, *Sry*, which encodes a sex-determining region Y protein in the placentas, in accordance with a previous study (Song et al., 2017). Placentas with no *Sry* gene expression (Ct value, >35 or similar to that in negative controls) were considered to be from female fetuses, whereas those with expression (Ct value, <30) were considered to be from male fetuses.

2.5. VPA and 4-ene-VPA quantification in rat plasma using liquid chromatography-tandem mass spectrometry.

For standard curve generation, blank plasma from control pregnant rats was spiked with VPA and 4-ene-VPA to final concentrations of 10–500 and 0.2–100 μg/mL, respectively. VPA and 4-ene-VPA levels in plasma were determined as previously described (Wu and Lu, 2014; Gao et al., 2011), with some modification. Briefly, 10 μL of VPA-d_6 (internal standard (IS), 100 μg/mL) and 25

150 μL of hydrochloric acid (1 M) were added to 50 μL of rat plasma. Next, 1 mL of toluene was added
151 to the solution and mixed for 1 min. The mixture was centrifuged at 13,000 *g* and 4℃ for 10 min,
152 and 800 μL of supernatant collected and distilled at 37℃ under gentle nitrogen steam. The residue
153 was reconstituted in 50 μL of mobile phase (methanol:10 mM ammonium formate solution, 80:20
154 (v/v)) and 2 μL injected for HPLC.
155 HPLC was performed using a completely equipped Prominence 20A system (Shimadzu,
156 Kyoto, Japan). VPA and 4-ene-VPA were separated using an Inertsil ODS-3 column (2.1 × 150 mm,
157 3 μm; GL Science Inc., Tokyo, Japan). The column temperature was maintained at 40℃ and the
158 mobile phase flow rate set at 0.2 mL/min. Multiple reaction monitoring was performed using an
159 API3200^{TM} LC-MS/MS system (Applied Biosystems, Foster City, CA). This system was operated in
160 the negative ion electrospray mode. Monitoring ions were *m/z* 143.1 → 143.1 for VPA, *m/z* 141.1 →
161 141.1 for 4-ene-VPA, and *m/z* 149.1 → 149.1 for IS. Data were analyzed using Analyst software
162 (Applied Biosystems).
163
164 **2.6. Western blotting**
165 Whole proteins were extracted from placentas of G20 pregnant rats that were repetitively
166 administrated VPA or water (control). Placentas were homogenized in ice-cold RIPA buffer (Cell
167 Signaling Technology, Danvers, MA, USA). The lysates were kept on ice for 5 min and sonicated
168 briefly, then centrifuged at 14,000 g and 4℃ for 10 min. The supernatant was used for western
169 blotting. The total protein concentration was measured using a Pierce® BCA Protein Assay Kit

(Thermo Fisher Scientific). Total protein was mixed with Blue Loading Buffer Pack (Cell signaling Technology, Danvers, MA, USA) and denatured at 100℃ for 3 min. The mixture was subjected to SDS-PAGE at 20 ug protein per well and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were blocked with 5% non-fat dry milk in TBS containing 0.05% Tween 20 (TBST), at room temperature for 1 h, then incubated with primary antibodies diluted with Can Get Signal® Solution 1 (Toyobo) overnight at 4℃. The following primary antibodies were used: mouse anti-P-glycoprotein (1:200, Merck Millipore, Burlington, MA, #517310) and mouse anti-actin (1:1000, Chemicon, Temecula, CA, #MAB1501) monoclonal antibodies. The bands were detected using HRP-conjugated secondary antibodies (anti-mouse IgG, 1 : 4000, Southern Biotech, Birmingham, AL, #1070-05), and ECL prime™ Western blotting detection reagent (GE Healthcare, Buckinghamshire, England), with Image Quant LAS 4000 (GE Healthcare). The band Intensities were analyzed with ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical analyses.

Maternal body weight gain and placental weight were analyzed using Student's *t*-test for comparison of the control and VPA groups. Gene expression was analyzed using a two-way analysis of variance (ANOVA), with "gestational day (G)" and "VPA" or "sex" and "VPA" as factors. If an interaction was present, the Tukey–Kramer test was used for multiple comparisons. Two-way ANOVA and Tukey–Kramer test were performed using JMP Pro (SAS Institute, Cary, NC, USA).

All data are shown as mean with standard deviation (SD), and $P < 0.05$ was assessed as statistically significant.

3. Results

3.1. Effects of single VPA Na administration on placental transporters at G13 and G20.

First, we confirmed the presence of VPA and 4-ene-VPA in the plasma from the pregnant rats 30 min (peak) post oral VPA Na administration. In a previous study in pregnant rats, the VPA level peaked at 0.5–0.9 h post-oral VPA Na administration (Binkerd et al., 1988). The plasma levels of VPA and its toxic active metabolite, 4-ene-VPA, 30 min post-administration were approximately 200 and 1.5 μg/mL, respectively (Table 1); this level was similar to that reported in a previous study (Binkerd et al., 1988). After 24 h, their concentrations were less than or close to the lower limit of quantification. There were no differences in the concentrations between mid- and late gestation. Body weight gain of the pregnant rats did not differ significantly between VPA Na single administration and control groups (data not shown).

3.1.1. Alteration of placental ABC transporter expression by single VPA Na administration.

The expression of 8 ABC transporter mRNAs in the placentas were assessed (Figure 1). Interactions between gestational day and VPA Na administration were statistically significant for rMrp1, rMrp4, and rMrp5. Multiple comparisons revealed that VPA Na decreased rMrp4 by 22 % at G20, whereas increased rMrp5 by 22 % at G13 (Figure 1F and 1G). No significant difference was noted in rMrp1expression (Figure 1C). With the mRNA expression alterations associated with the gestational stage, multiple comparisons showed that rMrp4 increased by2.84-folds (Figure 1F),

whereas rMrp5 decreased by0.67-folds (Figure 1G) at G20, compared with the G13 control groups. The main gestational day effects were significant for rMdr1a, rMdr1b, rMrp3, and rBcrp; rMdr1a and rMdr1b increased to 295 % and 262%, respectively (Figure 1A and 1B), whereas rMrp3 and rBcrp decreased to 33% and 49%, respectively (Figure 1E and 1H). rMrp2 could not be evaluated because it was scarcely expressed at G13 (Figure 1D).

3.1.2. Alteration of placental SLC transporter expression by single VPA Na administration.

Next, we assessed the expression of 10 SLC transporter mRNAs in the placentas (Figure 2). Interactions between gestational day and VPA Na administration were statistically significant for rLat1, rLat2, rOctn1, rOatp2a1, rOatp2b1, and rOatp4a1. Multiple comparisons revealed that VPA Na increased rLat2 by 36 % and rOatp2a1 by 75 % at G13 (Figure 2B and 2H). In contrast, it decreased rLat1 by 21 %, rOctn1 by 20 %, rOatp2b1 by 21 %, and rOatp4a1 by 18 % at G20 (Figure 2A, 2E, 2I, and 2J). No interactions were noted for rMct4, rOct3, and rEnt1(Figure 2C, 2D, and 2G). When compared with levels in the G13 control by multiple comparisons, rLat1, rOctn1, rOatp2b1, and rOatp4a1 increased to 783%, 1660%, 485%, and 585% at G20, respectively (Figure 2A, 2E, 2I, and 2J). rMct4 tended to increase to 210 %, whereas rEnt1 tended to decrease to 46 % (Figure 2C and 2G).

3.2. Effects of repetitive VPA Na administration on placental transporters at G13 and G20.

The plasma levels of VPA and 4-ene-VPA 30 min post each administration were approximately 100–300 μg/mL and 1.5–2.0 μg/mL, respectively (Table 1). After 24 h, the concentrations of both VPA and 4-ene-VPA were less than or close to the lower limit of quantification. There were no substantial differences between mid- and late gestation as well as between single and repetitive administration. Body weight gain of VPA-administered dams was approximately half of that of control dams at G20 in the late gestation group, whereas no difference was noted in the mid-gestation group (Figure 3A). Placental weight was significantly decreased by VPA Na at both G13 and G20 (Figure 3B).

3.2.1. Alteration of placental ABC transporter expression by repetitive administration of VPA Na.

Interactions between gestational day and VPA Na administration were statistically significant for rMdr1a, rMrp1, rMrp3, rMrp4, rMrp5, and rBcrp, among the eight ABC transporters (Figure 1). Multiple comparisons revealed that VPA Na increased rMrp1 by 43 % and rMrp5 by 67 % at G13 (Figure 1C and 1G) and rMdr1a by 34 % at G20 (Figure 1A), whereas decreased rMrp3 by 17 % at G13 and rMrp4 by 46 % at G20 (Figure 1E and 1F). With the mRNA expression alterations associated with gestational stage, multiple comparisons showed that rMdr1a and rMrp4 increased to 277 % and 212 % (Figure 1A and 1F), whereas rMrp3, rMrp5, and rBcrp decreased to 35 %, 59 %,

and 56 %, respectively (Figure 1E, 1G, and 1H) at G20, compared with at G13. These alterations were similar to those of the single administration. The main effects of VPA and gestational day on rMdr1b were significant, although they did not have an interaction: rMdr1b increased to 247 % through gestation and decreased by 25 % with VPA Na.

3.2.2. Alteration of placental SLC transporter expression by repetitive VPA Na administration.

Interactions between gestational day and VPA Na administration were statistically significant for Lat1, rLat2, rOctn1, rOctn2, rEnt1, rOatp2a1, and rOatp4a1 (Figure 2). Multiple comparisons showed that VPA Na increased rLat2 by 95 %, rOctn2 by 78 %, rEnt1 by 61 %, and rOatp2a1 by 131 % at G13 (Figure 2B, 2F, 2G, and 2H), whereas decreased rLat1 by 18 %, rOctn1 by 51 %, and rOatp4a1 18 % at G20 (Figure 2A, 2E, and 2J). With regards to gene expression alterations during gestation, multiple comparisons showed that rLat1, rOctn1, and rOatp4a1 increased by 7.6-, 17.2-, and 5.7-folds, respectively (Figure 2A, 2E, and 2J). Conversely, rEnt1expression at G20 was 0.49-folds lower than at G13 (Figure 2G). These alterations associated with the gestational stages were the same as those of single administration.

3.3. Analysis of fetal sexual effects on gene expression.

A previous study evaluating placental response to maternal metabolic changes indicated that the expression of signaling factors and nutrient transporters was altered in a sex-specific manner

(Song et al., 2017). We secondarily analyzed the data shown in Figures 1 and 2 by fetal sex to clarify whether differences existed between male and female placentas. The analysis results of G13 and G20 placentas are shown in Supplemental Tables 3 and 4, respectively. Interactions between sex and VPA Na were significant for rMdr1a in the G13 single administration groups and rOatp2a1 and rOatp2b1 in G20 repetitive administration groups. Alteration of the three transporter mRNAs is shown in Supplemental Figure 1. Multiple comparisons revealed that VPA Na increased rMdr1a by 27 % and decreased rOatp2a1 by 46 % in male placentas, whereas no significant changes were noted in female placentas (Supplemental Figure 1A and 1B). There were no significant changes in rOatp2b1 expression in both male and female placentas (Supplemental Figure 1C).

4. Discussion

VPA use during pregnancy poses risks to the fetus such as malformations, autism spectrum disorders, and cognitive defects (Baker et al., 2015; Christensen et al., 2013; Jentink et al., 2010; Tomson et al., 2011). Therefore, evaluation of its effects on the placenta, which supports fetal development and health maintenance in pregnancy, is important. In the present study, we comprehensively evaluated the effects of VPA administration on the expression of rat placental transporters.

In the study, we determined the experimental dose to observe fetal effects and not cause maternal death, based on the findings of a previous study (Vorhees, 1987). In addition, previous studies that have investigated the effects of prenatal VPA exposure on rat pups, chose the administration dose of approximately 300–800 mg/kg (Roullet FI et al., 2013). No considerable differences between VPA levels and the therapeutic VPA range, which is 42–114 μg/mL in humans (Kim et al., 2011), were observed in this study. Additionally, accumulation by repetitive administration and differences due to the gestational stage were not observed for VPA or 4-ene-VPA (Table 1). It has been reported that 4-ene-VPA was detected in both humans and rats after administration of VPA, and has been suggested to be involved in toxicity, such as hepatoxicity (Kesterson JW et al., 1984; Tennison MB et al., 1998).

VPA administration is known to affect fetal growth and cognitive development in rats as well as humans (Ornoy, 2009; Schneider and Przewłocki, 2005). Maternal body weight loss was shown to

293 occur after the administration of 400 mg/kg VPA on G7–18 (Vorhees, 1987). Our results showed no
294 significant difference in maternal body weight (data not shown); however, body weight gain showed
295 a similar trend (Figure 3). Fetal weight also decreased in the VPA-treated group by approximately
296 1.5 g in a previous study (Vorhees, 1987). Hence, fetal growth restriction might also contribute to the
297 decreased maternal body weight gain. In humans, contradictory studies exist regarding the
298 relationship between VPA and fetal growth. A population-based cohort study indicated that VPA use
299 did not induce fetal growth restriction (Veiby et al., 2014), whereas another cohort study showed that
300 the maternal and umbilical cord levels of VPA were negatively correlated with birth length
301 (Kacirova et al., 2015). Therefore, VPA concentration in the plasma might be important for normal
302 fetal growth.

303 Mdr1a, Mdr1b, Mrp1–3, and Bcrp, which play a key role in predominantly transferring
304 xenobiotics. Mdr1a, Mdr1b, Mrp2, Mrp3 and Bcrp localize to the apical membrane of placenta.
305 Mrp1 localize to the basolateral membrane. Furthermore, Mrp1 and Mrp3 are expressed in fetal
306 vessels (Joshi et al., 2016; Ni and Mao, 2011). In this study, Mdr1a, Mdr1b, and Mrp2 were
307 increased, whereas Mrp3 and Bcrp decreased through gestation (Figure 1). It has been reported that
308 Mdr1a and Mdr1b increased with gestational stage, which was consistent with our results (Novotna
309 M et al., 2004). In regard to Mrp1-3, St-Pierre et al. showed that Mrp1 mRNA was abundantly
310 expressed in the placenta throughout gestation (St-Pierre MV et al., 2004). The group reported that
311 Mrp3 mRNA was expressed throughout gestation, whereas Mrp2 was expressed at a low level in the

312 rat placenta. Repetitive VPA administration decreased Mdr1b throughout gestation and affected
313 Mdr1a, Mrp1, and Mrp3 in a gestational age-specific manner (Figure 1). In the G20 placenta,
314 repetitive VPA administration increased Mdr1a, whereas it tended to decrease Mdr1b. Therefore, we
315 examined the expression of P-gp protein, which is encoded by *Abcb1a* and *Abcb1b*, by western
316 blotting (Supplemental Figure 2). P-gp protein tended to increase by repetitive VPA administration,
317 although the difference was not statistically significant. The result suggested that change in *Abcb1a*
318 affected the P-gp protein expression in rat placenta, and variations of gene expression by VPA can
319 alter protein levels.

320 Bcrp on the apical side and Oatp2b1 on the basolateral side collaborate to transport sulfate
321 conjugates from fetal to maternal circulation (Grube et al., 2007). As gestation progresses, these two
322 genes showed opposite changes as follows: Bcrp decreased to half the initial level (Figure 1H),
323 whereas Oatp2b1 increased by 4-folds (Figure 2I). It has been reported that Bcrp mRNA in the rat
324 placenta peaks on G15 and declines significantly to one third at term (Cygalova L et al., 2008).
325 Oatp2b1 mRNA increased throughout gestation (St-Pierre MV et al., 2004). The tendency was
326 consistent with the present results. Oatp2b1 was decreased on G20 by single VPA administration;
327 however, no significant differences were noted after repetitive treatment (Figure 2I).

328 Mrp4 on the apical side and Mrp5 on the basolateral side contribute to the transport of cyclic
329 nucleotides (Joshi et al., 2016; Wielinga et al., 2003). In this study, Mrp4 increased and Mrp5
330 decreased, following fetal development (Figure 1). Moreover, VPA remarkably decreased Mrp4 on

G20 (Figure 1F) and increased Mrp5 on G13 (Figure 1G). Considering these findings, VPA is thought to increase cAMP efflux from the placenta to the fetus on G13, whereas decreasing it from the placenta to mother on G20. In the placentas, one of the cyclic nucleotides, cAMP, is involved in cell fusion and cytotrophoblast syncytialization, which is caused by an increase in Syncytin-1 via cAMP/Protein kinase A signaling (Gupta et al., 2016). Hence, the altered expression of cAMP transporters might affect placental cell differentiation and function.

Mrp4 is also involved in transporting prostaglandins (Reid et al., 2003). Additionally, Oatp2a1 has been shown to transport prostaglandins (Gose et al., 2016), which was increased by VPA on G13 in the present study (Figure 2H). Prostaglandin E2 is involved in cell invasion and migration in the placenta (Nicola et al., 2005). Thus, VPA might alter placental kinetics of prostaglandins at both G13 and G20.

Lat1 on the apical membrane and Lat2 on both apical and basal membranes are transporters exchanging large neutral amino acids (del Amo et al., 2008; Gaccioli et al., 2015). The expression of Lat1 increased remarkably as gestation progressed (Figure 2A). VPA treatment decreased Lat1 on G20 (Figure 2A), whereas increased Lat2 on G13 (Figure 2B). A previous study indicated the involvement of Lat1 in the transport of branched-chain amino acids in mice fetal brain, and impairment of this transporter led to neurological and behavioral abnormalities associated with autism spectrum disorders (Tărlungeanu et al., 2016). Since VPA induces fetal autism spectrum disorders, evaluation of its effect on placental Lat1 might have important implications. Furthermore,

Lat1, Lat2, and Oatp4a1 transport thyroid hormones, which are necessary for fetal growth (Bernal et al., 2015; Forhead and Fowden, 2014). Oatp4a1 was shown to have increased following gestation and decreased by VPA on G20 (Figure 2J). The change in Oatp4a1 mRNA throughout gestation was consistent with the previous results (St-Pierre MV et al., 2004).

Ent1 transports pyrimidine nucleosides (Nishimura et al., 2012). Ent1 decreased with gestational stage and was increased by VPA on G13 (Figure 2J). The expression of other nucleoside transporters, Cnt1 and Ent2, was found to be low on G20 (data not shown).

Mct4 mediates proton-dependent transport of monocarboxylates. Mct4 was increased by gestational development. It has been reported that the MCT4 protein was strongly detected in rat placentas throughout gestation (Moore et al., 2016). VPA did not affect the expression levels across gestation (Figures 2C).

Higher levels of Oct3 in the placenta, rather than in other organs, play a role in the efflux of organic cations from the fetus (Leazer and Klaassen, 2003; Sata et al., 2005). In contrast, Oct2 was present at low levels in the placenta on G20 (data not shown). Oct3 was increased by gestational development or VPA single administration; however, the differences were subtle (Figure 2D). It has been reported that Oct3 mRNA in the rat placenta increased throughout gestation (Ahmadimoghaddam et al., 2013). The tendency was consistent with our results.

In this study, Octn1 increased markedly following gestational development and decreased by VPA administration on G20 (Figure 2E). Octn2 increased by VPA repetitive administration on G13

(Figure 2F). Octn1 and Octn2 play a crucial role in the uptake of L-carnitine by the placenta (Grube et al., 2005; Wu et al., 2000). The defect in Octn2 was shown to induce embryonic lethality (Shekhawat et al., 2018). Although the role of placental Octn1 has been poorly understood, the decline in Octn1, whose substrates are similar to those of Octn2, might affect fetal growth. The remarkable increase in Octn1 during pregnancy might have a critical impact; thus, further investigation is needed to elucidate its role. Furthermore, previous studies showed that serum carnitine levels were altered after VPA treatment (Moreno et al., 2005). Carnitine was also reported to alter Octn2 expression, which might be involved in Octn2 alteration in the present study (Schürch et al., 2010).

In the present study, variation in transporters in the placenta by VPA administration were observed. As described above, repetitive VPA administration increased Mdr1a, whereas it tended to decrease Mdr1b in the G20 placenta. Furthermore, VPA administration significantly decreased Mrp4 expression at G20. Because these are efflux transporters at the apical membrane, the changes might cause variation in placental barrier function. In regard to cAMP transporters, Mrp4 and Mrp5 were changed in VPA; Mrp4 decreased at G20, whereas Mrp5 increased at G13. Because cAMP signaling is involved in cell fusion and syncytialization (Gupta et al., 2016), the altered expression of cAMP transporters might affect placental cell differentiation and function. VPA administration affected the expression SLC transporters that mediate transport of nutrition. VPA decreased Lat1, Octn1, and Oatp4a1 in the G20 placenta. These are influx transporters at the apical membrane, and are important

388 for fetal development, such as amino acids, carnitine, and thyroid hormones. Additionally,
389 prostaglandin transporter Oatp2a1 was increased by VPA during G13 in the present study.
390 Prostaglandin E2 was involved in cell invasion and migration in the placenta (Nicola et al., 2005).
391 Therefore, these changes might contribute to impairment of the placental and fetal development by
392 VPA. The present results and previous knowledge suggest that the changes might be linked to
393 placental function and fetal nutrition/development. However, we have not directly evaluated the links
394 between changes in transporters and adverse effects by VPA in the present study. Future studies are
395 required to assess whether the changes of placental transporters affect placental function, fetal
396 nutrition, and development. In addition, we have not revealed changes at the protein levels, except
397 for P-gp. It is essential to evaluate protein levels for precisely justifying placental transport function.
398 Future studies should investigate the changes in proteins and function levels by VPA administration.
399 It has been reported that transporters can be affected in a sex-specific manner in some cases.
400 For instance, a maternal high-fat diet has been shown to increase GLUT3 and system A amino acid
401 transporter 2 only in the placentas from male fetuses (Song et al., 2017). Previous studies evaluating
402 the effects of VPA on the expression of placental transporters did not elucidate fetal sex differences.
403 Therefore, we secondarily analyzed by fetal sex. In this study, Mdr1a on G13 and Oatp2a1 on G20
404 were shown to have higher susceptibilities to VPA in the male placenta (Supplemental Figure 1). A
405 study showed that sex differences altered the behavior of VPA-treated rats; thus, VPA has the
406 potential to affect various aspects in a sex-specific manner (Anshu et al., 2017). However, the present

study had a limited number of placentas for each sex because randomized placentas were divided into male or female. Thus, further investigations are needed to determine the effect of fetal sex.

In the present study, we used rats as the pregnant animal model to investigate the effects of VPA on placental transporters because an *in vivo* study is suited to the investigation of changes throughout gestation. However, species difference between human and rat placentas should be considered to understand the results. First, there are structural differences in the placental barrier. The human placental barrier consists of a syncytiotrophoblast layer. In rats, the placental labyrinth has three trophoblastic layers. Layer II and III are thought to be the syncytium, whereas the first layer that faces the maternal side does not represent a barrier (Joshi AA et al., 2016). Second, there are some transporters whose localization are different between humans and rats, although the transporters or orthologs we investigated in the present study have been reported to be expressed in human placentas (Bleasby K et al., 2006; Gaccioli F et al., 2015; Joshi AA et al., 2016). For instance, it has been reported that human MCT4 (*SLC16A3*) is expressed on the maternal side of placenta, whereas rat Mct4 (*Slc16a3*) is expressed on the fetal side (Moore NP et al., 2016; Settle P et al., 2004). Other transporter localizations have been reported. It has been reported that both human P-gp (MDR1, *ABCB1*) and rat P-gp (Mdr1a/1b, *Abcb1a/1b*), BCRP (*ABCG2*), Bcrp (*Abcg2*), ENT1 (*SLC29A1*), Ent1 (*Slc29a1*), OATP4A1 (*SLCO4A1*), and Oatp4a1 (*Slco4a1*) express apical membranes (Akashi T et al., 2016; Joshi AA et al., 2016; Nishikawa M et al., 2010; Nishimura T et al., 2019; Sato K et al., 2003). In addition, human MRP1 (*ABCC1*) expression at the basolateral side

426 of the syncytiotrophoblast of human placental villi agrees with Mrp1 expression on the basolateral
427 side of the syncytiotrophoblast in the rat labyrinth zone (St-Pierre MV et al., 2004). Human MRP2, 4
428 (*ABCC2*, *4*) and OATP2A1 (*SLCO2A1*) have been detected in the apical membrane of the placenta
429 (St-Pierre MV et al., 2002; Joshi AA et al., 2016), whereas their localization in the rat placenta have
430 not been determined. Human MRP5 (*ABCC5*) have been shown to be expressed in the basolateral
431 membrane of the syncytiotrophoblast (Joshi AA et al., 2016), whereas its localization in the rat
432 placenta remains unclear. It has been reported that rat Octn1, 2 (*Slc22a4, 5*) is expressed in the apical
433 membrane (St-Pierre MV., 2002). Human LAT1, 2 (*SLC7A5, 8*) have been reported to be present on
434 the apical side of the syncytiotrophoblast, whereas LAT2 is also expressed in the basolateral
435 membrane and in the fetal capillary endothelium (Gaccioli F et al., 2015). Lat1, 2 (*Slc7a5, 8*) is also
436 expressed in the apical membrane in the rat placenta (Rosario FJ et al., 2011). Third, variation in
437 placental expression of some transporters throughout gestation differs. For instance, human P-gp
438 (MDR1, *ABCB1*) expression was reported to decrease with advancing gestation, whereas rat P-gp
439 (Mdr1a/1b, *Abcb1a/1b*) increased (Joshi AA et al., 2016). Human placental OCT3 (*SLC22A3*)
440 expression during the first trimester was reported to be higher than that at term, whereas rat Oct3
441 (*Slc22a3*) increased with advancing gestation (Ahmadimoghaddam et al., 2013). Human placental
442 OCTN2 (*SLC22A5*) expression during the first trimester was reported to be lower than that at term
443 (Bai et al., 2019), whereas rat Octn2 (*Slc22a5*) was not changed with advancing gestation in the
444 present study.

In conclusion, we assessed the effect of VPA on the expression of rat placental transporters during pregnancy and revealed that sensitivity to VPA differed following gestational development. Our findings also indicated that sex differences might exist in these alterations of transporters. To the best of our knowledge, this is the first study to longitudinally and systematically investigate the influence of VPA on various placental transporters. As described above, prenatal exposure to VPA increases various risks. Future studies are required to better understand the association of the effects of VPA with fetal risks and provide an approach for relieving these risks.

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Conflicts of Interest

The authors declare no conflicts of interest.

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657

658 **Figure legends**

659 **Figure 1** Effects of single and repetitive VPA Na administration on the expression of rMdr1a (A),
660 rMdr1b (B), rMrp1 (C), rMrp2 (D), rMrp3 (E), rMrp4 (F), rMrp5 (G), and rBcrp (H) mRNAs in rat
661 placentas. Pregnant rats were orally administered 400 mg/kg VPA Na (black) or distilled water as a
662 control (white). Rats in the single administration groups were treated during G12 (mid-gestation) or
663 G19 (late gestation), and those in the repetitive groups were treated for 4 d during G9–12
664 (mid-gestation) or G16–19 (late gestation). Expression of transporters was assessed by real-time PCR.
665 Each column represents the mean with SD (Single administration group: n = 9–12 placentas from
666 three dams; Repetitive administration group: n = 10–12 placentas from three dams). *: $P < 0.05$
667 when compared to the G20 control. **: $P < 0.01$ when compared to the G20 control. †: $P < 0.05$
668 when compared to the G13 control. ††: $P < 0.01$ when compared to the G13 control. ND: not
669 determined because of low expression. G: gestational day. Gene expression was analyzed using an
670 ANOVA, with “G” and “VPA” as factors. If an interaction was present, the Tukey–Kramer test was
671 used for multiple comparisons.

672

673 **Figure 2** Effects of single and repetitive VPA Na administration on the expression of rLat1 (A),
674 rLat2 (B), rMct4 (C), rOct3 (D), rOctn1 (E), rOctn2 (F), rEnt1(G), rOatp2a1 (H), rOatp2b1 (I), and
675 rOAtp4a1 (J) mRNAs in rat placentas. Pregnant rats were orally administered 400 mg/kg VPA Na
676 (black) or distilled water as a control (white). Rats of the single administration groups were treated

during G12 (mid-gestation) or G19 (late gestation), and those in the repetitive groups were treated for 4 d during G9–12 (mid-gestation) or G16–19 (late gestation). Expression of transporters was assessed by real-time PCR. Each column represents the mean with SD (Single administration group: n = 9–12 placentas from three dams; Repetitive administration group: n = 10–12 placentas from three dams). *: $P < 0.05$ when compared to the G20 control. **: $P < 0.01$ when compared to the G20 control. ††: $P < 0.01$ when compared to the G13 control. G: gestational day. Gene expression was analyzed using an ANOVA, with "G" and "VPA" as factors. If an interaction was present, the Tukey–Kramer test was used for multiple comparisons.

Figure 3 Body weight gain (A) and placental weight (B) of pregnant rats orally administered 400 mg/kg VPA Na or distilled water as control for 4 days on G9–12 or G16–19, respectively. (A) Maternal body weight gain was calculated by subtracting the body weight on the first day of administration from those of each gestational day. Each point represents the mean with SD (n = 3 dams). *: $P < 0.05$ when compared to the control. (B) Each column represents the mean with SD (n = 40 placentas for G13 control, 34 for G13 VPA, 32 for G20 control, 33 for G20 VPA placentas from three dams. **: $P < 0.01$ when compared to each control. G: gestational day. Student's t-test was used for comparison of the control and VPA groups.

Supplemental Figure 1 Interactive effects of VPA Na administration and fetal sex on rMdr1a expression in G13 rat placentas (A), and those of rOatp2a1 (B) and rOatp2b1 (C) in G20 rat placentas. Pregnant rats were orally administered 400 mg/kg/day VPA Na (black) or distilled water as a control (white) on G12 (A) or G16–19 (B, C). Expression of transporters was assessed by real-time PCR. Each column represents the mean with SD (n = 3–7 placentas from two or three dams). *: $P < 0.05$ when compared to the male control. **: $P < 0.01$ when compared to the male control. G: gestational day. Gene expression was analyzed using a two-way analysis of ANOVA, with "sex" and "VPA" as factors. If an interaction was present, the Tukey–Kramer test was used for multiple comparisons.

Supplemental Figure 2 Effects of repetitive VPA Na administration on P-gp protein expression in rat placentas (G20). Pregnant rats were orally administered 400 mg/kg/day VPA Na (black) or distilled water as a control (white), for 4 d. Whole proteins were extracted from placentas of G20 pregnant rats with repetitive administrated of VPA or water (control). Expression of P-gp was assessed by western blotting. Fetal sex was not determined for the protein samples. (A) Data shown are typical results of three independent experiments. (B) Each column represents the mean with SD (n = 12 placentas from three dams).

713 **Table 1** Concentration of VPA and 4-ene-VPA in the plasma of pregnant rats orally administered 400 mg/kg VPA Na.

			Mid-gestation					Late gestation				
		Time after administration		VPA (μg/mL)		4-ene-VPA (μg/mL)			VPA (μg/mL)		4-ene-VPA (μg/mL)	
				Mean	SD	Mean	SD		Mean	SD	Mean	SD
Single administration		30 min	G12	211	28.9	1.49	0.288	G19	175	57.2	1.50	0.165
		24 h	G13	a		a		G20	a		0.827	0.543
Repetitive administration	Day 1	30 min	G9	159	38.8	1.62	0.0858	G16	241	4.93	1.41	0.230
	Day 2	24 h	G10	a		a		G17	a		a	
		30 min		160	14.5	2.02	0.680		261	157	1.36	0.361
	Day 3	24 h	G11	a		a		G18	a		a	
		30 min		286	151	1.61	0.205		200	30.1	1.76	0.301
	Day 4	24 h	G12	a		a		G19	a		a	
		30 min		98.8	67.0	2.08	0.544		146	102	1.95	0.151
	Day 5	24 h	G13	a		a		G20	a		0.437	0.308

714

715 Concentration of VPA and 4-ene-VPA was quantified by LC/MS/MS. Data are shown as the mean with SD (n = 3 dams). a: less than the lower
716 limit of quantification (LLOQ). LLOQs of VPA and 4-ene-VPA were 10 μg/mL and 0.2 μg/mL, respectively.

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722 **Supplemental Table 1** Primer sequences for real-time PCR.

723

Name	Primer sequence (5' to 3')	Product size (bp)
rBeta-actin (*Actb*)	Forward : CTATCGGCAATGAGCGGTTC Reverse : GAGGTCTTTACGGATGTCAACG	134
rBcrp (*Abcg2*)	Forward : GTTTGGACTCAAGCACAGCA Reverse : TGAGTTTCCCAGAAGCCAGT	150
rMrp1 (*Abcc1*)	Forward : CGAATGTCCTCTGAGATGGAGAC Reverse : CTCTACACGGCCTGAATGGG	138
rMrp2 (*Abcc2*)	Forward : TGATCGGTTTCGTGAAGAGCT Reverse : ACGCACATTCCCAACACAAA	139
rMrp3 (*Abcc3*)	Forward : CGTTCCGATTCACCACTTTC Reverse : TCTGGGCAAGGATTTGTGTC	124
rMrp4 (*Abcc4*)	Forward : GGACACTGAACTAGCAGAATC Reverse : TGTATTAACTCGTCAGTTCTCG	150
rMrp5 (*Abcc5*)	Forward : CCACCATCCATGCCTATAACAA Reverse : CCCCGTGGTGGTGATCAG	158
rMdr1a (*Abcb1a*)	Forward : GCAGGTTGGCTGGACAGATT Reverse : GGAGCGCAATTCCATGGATA	70
rMdr1b (*Abcb1b*)	Forward : CTGCTATCATCCACGGAACC Reverse : GCTGACGGTCTGTGTACTGTTG	140
rLat1 (*Slc7a5*)	Forward : CCTACGGAGGATGGAACTATCTGA Reverse : TGGGCAAGGAGATGATGATG	93
rLat2 (*Slc7a8*)	Forward : TCCACATTTGGTGGAGTCAA Reverse : TGGATCATGGCTAACACGCT	101
rOct3 (*Slc22a3*)	Forward : TATGCAGCGGACAGATACGG Reverse : AAAATTCGGTGCAAACGCCA	96
rOctn1 (*Slc22a4*)	Forward : TGATGTCTGTGATGCTGTGG Reverse : ATATATCTCCGAGGCAGGGTTC	172
rOctn2 (*Slc22a5*)	Forward : CGATCCCAGTGAGTTACAAGAC Reverse : GAGAAAGTCCGAAGTAGCCC	149
rOatp2a1 (*Slco2a1*)	Forward : GCCAGATACCCACAAGGAGA Reverse : GATGGCGAATAGGATGGAGA	166
rOatp2b1 (*Slco2b1*)	Forward : ACGACTTTGCCCACCATAGC Reverse : CCACGTAAAGGCGTAGCATGA	117
rOatp4a1 (*Slco4a1*)	Forward : AGAACGTCAAGTCGAGCTATTCG Reverse : GGCCCACTTCTGTGTAAACATTT	123
rEnt1 (*Slc29a1*)	Forward : GGCCTGTGCAGTTGTCATTC Reverse : CCTCCTCTTGGCTCCTCTCC	153
rMct4 (*Slc16a3*)	Forward : GGGTCATCACTGGCTTGGGT Reverse : GGAACACGGGACTGCCTGC	123
r*Sry*	Forward : AAGCCTTACAGAAGCCGAAAAA Reverse : TGTGGCACTTTAACCCTTCGA	120

Supplemental Table 2 Ct value of rat placental samples.

Gene		Ct value	
		Single administration (G20 Control)	
		Mean	SD
ABC transporters	rBcrp	29.5	0.6
	rMdr1a	27.8	0.2
	rMdr1b	25.1	0.6
	rMrp1	27.2	0.5
	rMrp2	30.8	1.3
	rMrp3	28.7	0.4
	rMrp4	28.4	0.3
	rMrp5	27.2	0.3
SLC transporters	rLat1	24.6	0.4
	rLat2	30.6	0.6
	rOctn1	26.6	0.3
	rOctn2	27.2	0.3
	rOct3	26.2	0.3
	rOatp2a1	24.7	0.4
	rOatp2b1	29.6	0.5
	rOatp4a1	23.9	0.3
	rEnt1	29.3	0.6
	rMct4	23.1	0.2

Data are shown as the mean with SD from placentas of G20 control rats of single administration groups (n = 9–12 placentas from three dams). ND: not determined because of low expression. G: gestational day. For measurements of each gene expression, 50-fold (rMrp2, rMrp3 G20, rMrp5, and rOatp2b1), 100-fold (rBcrp, rMdr1a, rMdr1b, rMrp1, rMrp4, rOctn1, rOctn2, rOct3, rOatp2a1, rOatp4a1, and rMct4) 200-fold (rMrp3 G13, rLat2), or 500-fold (rLat1 and rEnt1) diluted samples were amplified by real-time PCR.

Supplemental Table 3 Analysis of fetal sexual effects on gene expression (G13 rat placentas).

Gene	Single administration									Repetitive administration								
	Target gene / beta-actin mRNA (relative to male control)								P value	Target gene / beta-actin mRNA (relative to male control)								
	Male				Female					Male				Female				
	Control		VPA		Control		VPA		Interaction	Control		VPA		Control		VPA		Interaction
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD	Mean	SD	(*P* value)
rBcrp	1.000	0.218	0.950	0.205	0.947	0.265	0.768	0.111	0.4797	1.000	0.215	0.804	0.211	0.984	0.233	0.818	0.062	0.8587
rMdr1a	1.000	0.081	1.273	0.203	1.001	0.136	1.007	0.093	**0.0361**	1.000	0.137	0.990	0.200	0.957	0.194	0.931	0.175	0.9103
rMdr1b	1.000	0.264	1.020	0.202	1.064	0.368	1.079	0.345	0.9871	1.000	0.199	0.624	0.112	0.981	0.170	0.907	0.203	0.0549
rMrp1	1.000	0.124	1.118	0.143	0.940	0.115	1.104	0.031	0.6424	1.000	0.189	1.555	0.330	1.059	0.080	1.361	0.259	0.2172
rMrp2	ND		ND		ND		ND			ND		ND		ND		ND		
rMrp3	1.000	0.112	1.252	0.319	1.068	0.220	1.042	0.150	0.1536	1.000	0.129	0.842	0.073	1.001	0.099	0.797	0.054	0.4180
rMrp4	1.000	0.103	1.024	0.210	0.925	0.128	0.993	0.110	0.7236	1.000	0.145	1.118	0.218	0.872	0.447	1.076	0.117	0.5272
rMrp5	1.000	0.207	1.274	0.249	1.034	0.313	1.215	0.170	0.6605	1.000	0.311	1.751	0.670	0.913	0.104	1.433	0.590	0.5697
rLat1	1.000	0.090	0.919	0.123	1.070	0.146	0.966	0.192	0.8769	1.000	0.092	0.893	0.186	1.221	0.336	1.150	0.425	0.8733
rLat2	1.000	0.240	1.221	0.349	0.927	0.268	1.405	0.294	0.3096	1.000	0.275	2.198	0.920	1.047	0.301	1.826	0.924	0.5018
rOctn1	1.000	0.143	1.268	0.246	1.238	0.090	1.660	0.489	0.5035	1.000	0.235	1.175	0.365	0.926	0.078	1.035	0.297	0.7831
rOctn2	1.000	0.309	1.093	0.471	0.951	0.342	1.204	0.185	0.5959	1.000	0.339	1.794	0.753	0.979	0.326	1.726	0.241	0.9065
rOct3	1.000	0.155	1.382	0.281	1.079	0.209	1.106	0.292	0.0935	1.000	0.100	0.855	0.352	0.985	0.161	1.141	0.216	0.1289
rOatp2a1	1.000	0.215	1.856	0.743	1.039	0.197	1.721	0.287	0.6349	1.000	0.218	2.534	1.525	0.931	0.115	1.893	0.783	0.4455
rOatp2b1	1.000	0.151	1.200	0.262	1.047	0.267	1.273	0.406	0.9088	1.000	0.157	1.290	0.274	1.010	0.118	1.071	0.582	0.4055
rOatp4a1	1.000	0.104	1.091	0.162	1.096	0.143	0.988	0.227	0.1613	1.000	0.132	1.180	0.222	0.976	0.112	1.226	0.341	0.7034
rEnt1	1.000	0.221	1.204	0.478	1.031	0.448	1.358	0.168	0.7005	1.000	0.278	1.664	0.612	0.965	0.228	1.490	0.439	0.6974
rMct4	1.000	0.108	1.237	0.173	1.067	0.166	1.095	0.133	0.1174	1.000	0.035	0.862	0.155	0.990	0.051	1.003	0.103	0.0810

Data are shown as the mean with SD (n = 5–7 placentas from three dams). ND: not determined because of low expression. The data shown in Figures 1 and 2 of G13 rat placentas were analyzed by fetal sex. Gene expression was analyzed using a two-way ANOVA, with “sex” and “VPA” as factors. If p value was < 0.05, an interaction was considered to be present.

Supplemental Table 4 Analysis of fetal sexual effects on gene expression (G20 rat placentas).

Gene	Single administration									Repetitive administration								
	Target gene / beta-actin mRNA (relative to male control)									Target gene / beta-actin mRNA (relative to male control)								
	Male				Female					Male				Female				
	Control		VPA		Control		VPA		Interaction	Control		VPA		Control		VPA		Interaction
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(*P* value)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	(*P* value)
rBcrp	1.000	0.144	1.180	0.601	0.906	0.294	0.844	0.086	0.4835	1.000	0.290	1.386	0.451	0.924	0.187	0.756	0.117	0.0701
rMdr1a	1.000	0.091	0.985	0.148	0.885	0.070	0.949	0.177	0.5206	1.000	0.282	1.319	0.180	0.957	0.271	1.247	0.135	0.9003
rMdr1b	1.000	0.169	0.950	0.257	1.248	0.486	1.059	0.158	0.6200	1.000	0.121	0.832	0.134	1.274	0.203	1.026	0.022	0.5633
rMrp1	1.000	0.160	0.942	0.190	1.044	0.112	0.957	0.155	0.8428	1.000	0.190	0.764	0.171	0.893	0.133	0.896	0.003	0.1023
rMrp2	1.000	0.399	0.761	0.568	0.794	0.612	0.528	0.333	0.9536	1.000	0.933	1.254	0.923	0.693	0.241	0.455	0.186	0.4522
rMrp3	1.000	0.161	1.218	0.402	1.225	0.393	1.405	0.219	0.8993	1.000	0.158	0.894	0.261	1.017	0.206	0.951	0.231	0.8409
rMrp4	1.000	0.095	0.742	0.180	1.203	0.330	1.030	0.222	0.6864	1.000	0.186	0.570	0.222	1.118	0.226	0.601	0.113	0.6502
rMrp5	1.000	0.088	0.914	0.162	0.966	0.169	0.873	0.106	0.9622	1.000	0.219	1.181	0.266	1.022	0.231	0.971	0.135	0.2840
rLat1	1.000	0.107	0.801	0.044	1.046	0.157	0.812	0.105	0.7224	1.000	0.164	0.745	0.103	0.851	0.090	0.770	0.083	0.1100
rLat2	1.000	0.137	0.859	0.104	0.967	0.171	0.858	0.229	0.8340	1.000	0.116	0.831	0.213	0.845	0.100	0.943	0.132	0.0702
rOctn1	1.000	0.111	0.771	0.116	0.927	0.143	0.761	0.185	0.6353	1.000	0.131	0.479	0.074	1.086	0.155	0.601	0.041	0.7378
rOctn2	1.000	0.107	0.996	0.145	0.871	0.205	0.854	0.194	0.9376	1.000	0.083	0.911	0.200	1.118	0.156	1.277	0.081	0.0916
rOct3	1.000	0.083	1.032	0.166	1.052	0.134	1.189	0.099	0.3865	1.000	0.398	1.448	0.256	1.282	0.228	1.621	0.577	0.7230
rOatp2a1	1.000	0.183	0.861	0.159	1.074	0.264	0.779	0.408	0.5357	1.000	0.101	0.541	0.070	1.061	0.149	0.862	0.256	**0.0494**
rOatp2b1	1.000	0.118	0.819	0.151	1.003	0.156	0.755	0.129	0.6052	1.000	0.184	1.262	0.156	1.054	0.208	0.928	0.049	**0.0240**
rOatp4a1	1.000	0.181	0.812	0.099	0.968	0.151	0.792	0.180	0.9302	1.000	0.240	0.772	0.109	0.973	0.078	0.883	0.049	0.2805
rEnt1	1.000	0.324	0.879	0.172	1.191	0.091	1.063	0.136	0.9637	1.000	0.113	0.657	0.127	0.828	0.248	0.684	0.184	0.2387
rMct4	1.000	0.064	0.868	0.167	0.887	0.181	0.926	0.481	0.5004	1.000	0.204	1.146	0.183	1.123	0.181	1.178	0.093	0.5819

Data are shown as the mean with SD (n = 3–7 placentas from one to three dams). The data shown in Figures 1 and 2 of G20 rat placentas were analyzed by fetal sex. Gene expression was analyzed using a two-way ANOVA, with “sex” and “VPA” as factors. If p value was < 0.05, an interaction was considered to be present.

Figure 1

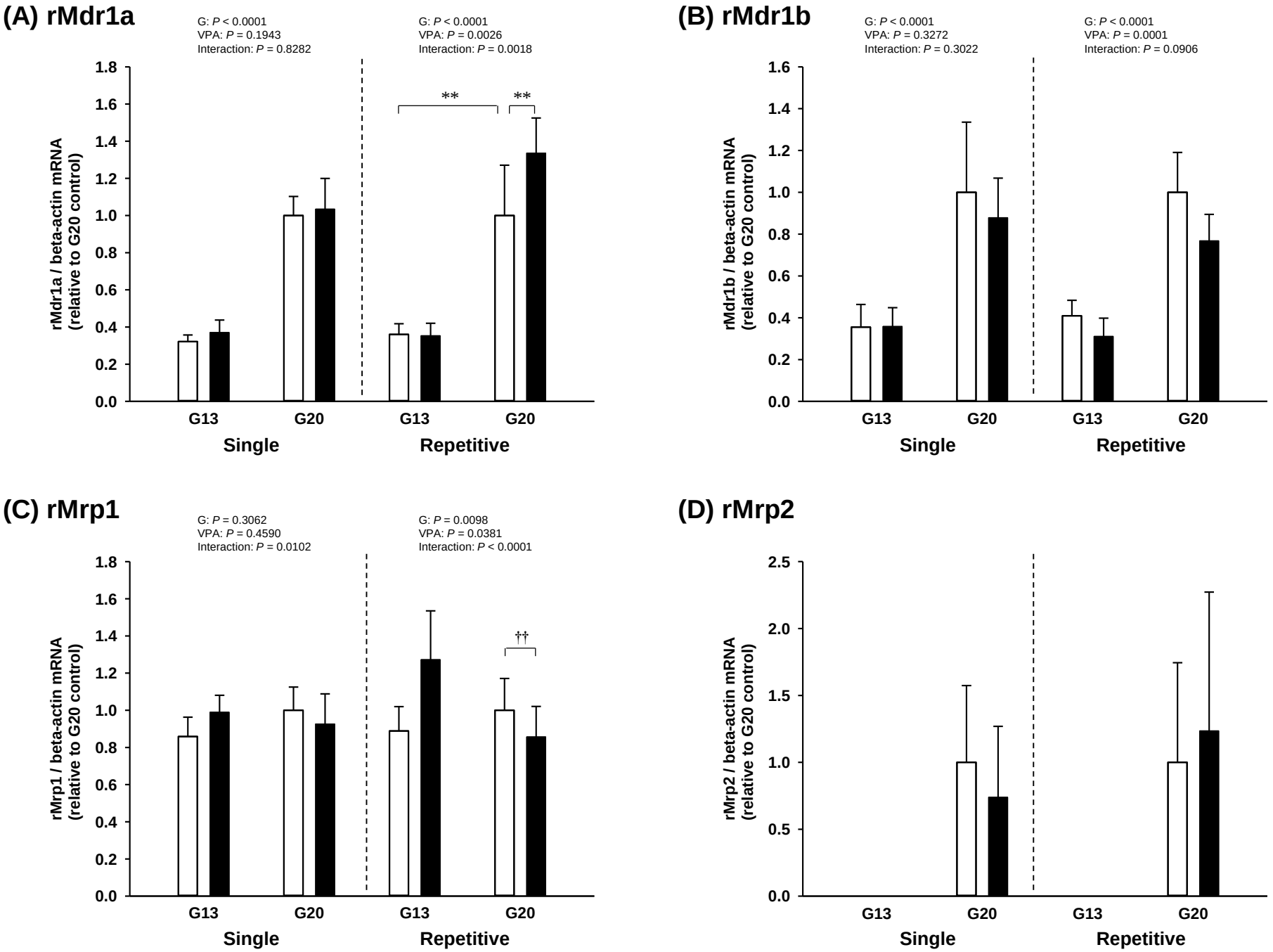
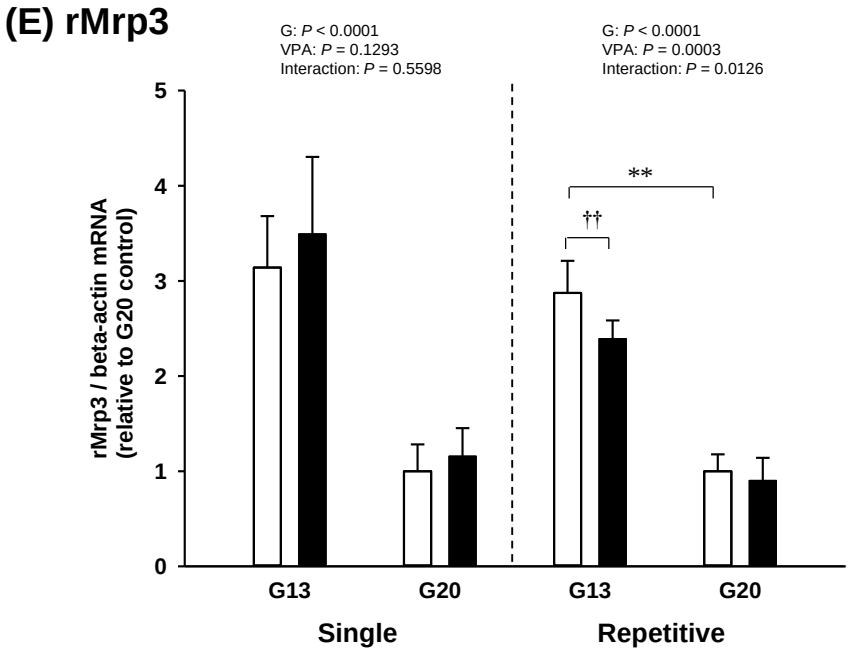
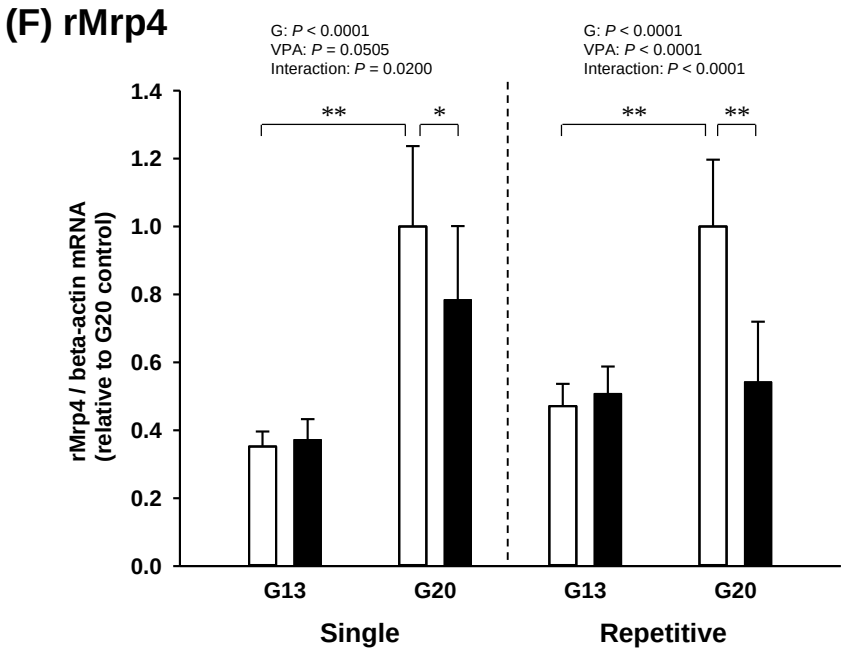
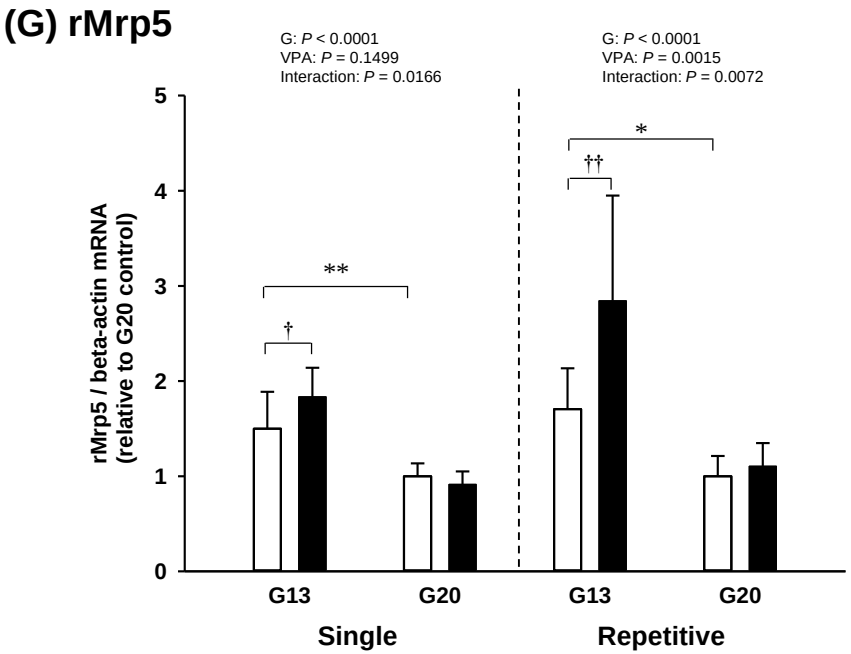

(A) rMdr1a
G: P < 0.0001
VPA: P = 0.1943
Interaction: P = 0.8282
G: P < 0.0001
VPA: P = 0.0026
Interaction: P = 0.0018
**
**
rMdr1a / beta-actin mRNA (relative to G20 control)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G13
G20
G13
G20
Single
Repetitive
(B) rMdr1b
G: P < 0.0001
VPA: P = 0.3272
Interaction: P = 0.3022
G: P < 0.0001
VPA: P = 0.0001
Interaction: P = 0.0906
rMdr1b / beta-actin mRNA (relative to G20 control)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G13
G20
G13
G20
Single
Repetitive
(C) rMrp1
G: P = 0.3062
VPA: P = 0.4590
Interaction: P = 0.0102
G: P = 0.0098
VPA: P = 0.0381
Interaction: P < 0.0001
††
rMrp1 / beta-actin mRNA (relative to G20 control)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G13
G20
G13
G20
Single
Repetitive
(D) rMrp2
rMrp2 / beta-actin mRNA (relative to G20 control)
2.5
2.0
1.5
1.0
0.5
0.0
G13
G20
G13
G20
Single
Repetitive

Figure 1


(E) rMrp3
G: P < 0.0001
VPA: P = 0.1293
Interaction: P = 0.5598
G: P < 0.0001
VPA: P = 0.0003
Interaction: P = 0.0126
rMrp3 / beta-actin mRNA
(relative to G20 control)
0
1
2
3
4
5
**
††
G13
G20
G13
G20
Single
Repetitive


(F) rMrp4
G: P < 0.0001
VPA: P = 0.0505
Interaction: P = 0.0200
G: P < 0.0001
VPA: P < 0.0001
Interaction: P < 0.0001
rMrp4 / beta-actin mRNA
(relative to G20 control)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
**
*
**
**
G13
G20
G13
G20
Single
Repetitive


(G) rMrp5
G: P < 0.0001
VPA: P = 0.1499
Interaction: P = 0.0166
G: P < 0.0001
VPA: P = 0.0015
Interaction: P = 0.0072
rMrp5 / beta-actin mRNA
(relative to G20 control)
0
1
2
3
4
5
**
†
*
††
G13
G20
G13
G20
Single
Repetitive

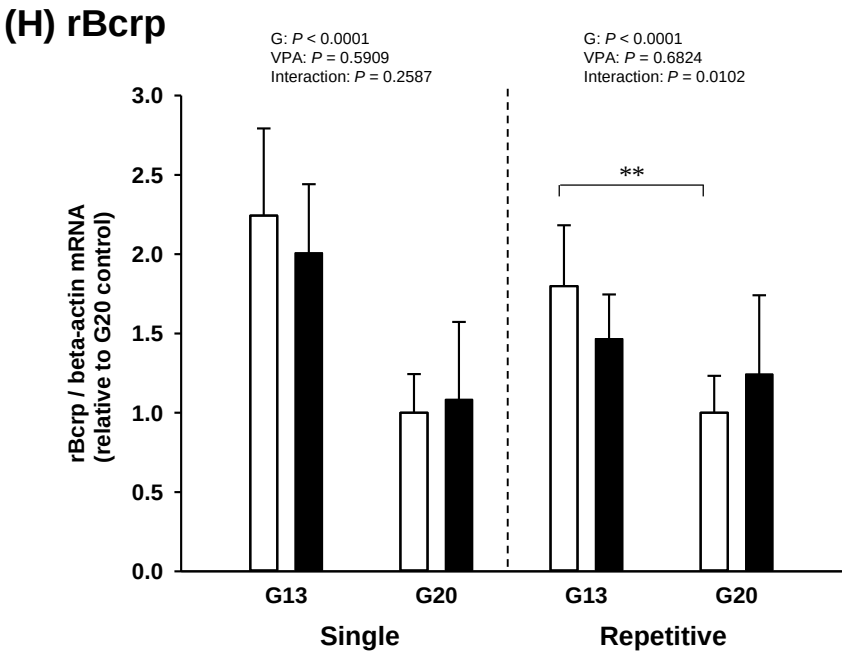
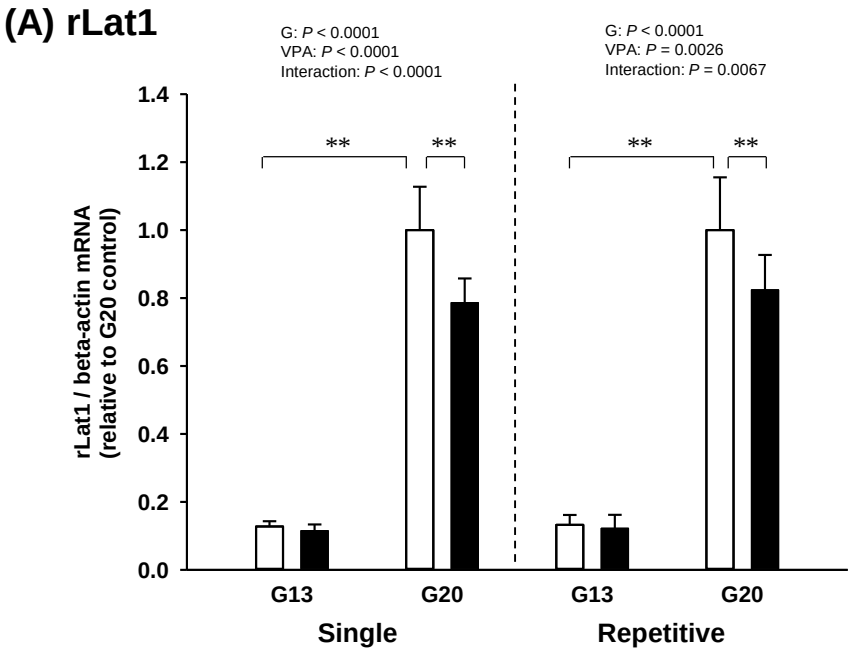
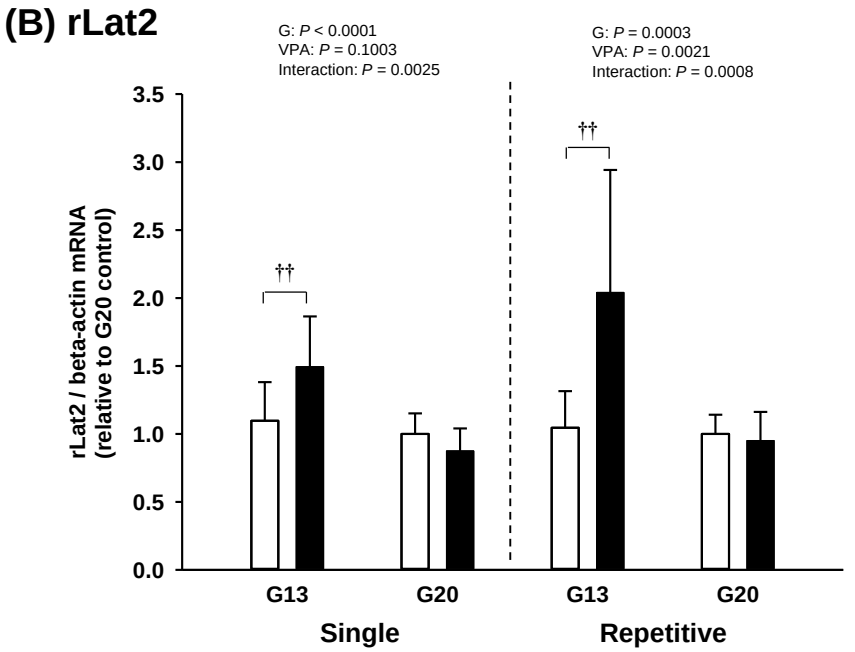
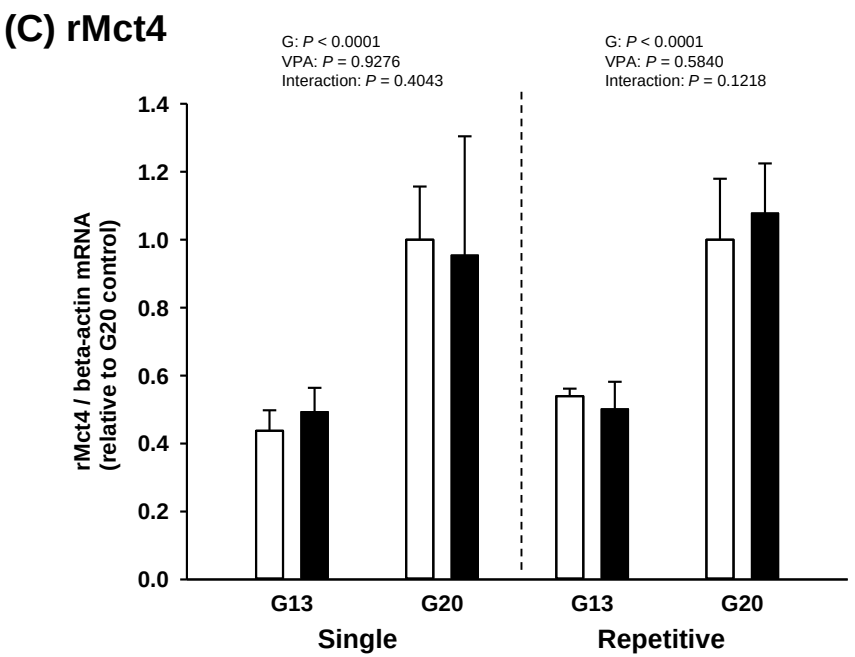

(H) rBcrp
G: P < 0.0001
VPA: P = 0.5909
Interaction: P = 0.2587
G: P < 0.0001
VPA: P = 0.6824
Interaction: P = 0.0102
rBcrp / beta-actin mRNA
(relative to G20 control)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
**
G13
G20
G13
G20
Single
Repetitive

Figure 2


(A) rLat1
G: P < 0.0001
VPA: P < 0.0001
Interaction: P < 0.0001
G: P < 0.0001
VPA: P = 0.0026
Interaction: P = 0.0067
rLat1 / beta-actin mRNA (relative to G20 control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
G13
G20
G13
G20
Single
Repetitive


(B) rLat2
G: P < 0.0001
VPA: P = 0.1003
Interaction: P = 0.0025
G: P = 0.0003
VPA: P = 0.0021
Interaction: P = 0.0008
rLat2 / beta-actin mRNA (relative to G20 control)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
††
††
G13
G20
G13
G20
Single
Repetitive


(C) rMct4
G: P < 0.0001
VPA: P = 0.9276
Interaction: P = 0.4043
G: P < 0.0001
VPA: P = 0.5840
Interaction: P = 0.1218
rMct4 / beta-actin mRNA (relative to G20 control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G13
G20
G13
G20
Single
Repetitive

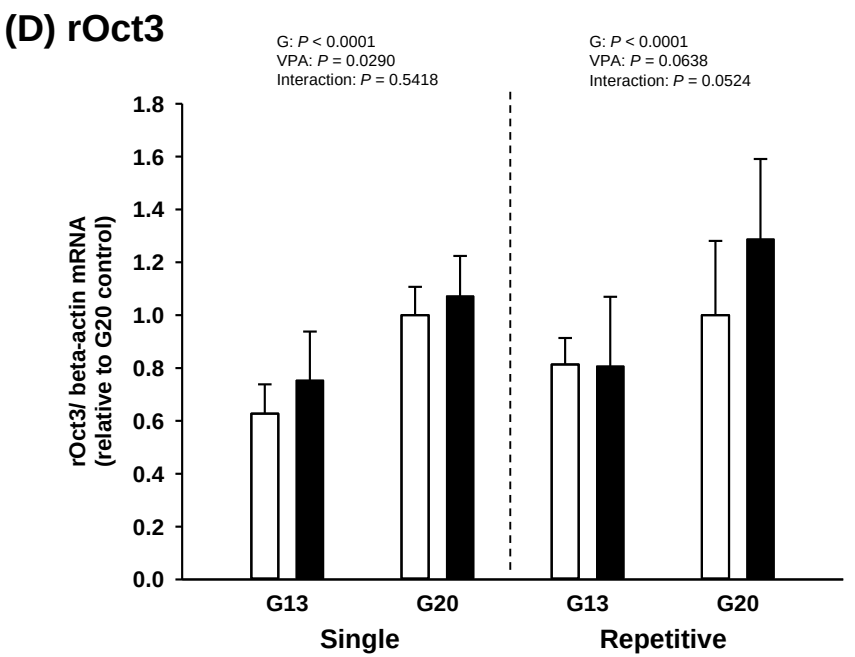
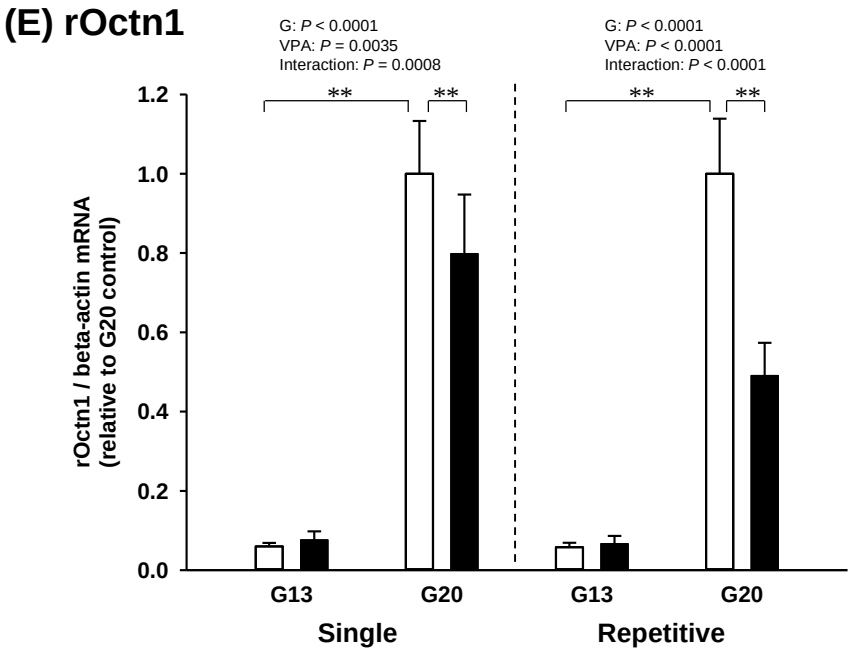
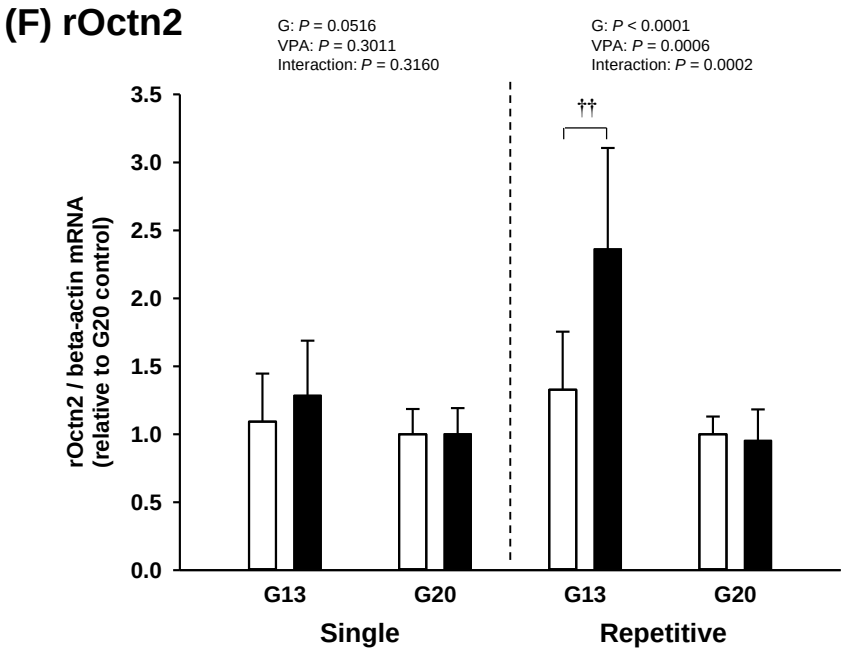
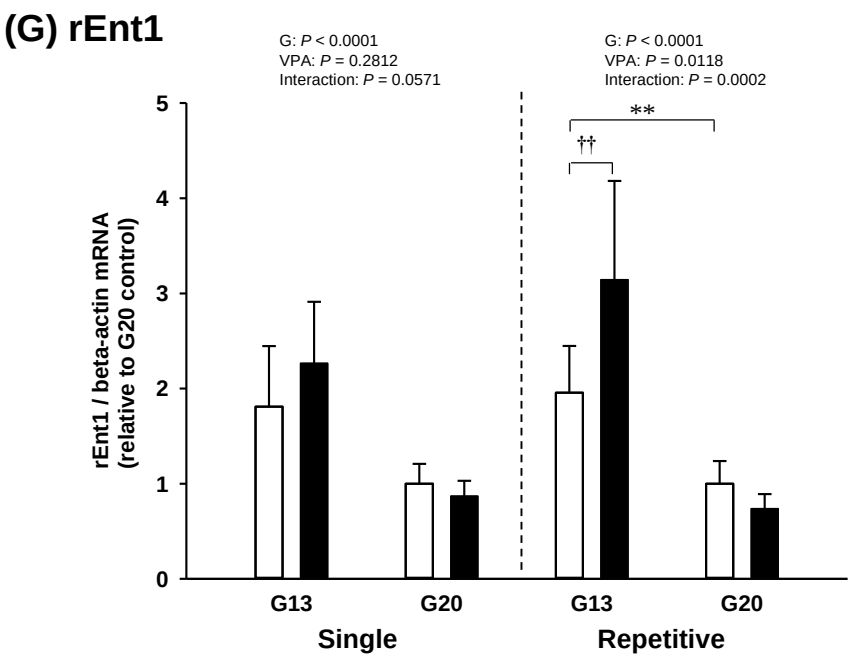

(D) rOct3
G: P < 0.0001
VPA: P = 0.0290
Interaction: P = 0.5418
G: P < 0.0001
VPA: P = 0.0638
Interaction: P = 0.0524
rOct3/ beta-actin mRNA (relative to G20 control)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
G13
G20
G13
G20
Single
Repetitive

Figure 2


(E) rOctn1
G: P < 0.0001
VPA: P = 0.0035
Interaction: P = 0.0008
G: P < 0.0001
VPA: P < 0.0001
Interaction: P < 0.0001
rOctn1 / beta-actin mRNA
(relative to G20 control)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
**
G13
G20
G13
G20
Single
Repetitive


(F) rOctn2
G: P = 0.0516
VPA: P = 0.3011
Interaction: P = 0.3160
G: P < 0.0001
VPA: P = 0.0006
Interaction: P = 0.0002
rOctn2 / beta-actin mRNA
(relative to G20 control)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
††
G13
G20
G13
G20
Single
Repetitive


(G) rEnt1
G: P < 0.0001
VPA: P = 0.2812
Interaction: P = 0.0571
G: P < 0.0001
VPA: P = 0.0118
Interaction: P = 0.0002
rEnt1 / beta-actin mRNA
(relative to G20 control)
5
4
3
2
1
0
**
††
G13
G20
G13
G20
Single
Repetitive

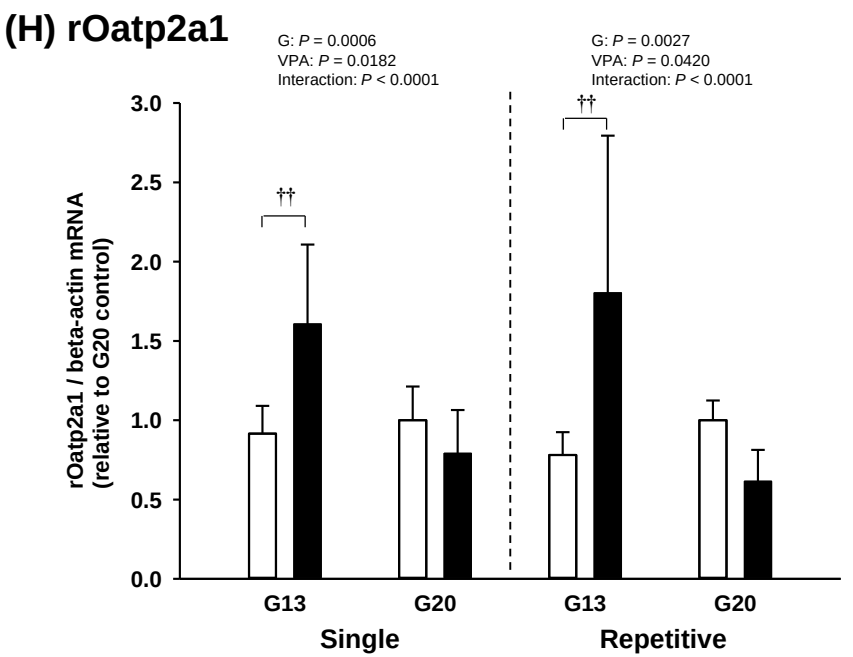
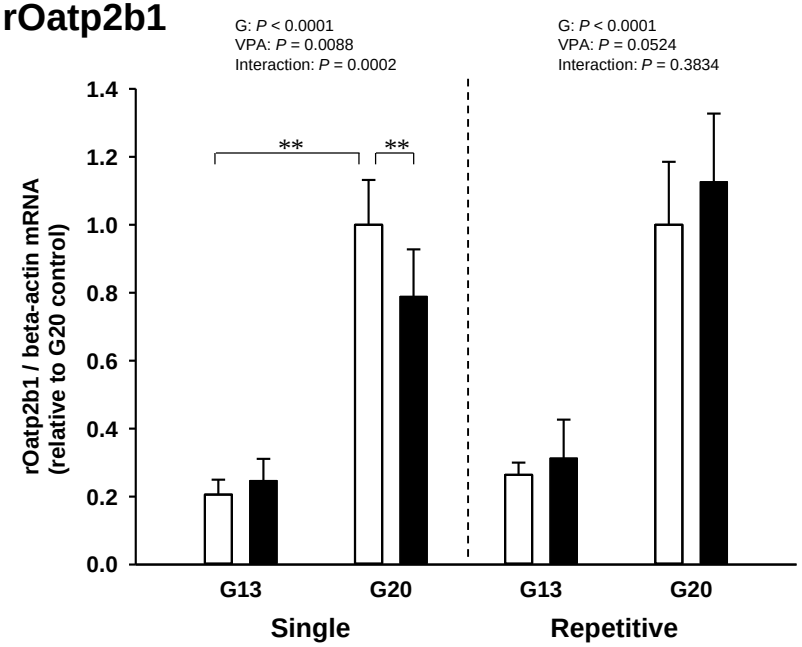

(H) rOatp2a1
G: P = 0.0006
VPA: P = 0.0182
Interaction: P < 0.0001
G: P = 0.0027
VPA: P = 0.0420
Interaction: P < 0.0001
rOatp2a1 / beta-actin mRNA
(relative to G20 control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
††
††
G13
G20
G13
G20
Single
Repetitive

Figure 2

(I) rOatp2b1



(J) rOatp4a1

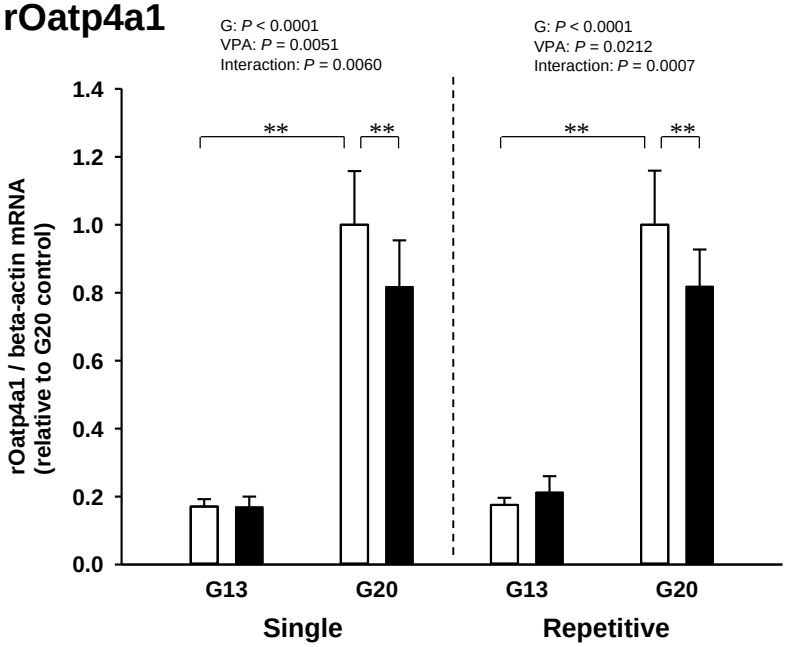
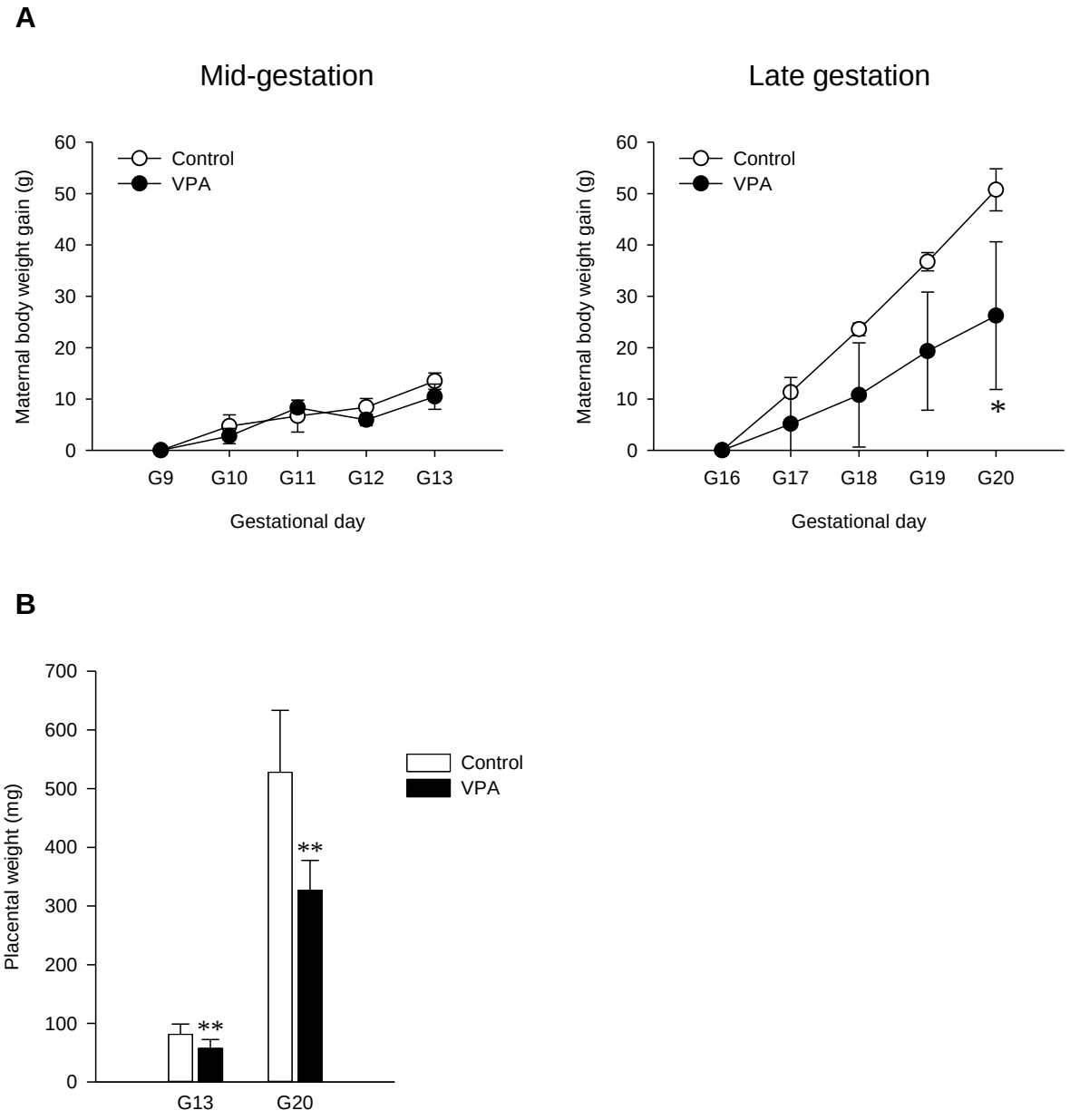
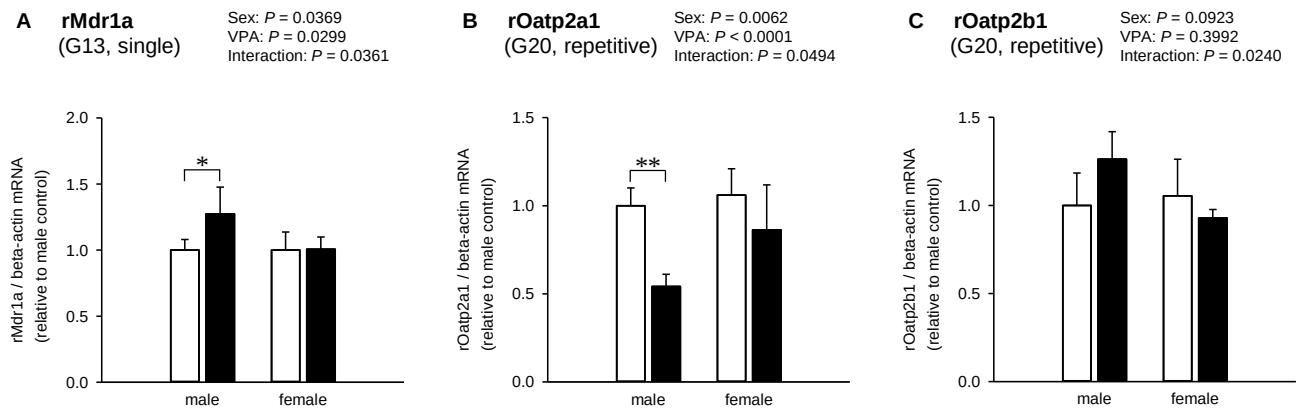


Figure 3


A
Mid-gestation
Late gestation
Control
VPA
Maternal body weight gain (g)
0
10
20
30
40
50
60
G9
G10
G11
G12
G13
G16
G17
G18
G19
G20
Gestational day
*
B
Placental weight (mg)
0
100
200
300
400
500
600
700
**
**
G13
G20
Control
VPA

Supplemental Figure 1



Supplemental Figure 2