



Title	Uptake of antiepileptic drugs in forskolin-induced differentiated BeWo cells: alteration of gabapentin transport
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Citation	Xenobiotica, 52(4), 405-412 <a href="https://doi.org/10.1080/00498254.2022.2085635">https://doi.org/10.1080/00498254.2022.2085635</a>
Issue Date	2022-06-16
Doc URL	<a href="http://hdl.handle.net/2115/89953">http://hdl.handle.net/2115/89953</a>
Rights	This is an Accepted Manuscript of an article published by Taylor & Francis in Xenobiotica on 16th Jun 2022, available online: <a href="http://www.tandfonline.com/10.1080/00498254.2022.2085635">http://www.tandfonline.com/10.1080/00498254.2022.2085635</a> .
Type	article (author version)
File Information	Koishikawa et al.pdf



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# **Uptake of antiepileptic drugs in forskolin-induced differentiated BeWo cells: Alteration of gabapentin transport**

1. Previous studies have indicated that the expression levels of several transporters are altered during placental trophoblast differentiation. However, changes in the transport activities of therapeutic agents during differentiation must be comprehensively characterised. Antiepileptic drugs, including gabapentin (GBP), lamotrigine (LTG), topiramate, and levetiracetam, are increasingly prescribed during pregnancy. The objective of this study was to elucidate differences in the uptake of antiepileptic drugs during the differentiation process.

2. Human placental choriocarcinoma BeWo cells were used as trophoblast models. For differentiation into syncytiotrophoblast-like cells, cells were treated with forskolin.

3. The uptake of GBP and LTG was lower in differentiated BeWo cells than in undifferentiated cells. In particular, the maximum uptake rate of GBP transport was decreased in differentiated BeWo cells. Furthermore, GBP transport was trans-stimulated by the amino acids His and Met. We investigated the profiles of amino acids in undifferentiated and differentiated BeWo cells. Supplementation with His and Met, which demonstrated trans-stimulatory effects on GBP uptake, restored GBP uptake in differentiated cells. The findings of this study suggest that drug transport in BeWo cells can be altered before and after differentiation, and that the altered GBP uptake could be mediated by the intracellular amino acid status.

Keywords: BeWo; placenta; trophoblast; differentiation; antiepileptic drug; gabapentin; transporter

## 1. Introduction

Epilepsy is one of the most common neurological complications in pregnant women (Laskowska et al. 2001). For drug therapy during pregnancy, it is necessary to assess the effects of drugs throughout the gestational period, from early teratogenicity to late foetal toxicity. Antiepileptic drugs, including gabapentin (GBP), lamotrigine (LTG), topiramate (TPM), and levetiracetam (LEV), are increasingly prescribed during the gestational period (Vajda et al. 2014); these drugs can pass from the mother to the foetus. Umbilical cord-to-maternal plasma GBP concentration ratios reportedly range from 1.3 to 2.1 (Ohman et al. 2005). Ohman et al. (2000) reported that umbilical cord-to-maternal LTG concentration ratios range between 0.6 and 1.3. Furthermore, studies have reported that umbilical cord-to-maternal plasma concentration ratios of LEV and TPM range from 0.56 to 2.0 and 0.85 to 1.06, respectively (Tomson et al. 2007; Ohman et al. 2002). Therefore, understanding the transport characteristics of antiepileptic drugs and variable factors in the placenta could help estimate the associated risks and provide a strategy to protect the foetus. We had previously investigated the accumulation of these drugs in human placental choriocarcinoma BeWo cells (Furugen et al. 2015), demonstrating that GBP and LTG are transported to the cells via carrier-mediated mechanisms (Furugen et al. 2017; Hasegawa et al. 2020). In particular, L-type amino acid transporter 1 (LAT1/*SLC7A5*) was found to contribute to this process, and GBP was present in high concentrations (Furugen et al. 2017).

In placental villi, nutrients, drugs, and waste products are exchanged between the mother and the foetus. On the villous surface, mononuclear cytotrophoblast cells (CT cells) are the predominant cells present in early pregnancy, and these cells differentiate into multinuclear syncytiotrophoblast cells (ST cells) as pregnancy progresses (Tetro et al. 2018). Both morphological and biochemical changes have been observed during the differentiation of CT cells into ST cells. For example, human chorionic gonadotropin (hCG) secretion and syncytin-1 expression increase during differentiation. hCG, a hormone produced during pregnancy, promotes progesterone production from the luteum, the fusion of CT cells, and their differentiation into ST cells (Cole 2010). Syncytin-1 (*HERV-W*) is an endogenous retrovirus envelope protein that plays a role in the membrane fusion of CT cells (Bastida-Ruiz et al. 2016). hCG and syncytin-1 are widely employed as markers of trophoblast differentiation. It has been observed that the differentiation of CT cells into ST cells can be reproduced *in vitro* by isolating and culturing CT cells from the human placenta (Kliman et al. 1986). Primary human trophoblast cells have the advantage of retaining the property of normal cells. Conversely, the experimental procedure is complicated, and the obtained primary human trophoblast cells possess a low proliferation capacity. Therefore, as an alternative, the human

placental choriocarcinoma cell line, BeWo cells, can be induced to differentiate into ST-like cells by stimulation with forskolin, an adenylate cyclase activator (Rothbauer et al. 2017).

Placental transporters play a role in nutrient and xenobiotic transport between the mother and the foetus (Tetro et al. 2018); hence, the expression and function of transporters affect the *in utero* environment. A previous study revealed that the mRNA expression of several transporters is associated with the differentiation of CT cells into ST cells (Kallol et al. 2018). Furthermore, several previous reports have demonstrated changes in nutrient transport during differentiation (Kudo and Boyd. 2002; Huang et al. 2009). However, changes in the transport function of xenobiotics during differentiation have not yet been comprehensively elucidated. Therefore, we investigated the uptake of antiepileptic drugs by forskolin-induced BeWo cells and the possible underlying mechanisms.

## **2. Materials and methods**

### ***2.1. Cell culture***

BeWo cells were cultured as previously described (Furugen et al. 2015). After culturing for 2 days, 20  $\mu\text{M}$  or 100  $\mu\text{M}$  forskolin and 0.1% dimethyl sulfoxide DMSO (control) were added to the medium for 48 h as detailed in a previous report (Huang et al. 2009).

### ***2.2. Chemicals***

Forskolin was purchased from Wako Pure Chemical Industries (Osaka, Japan). All amino acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antiepileptic drugs were purchased from the Tokyo Chemical Industry (Tokyo, Japan).

### ***2.3. hCG enzyme-linked immunosorbent assay***

BeWo cells ( $1.0 \times 10^5$  cells/well) were seeded on 24-well plastic plates and cultured as described in section 2.1. The cell culture medium was collected and centrifuged at 1,500 rpm at 4° C for 10 min. hCG enzyme-linked immunosorbent assay (ELISA) kits were purchased from Abcam (Cambridge, UK). ELISA was performed according to the manufacturer's instructions. Each sample was diluted 150 times with assay diluent B.

#### ***2.4. Real-time PCR***

BeWo cells ( $2.0 \times 10^5$  cells/well) were seeded on 12-well plastic plates and cultured. Total RNA was isolated from BeWo cells, and single-stranded cDNA was obtained from total RNA by reverse transcription, as previously described (Furugen et al. 2017). Real-time PCR was performed using the KAPA SYBR Fast qPCR Kit (KAPA Biosystems, Boston) using the Mx3000™ real-time PCR system (STRATAGENE) and specific primers (Supplemental Table 1) through 40 cycles of 95° C for 3 s and 60° C for 20 s. Relative mRNA levels of target genes were normalised to  $\beta$ -actin as a reference gene (Knerr et al. 2005).

#### ***2.5. Antiepileptic drug uptake***

BeWo cells ( $1.0 \times 10^5$  cells/well) were seeded on 24-well collagen-coated plastic plates and cultured. To examine the transport activities of four antiepileptic drugs (GBP, LEV, LTG, and TPM) in BeWo cells, cells cultured for 48 h with forskolin treatment were employed. Uptake experiments were performed as previously described (Furugen et al. 2017). Briefly, cells were incubated for 5 min at 37° C with transport buffer (pH 7.4) containing 100  $\mu$ M of each antiepileptic drug. A concentration-dependent GBP uptake study was performed using drug concentrations ranging from 3.1 and 500  $\mu$ M. The effect of LAT1 inhibitors, including 2-amino-2-norbornanecarboxylic acid (BCH) (Prasad et al. 1999) and JPH203 (Wempe et al. 2012), on GBP uptake were investigated by incubating cells with transport buffer containing 100  $\mu$ M GBP in the presence or absence of the inhibitors. To investigate the short-term effects of forskolin on GBP transport, BeWo cells were treated for 14.5 min with 20  $\mu$ M and 100  $\mu$ M forskolin, followed by the determination of GBP uptake. The treatment duration for short-term forskolin was reported in a previous study (Delidaki et al. 2011). The drug concentration was determined using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/MS/MS) as previously described (Furugen et al. 2015). The uptake amount was normalised to the protein level determined using a BCA assay.

#### ***2.6. Amino acid-preloaded GBP uptake***

BeWo cells ( $1.0 \times 10^5$  cells/well) were seeded on 24-well collagen-coated plastic plates and cultured. Cells were washed with transport buffer and preloaded at 37° C for 10 min with 0.5 mL transport buffer containing 1 mM of each amino acid. The buffer was then removed and the cells were immediately rinsed twice using ice-cold transport buffer. Next, the cells were incubated for 5 min at 37° C with transport buffer containing 100  $\mu$ M GBP. After incubation, the buffer was removed and the cells were rinsed using ice-cold transport buffer.

## **2.7. Western blotting**

BeWo cells ( $4.0 \times 10^5$  cells/well) were seeded on 6-well plastic plates for whole protein analysis. After induction of differentiation by forskolin, cells were lysed using ice-cold RIPA buffer (Cell Signaling Technology, Beverly, MA). For analysis of membrane proteins, BeWo cells ( $2.0 \times 10^6$  cells/dish) were seeded on a 60.1 cm<sup>2</sup> dish. After forskolin-induced differentiation, membrane protein extraction was performed using the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific). Western blotting was performed as previously described (Furugen et al. 2017). The bands were detected with Image Quant LAS 4000 (GE Healthcare, Buckinghamshire, England). The band intensities were analyzed using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

## **2.8. Intracellular amino acid concentrations in BeWo cells**

BeWo cells ( $2.0 \times 10^6$  cells/dish) were seeded on a 60.1 cm<sup>2</sup> dish and cultured. The cells were treated with forskolin (100 μM) or DMSO (control) for 48 h. After removal of the culture medium, the cells were washed four times using 10 mL of ice-cold phosphate-buffered saline (PBS). The cells were scraped, and the suspension was centrifuged at  $100 \times g$  for 2 min at 4° C. Quantification of free amino acids in cell pellets was performed by employing a pre-label method using high-pressure liquid chromatography (HPLC) by IDEA Consultants, Inc. (Tokyo, Japan). The amino acid amount was normalised to the protein level determined by the BCA assay.

## **2.9. Statistical analyses**

Data are presented as the mean  $\pm$  standard error (S.E.) of independent experiments. Experiments were repeated at least three times. Student's t-test was used for comparisons between two groups. Dunnett's test or Tukey–Kramer test was used for multiple comparisons. A  $p$ -value  $< 0.05$  was considered statistically significant. Nonlinear regression analysis was performed using SigmaPlot 12.5 (HULINKS). Kinetic parameters were calculated using the following equation:

$$v = V_{max} \times s / (K_m + s) + a \times s$$

where  $v$  is the uptake rate of the compound,  $s$  is the compound concentration,  $a$  is the passive diffusion coefficient,  $V_{max}$  is the maximum uptake rate, and  $K_m$  is the Michaelis-Menten constant. The parameters ( $K_m$ ,  $V_{max}$ ) were evaluated from each independent uptake experiment and the mean values of the parameters from three experiments are shown. The statistical differences in the parameters between the control and forskolin (100 μM) groups were analyzed using the Mann–Whitney U test.

### 3. Results

#### *3.1. Confirmation of forskolin-induced differentiation in BeWo cells*

We assessed the differentiation of BeWo cells after treatment with forskolin (20 and 100  $\mu\text{M}$ ) for 48 h using hCG secretion and syncytin-1 mRNA expression as differentiation markers. As shown in Figure 1, hCG secretion and syncytin-1 mRNA levels were significantly increased, as previously reported (Huang et al. 2009). Therefore, BeWo cells differentiated into ST-like cells under the present conditions.

#### *3.2. Uptake of antiepileptic drugs during differentiation of BeWo cells*

Changes in the accumulation of four antiepileptic drugs (GBP, LEV, LTG, and TPM) were investigated during differentiation. As shown in Figure 2A, after forskolin treatment, the uptake of GBP and LTG significantly decreased compared with the control, whereas LEV and TPM uptake did not change significantly. The uptake of GBP was reduced by 44% (20  $\mu\text{M}$  forskolin) and 55% (100  $\mu\text{M}$  forskolin), respectively, compared with the control (Figure 2B). Among the four antiepileptic drugs investigated, GBP transport was the most significantly altered. Hence, we assessed the mechanism underlying the reduction in GBP transport capacity induced by forskolin.

#### *3.3. Effect of forskolin treatment on concentration-dependent GBP uptake*

The effects of forskolin treatment on concentration-dependent uptake were investigated to determine the kinetics of GBP uptake. As shown in Figure 2C, GBP transport was saturated at high concentrations in both control and 100  $\mu\text{M}$  forskolin-treated groups. The values of  $K_m$  and  $V_{max}$  were  $52.3 \pm 10.2 \mu\text{M}$  and  $3813.6 \pm 927.8 \text{ pmol/mg protein/min}$ , respectively, for the control and  $29.1 \pm 4.8 \mu\text{M}$  ( $p = 0.10$ , compared to the control) and  $1260.1 \pm 201.8 \text{ pmol/mg protein/min}$  ( $p < 0.05$ , compared to the control), respectively, for 100  $\mu\text{M}$  forskolin-treated cells, showing that  $V_{max}$  decreased.

#### *3.4. Effect of short-term forskolin exposure on GBP transport*

Forskolin is an adenylate cyclase activator that increases intracellular cAMP levels and activates various signalling pathways (Delidakis et al. 2011). To assess whether the effect of forskolin was directly mediated by changes in transporter activity, the effect of short-term (14.5 min) forskolin exposure on GBP transport was investigated. The exposure time was based on a previously

reported condition that showed an increase in intracellular cAMP levels in BeWo cells after exposure to 100  $\mu$ M forskolin (Delidaki et al. 2011). As shown in Supplemental Figure 1, GBP uptake was not significantly altered following short-term forskolin exposure.

### ***3.5. Changes in LAT1 and 4F2hc (CD98) expression induced by forskolin***

Previously, we had reported that LAT1/*SLC7A5* is involved in GBP transport in undifferentiated BeWo cells (Furugen et al. 2017). Therefore, we investigated the effects of LAT1 inhibitors on GBP transport under forskolin stimulation were investigated. As shown in Figure 3A, an inhibitor of the L-type amino acid transporter (2-amino-2-norbornanecarboxylic acid, BCH) (Prasad et al. 1999) significantly reduced GBP uptake under control conditions. BCH reduced GBP uptake under forskolin treatment ( $P = 0.08$ ) compared to forskolin alone. Furthermore, the LAT1-specific inhibitor (JPH203) (Wempe et al. 2012) (reduced GBP uptake under both control and forskolin treatments Figure 3B). These results suggest that LAT1 contributes to GBP uptake in undifferentiated and differentiated BeWo cells. Next, LAT1 mRNA and protein expressions were investigated. LAT1 mRNA expression increased by 288% (20  $\mu$ M forskolin) and 286% (100  $\mu$ M forskolin), compared with the control (Figure 4A). Similarly, LAT1 whole protein expression was increased by 202% (20  $\mu$ M forskolin) and 169% (100  $\mu$ M forskolin), compared with the control (Figure 4C). Furthermore, the auxiliary protein 4F2hc (*SLC3A2*), reportedly involved in the membrane transfer of LAT1 (Yanagida et al. 2001), increased at the mRNA level by 204% (20  $\mu$ M forskolin) and 219% (100  $\mu$ M forskolin), compared with the control (Figure 4B). Although we considered the possibility of reduced membrane LAT1 expression, the expression of LAT1 protein was not reduced following forskolin exposure (Supplemental Figure 2). These results suggested that altered LAT1 and 4F2hc expression levels did not contribute to changes in GBP transport activity after forskolin treatment.

### ***3.6. Examination of exchange substrates in GBP uptake***

Subsequently, we focused on the transport characteristics of LAT1 by evaluating the mechanisms underlying reduced GBP uptake following differentiation. LAT1 is an  $\text{Na}^+$ -independent and pH-independent exchanger that influences the amino acid substrate entering cells by interacting with intracellular amino acids (Scalise et al. 2018; Yanagida et al. 2001). LAT1 transport has been reported to be functionally coupled with other amino acid transporters (Nicklin P et al. 2009). Leu transport mediated by LAT1 is dependent on Gln, with Leu actively delivered into cells by an  $\text{Na}^+$ -dependent amino acid transporter (*ASCT2/SLC1A5*) (Nicklin P et al. 2009). However, the amino acids that are highly exchanged with GBP and the transporter coupled with LAT1 in BeWo cells have not been comprehensively elucidated. To determine the exchanged



intracellular amino acids, GBP uptake after amino acid preloading was investigated. As shown in Figure 5, GBP uptake significantly increased after His and Met preloading, by 231% and 231%, respectively. These results indicated that GBP uptake in BeWo cells can be enhanced using intracellular amino acids, particularly His and Met.

### ***3.7. Concentration of amino acids in BeWo cells***

Because GBP influx was suggested to occur via an exchange with amino acids in BeWo cells, free amino acid concentrations in the cells were investigated. The relative amino acid concentrations in undifferentiated BeWo cells were as follows: Glu > Pro > Asp > Gly > Ala > Arg > Asn > Gln > Thr > Ser > His > Val > Leu > Lys > Trp > Met > Phe > Tyr > Ile (Table 1). Cys levels were below the lower limit of quantification. Pro in differentiated BeWo cells significantly decreased to 51% of the concentration in undifferentiated cells.

### ***3.8. Effects of amino acids on reduced GBP uptake in differentiated BeWo cells***

Finally, we investigated whether amino acid supplementation could restore the reduced GBP uptake in differentiated BeWo cells. His and Met, which demonstrated trans-stimulation effects on GBP uptake (Figure 5) restored it in differentiated BeWo cells (Figure 6). In contrast, the cationic amino acid Lys and the sulfur-containing amino acid Cys, which did not demonstrate trans-stimulatory effects on GBP uptake, showed no recovery effect in differentiated BeWo cells. These findings suggest that the altered GBP uptake in differentiated BeWo cells could be ameliorated by intracellular amino acid status.

## **4. Discussion**

In the present study, we found that GBP transport in BeWo cells decreased following forskolin-induced differentiation. We hypothesised that intracellular amino acid levels may contribute to this phenomenon. Furthermore, the amino acids (His and Met) may be exchanged for GBP influx in BeWo cells.

The relative intracellular concentrations of the antiepileptic drugs assessed in this study in undifferentiated BeWo cells is as follows: GBP > LTG > LEV  $\approx$  TPM (Furugen et al. 2015). Considering their intracellular volume, GBP and LTG can be transported into BeWo cells by carriers. The uptake of LTG into BeWo cells is mediated by a carrier that is sensitive to chloroquine, imipramine, quinidine, and verapamil (Hasegawa et al. 2020). In the present study, LTG uptake was decreased after BeWo differentiation. Although the detailed mechanism remains unclear, the

expression and function of a carrier may be involved in the observed alteration. Regarding GBP, LAT1 is reportedly involved in the uptake process in BeWo cells (Furugen et al. 2017). However, the mRNA expression levels of LAT1 and the auxiliary protein 4F2hc increased after forskolin-induced differentiation (Figure 4). The observation that LAT1 and 4F2hc expression increased after differentiation is consistent with previous reports (Balthasar et al. 2017; Kallol et al. 2018; Zaugg et al. 2020). Previously, it was reported that the expression of the carnitine transporter (OCTN2/*SLC22A5*) increases following forskolin-induced syncytialization; however, the activity of carnitine transport remains unchanged (Huang et al. 2009). Considering the possible discrepancy between expression and function, it is essential to assess the transport activity of targeted drugs.

In the present study, we focused on the transport characteristics of LAT1 as an exchanger in the mechanism of reduced GBP uptake after differentiation. Previously, we reported that the intracellular GBP concentration was markedly higher in the extracellular compartment in undifferentiated BeWo cells. However, LAT1 is a Na<sup>+</sup>-independent and pH-independent exchanger that influences the amino acid substrate entering cells via exchange with intracellular amino acids (Scalise et al. 2018; Yanagida et al. 2001), and the present study indicates that GBP uptake in undifferentiated BeWo cells can be stimulated by the preloading of several amino acids, with His and Met demonstrating the highest facilitatory effects. These amino acids were detected in undifferentiated BeWo cells (Table 1). Moreover, these amino acids have also been detected in human placental villi in the first and third trimesters and most amino acids are significantly decreased in placental villi at term (Sooranna et al. 1994). These findings suggest that GBP exchange with amino acids may occur in the placenta, and that the concentration of amino acids in placental villi is altered throughout gestation. Supplementation with His and Met, which can be exchanged for GBP, restored GBP uptake in differentiated BeWo cells (Figure 6). In contrast, another cationic amino acid (Lys) and sulfur-containing amino acid (Cys), which did not demonstrate trans-stimulatory effects on GBP uptake, failed to alleviate uptake. These results indicate that altered GBP uptake in differentiated BeWo cells could be ameliorated by the intracellular amino acid status.

As a candidate transporter that is functionally coupled with LAT1, ASCT2 function has been implicated in HeLa cells (Nicklin et al. 2009). Furthermore, ASCT2 knockdown reduced Leu uptake in human melanoma cells, suggesting that Leu transport is dependent on ASCT2-mediated Gln transport (Wang et al. 2017). Meanwhile, reports have indicated that ASCT2 knockdown does not affect Leu uptake in colon LS174T and lung A549 cell lines (Cormerais et al. 2018). Reportedly, the sodium-neutral amino acid transporter SNAT1/2 (*SLC38A1/2*) has been implicated in the maintenance of intracellular Gln levels in the absence of ASCT2 (Bröer et al. 2016). Furthermore, the present study suggests that His and Met are more favourable intracellular substrates than Gln for exchange with GBP. It has been reported that His is a bidirectionally transported substrate and a good exchange substrate for transport by LAT1 (Scalise et al. 2018). Furthermore, LAT1 mainly contributes to Met influx into human trophoblasts (Araújo et al. 2013). His and Met are essential

amino acids that are indispensable to the foetus. Met is required not only for foetal protein synthesis and as an energetic substrate for fetal oxidative catabolism, but also for the production of S-adenosyl methionine, which is the principal methyl group donor in mammalian cells and is thus essential for methylation reactions (Araújo et al. 2013). Further studies are required to comprehensively assess the contribution of these transporters and amino acids on GBP transport.

In addition to epilepsy, GBP is utilised for treating various disorders, including restless leg syndrome and pain. It has been reported that GBP may be effective for the treatment of hyperemesis gravidarum (Guttuso et al. 2021). Considering the increasing number of patients who may use this drug, information regarding foetal safety is crucial. A previous report indicated that GBP is transferred from the mother to the foetus via the placenta (Ohman et al. 2005). In addition, GBP plasma concentrations in infants range from 12% to 36% (mean, 27%) of umbilical cord plasma levels. We previously reported that GBP uptake is inhibited by amino acids transported by LAT1 (Furugen et al. 2017). In the present study, GBP uptake was found to be stimulated by several intracellular amino acids. These results imply that plasma and placental amino acid levels may affect GBP transfer to the foetus. Simultaneously, the amino acid concentrations in both the placenta and foetus may be altered following GBP administration. The use of GBP during pregnancy may increase the risk of preterm birth and low birth weight (Fujii et al. 2013). Recently, it was reported that there is a high risk of neonatal intensive care unit admission, small for gestational age, and preterm birth among women treated with GBP during pregnancy, particularly in late pregnancy (Patorno et al. 2020). Antiepileptic drugs are transported into the brain via the blood-brain barrier to exert their effects; however, there are several unknown aspects regarding their effects on the foetal brain (Goasdoué et al. 2017). Future studies are needed to assess the possibility of an association between these risks and the exposure level of GBP and the function of LAT1.

In conclusion, we demonstrated that GBP transport significantly decreased following BeWo differentiation. As a possible mechanism, intracellular amino acid status may be involved in the reduced GBP uptake after differentiation. Furthermore, the current study suggests that the transport function of xenobiotics varies according to trophoblastic differentiation stage. Further studies are required to investigate this phenomenon in normal trophoblasts.

## **Acknowledgments**

This study was supported by a grant from the Noastec Foundation (grant number H29-ST-14) (provided to A.F.).

## Conflicts of Interest

Shuhei Ishikawa has received personal fees from Janssen Pharmaceutical, Dainippon Sumitomo Pharma, Eisai, Meiji Seika Pharma, Takeda Pharmaceutical Company Limited, Lundbeck, Otsuka Pharmaceutical and has received research/grant support from Eli Lilly.

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## Figure Captions

Figure 1. Effects of forskolin on human chorionic gonadotropin (hCG) secretion (A) and syncytin-1 mRNA expression (B) in BeWo cells. Cells were treated for 48 h with culture medium containing forskolin (20  $\mu$ M or 100  $\mu$ M) or DMSO (control). After treatment, hCG concentration in the culture medium was quantified by enzyme-linked immunosorbent assay (ELISA). Syncytin-1 mRNA expression was evaluated by real-time PCR. Data are presented as mean with standard error (S.E.) for three independent experiments, each performed in triplicate. \*\* :  $p < 0.01$ , \* :  $p < 0.05$  compared with the control.

Figure 2. (A and B) Effects of forskolin treatment on the uptake of antiepileptic drugs in BeWo cells. The uptake of levetiracetam (LEV), topiramate (TPM), lamotrigine (LTG), and gabapentin (GBP). BeWo cells were treated for 48 h in a culture medium containing forskolin (20  $\mu$ M or 100  $\mu$ M) or DMSO (control). After forskolin treatment, cells were incubated in transport buffer containing 100  $\mu$ M of LEV, TPM, LTG, or TPM for 5 min at 37° C. Concentrations of antiepileptic drugs were quantified by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/MS/MS). Data are presented as the mean standard error (S.E.) of three independent experiments, each performed in triplicate. \*\* :  $p < 0.01$ , \* :  $p < 0.05$  compared with the control. (C) Effect of forskolin on concentration-dependence of GBP uptake. After the treatment with 100  $\mu$ M forskolin, BeWo cells were incubated with a transport buffer containing 3.1-500  $\mu$ M GBP for 5 min at 37° C. Data are presented as mean with S.E. for three independent experiments, each performed in triplicate.

Figure 3. Effects of L-type amino acid transporter (LAT) inhibitors, 2-Amino-2-norbornanecarboxylic acid (BCH) (A) and JPH203 (B), on the uptake of gabapentin (GBP) in BeWo cells. Cells were treated for 48 h with culture medium containing forskolin (100  $\mu$ M) or DMSO (control). After forskolin treatment, cells were incubated in transport buffer containing 100  $\mu$ M of GBP in the presence or absence of 10 mM BCH (A) or 10  $\mu$ M JPH203 (B) for 5 min at 37° C. The concentration of GBP was quantified by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/MS/MS). Data are presented as the mean standard error (S.E.) of three or five independent experiments, each performed in triplicate. \*\* :  $p < 0.01$  compared with the control. †† :  $p < 0.01$  compared with forskolin alone.

Figure 4. Effects of forskolin treatment on L-type amino acid transporter 1 (LAT1) mRNA (A), 4F2hc mRNA (B), and LAT1 protein (C) in BeWo cells. (A and B) Cells were treated for 48 h with culture medium containing forskolin (20  $\mu$ M or 100  $\mu$ M) or DMSO (control). After treatment, mRNA expression was evaluated by real-time PCR. Data are presented as mean with standard error (S.E.) for three independent experiments, each performed in triplicate. (C) After treatment, whole



LAT1 protein was evaluated by western blotting. Data are presented as mean with S.E. for three independent experiments. \*\* :  $p < 0.01$ , \* :  $p < 0.05$  compared with the control.

Figure 5. Effects of preloading with amino acids on gabapentin (GBP) uptake. After preloading with 1 mM of each amino acid for 10 min, undifferentiated BeWo cells were incubated in transport buffer containing 100  $\mu$ M GBP for 5 min. The concentration of GBP was quantified by liquid chromatography/electrospray ionization tandem mass spectrometry (LC/MS/MS). Data are presented as mean with standard error (S.E.) for three or four independent experiments. \*\* :  $p < 0.01$ , \* :  $p < 0.05$  compared with the control.

Figure 6. Effects of preloading with amino acids on decreased gabapentin (GBP) uptake in differentiated BeWo cells. Cells were treated for 48 h in culture medium containing forskolin (100  $\mu$ M) or DMSO (control). After differentiation, cells were treated with 1 mM of amino acid (His, Lys, Met, or Cys) for 10 min, and then incubated with a transport buffer containing 100  $\mu$ M GBP for 5 min. Data are presented as mean with standard error (S.E.) for three to four independent experiments, each performed in triplicate. \*\* :  $p < 0.01$  compared to the control. †† :  $p < 0.01$  compared with forskolin alone.

Supplemental Figure 1. Effect of short-term forskolin treatment on gabapentin (GBP) uptake. After BeWo cells were cultured, they were incubated in transport buffer containing forskolin (20  $\mu$ M or 100  $\mu$ M) or DMSO (control) for 14.5 min and subsequently incubated with 100  $\mu$ M GBP for 5 min. Data are presented as mean with standard error (S.E.) for three independent experiments, each performed in triplicate.

Supplemental Figure 2. Effects of forskolin treatment on the membrane expression of L-type amino acid transporter 1 (LAT1) protein. (A) Western blotting of LAT1 protein in the cytosol and plasma membrane. (B) Cells were treated for 48 h in a culture medium containing forskolin (20  $\mu$ M or 100  $\mu$ M) or DMSO (control). After treatment, the membrane LAT1 protein level was evaluated by western blotting. Data are presented as mean with standard error (S.E.) for three independent experiments.

**Table 1 Quantification of intracellular amino acids in BeWo cells.**

	Amino acids (nmol/mg protein)						<i>p</i> -value
	Control			Forskolin			
Trp	1.17	±	0.89	1.15	±	1.0	0.989
Phe	1.02	±	0.42	0.58	±	0.24	0.402
Tyr	0.85	±	0.36	0.51	±	0.20	0.436
Leu	1.92	±	0.81	1.14	±	0.41	0.424
Ile	0.54	±	0.26	0.43	±	0.17	0.743
Val	2.20	±	0.55	1.46	±	0.34	0.300
Met	1.02	±	0.42	0.70	±	0.24	0.533
Cys	< LLOQ			< LLOQ			-
Thr	3.05	±	0.50	1.72	±	0.31	0.066
Ser	2.83	±	1.21	1.40	±	0.63	0.337
Gln	3.71	±	1.44	4.06	±	0.66	0.829
Asn	4.27	±	0.75	2.17	±	0.45	0.052
Arg	5.96	±	0.67	5.24	±	0.55	0.434
Lys	1.56	±	0.42	1.14	±	0.20	0.408
His	2.78	±	0.37	1.75	±	0.28	0.069
Gly	9.68	±	1.41	6.60	±	0.97	0.123
Ala	7.32	±	0.94	6.38	±	0.54	0.419
Asp	13.3	±	2.48	10.2	±	1.18	0.299
Glu	31.8	±	7.29	34.7	±	2.99	0.727
Pro	13.5	±	1.32	6.82	±	1.43	0.014

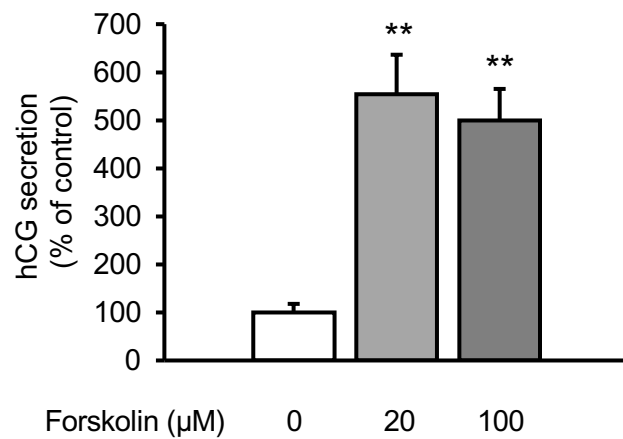
Cells were treated for 48 h with culture medium containing forskolin (100  $\mu$ M) or DMSO (control). Quantification of free amino acids in cell pellets was performed using a pre-label method using high-pressure liquid chromatography (HPLC). LLOQ, lower limits of quantification. Data are presented as mean with standard error (S.E.) for four independent experiments.

**Supplemental Table 1 Primer sequences**

Name	Sequence		Product size
Syncytin-1 ( <i>ERVW-1</i> )	Forward	5'-GGTACATGGCACCTCTAGCCCCTAC -3'	191
	Reverse	5'-CAGGGATTGAAACATATGGCCTGAAGTTC -3'	
LAT1 ( <i>SLC7A5</i> )	Forward	5'-TCATCATCCGGCCTTCATCG -3'	159
	Reverse	5'-TCACGCTGTAGCAGTTCACG -3'	
4F2hc ( <i>SLC3A2</i> )	Forward	5'-TTGGCTGAGTGGCAAATATCA -3'	104
	Reverse	5'-GATTCGAGTAGGCTCAGGATCTG -3'	
$\beta$ -actin ( <i>ACTB</i> )	Forward	5'-TGGCACCCAGCACAATGAA -3'	186
	Reverse	5'-CTAAGTCATAGTCCGCCTAGAAGCA -3'	

Figure 1

(A)



(B)

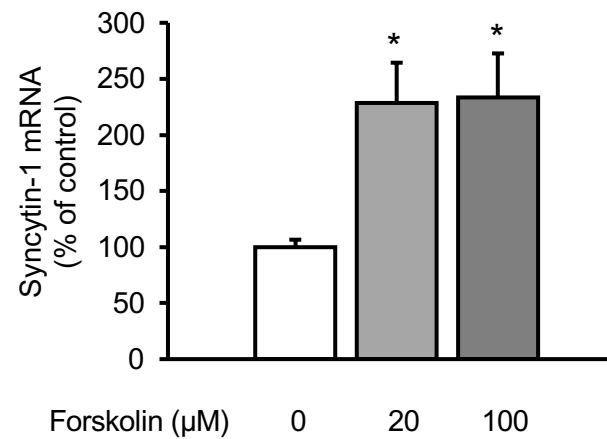
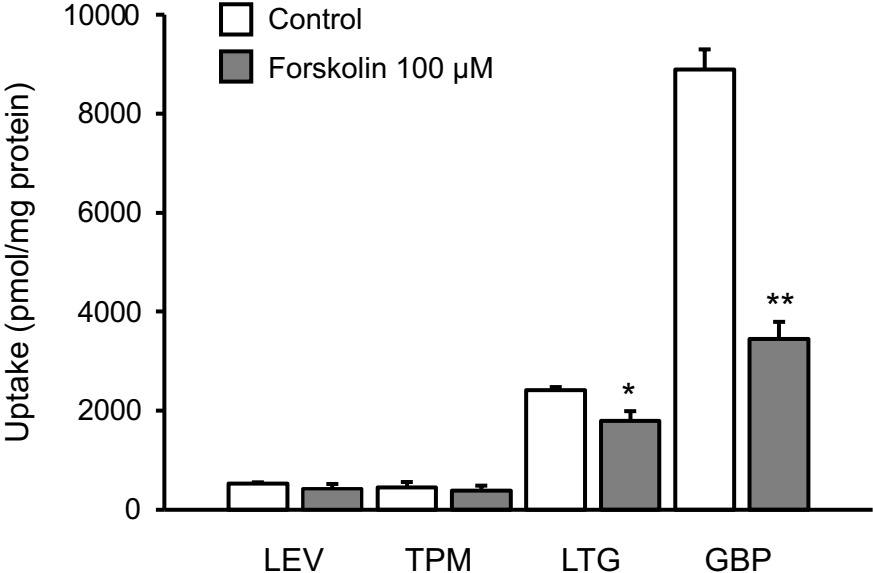
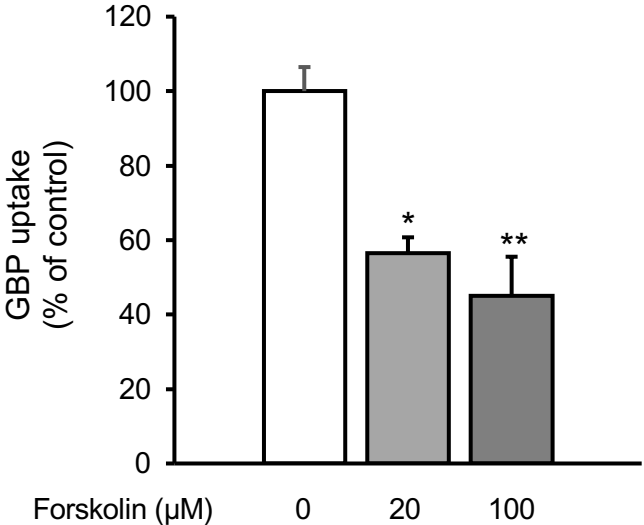


Figure 2

(A)



(B)



(C)

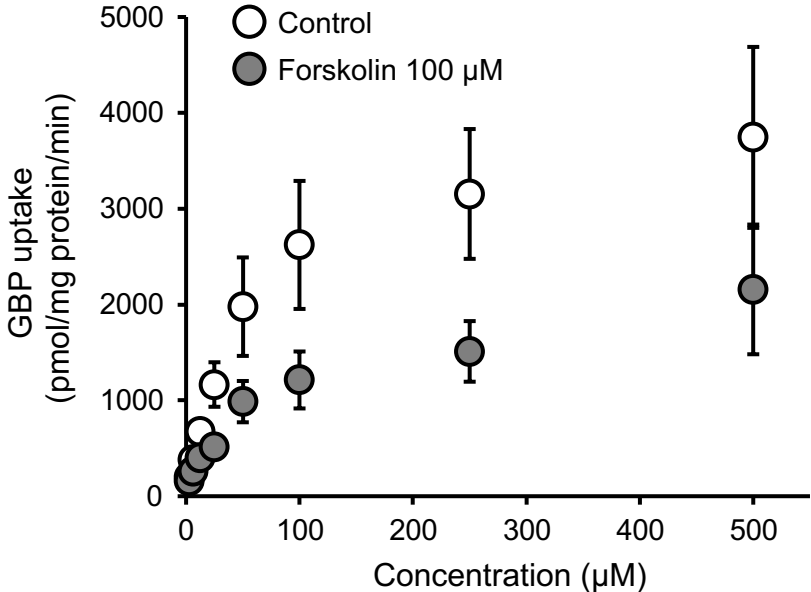
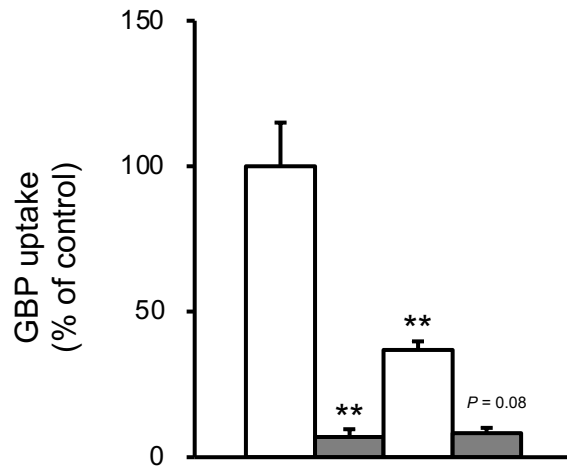


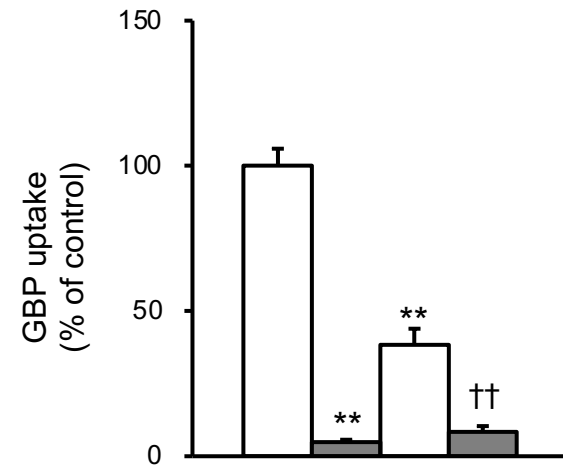
Figure 3

(A)



Forskolin (100 μM)	-	-	+	+
BCH (10 mM)	-	+	-	+

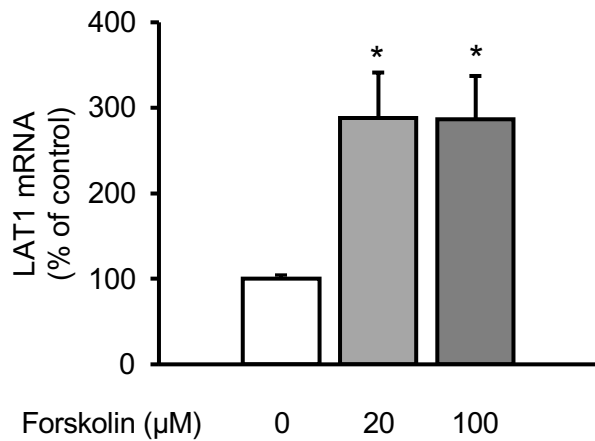
(B)



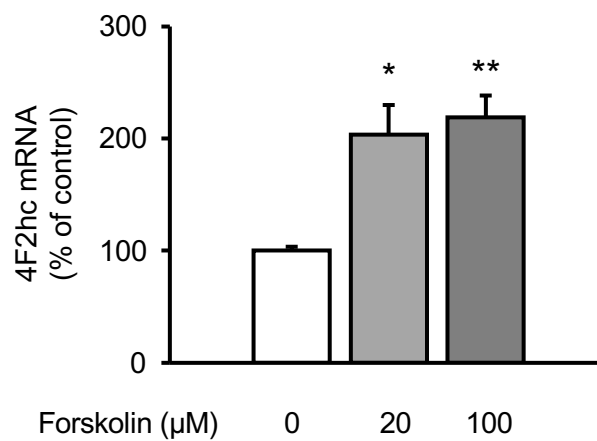
Forskolin (100 μM)	-	-	+	+
JPH203 (10 μM)	-	+	-	+

Figure 4

(A)



(B)



(C)

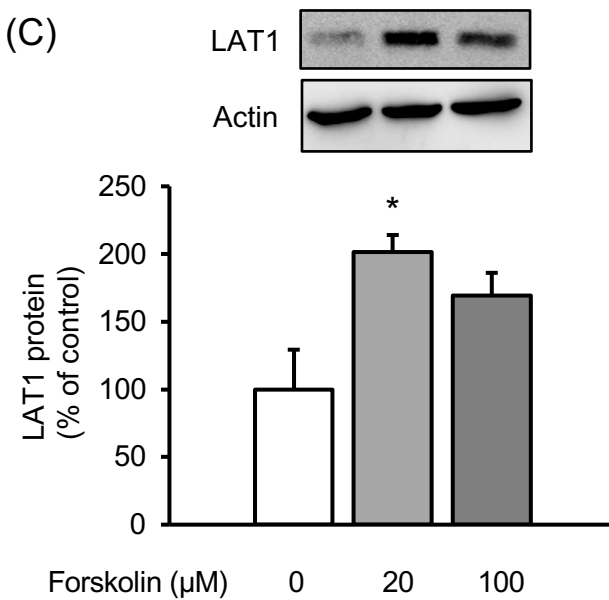


Figure 5

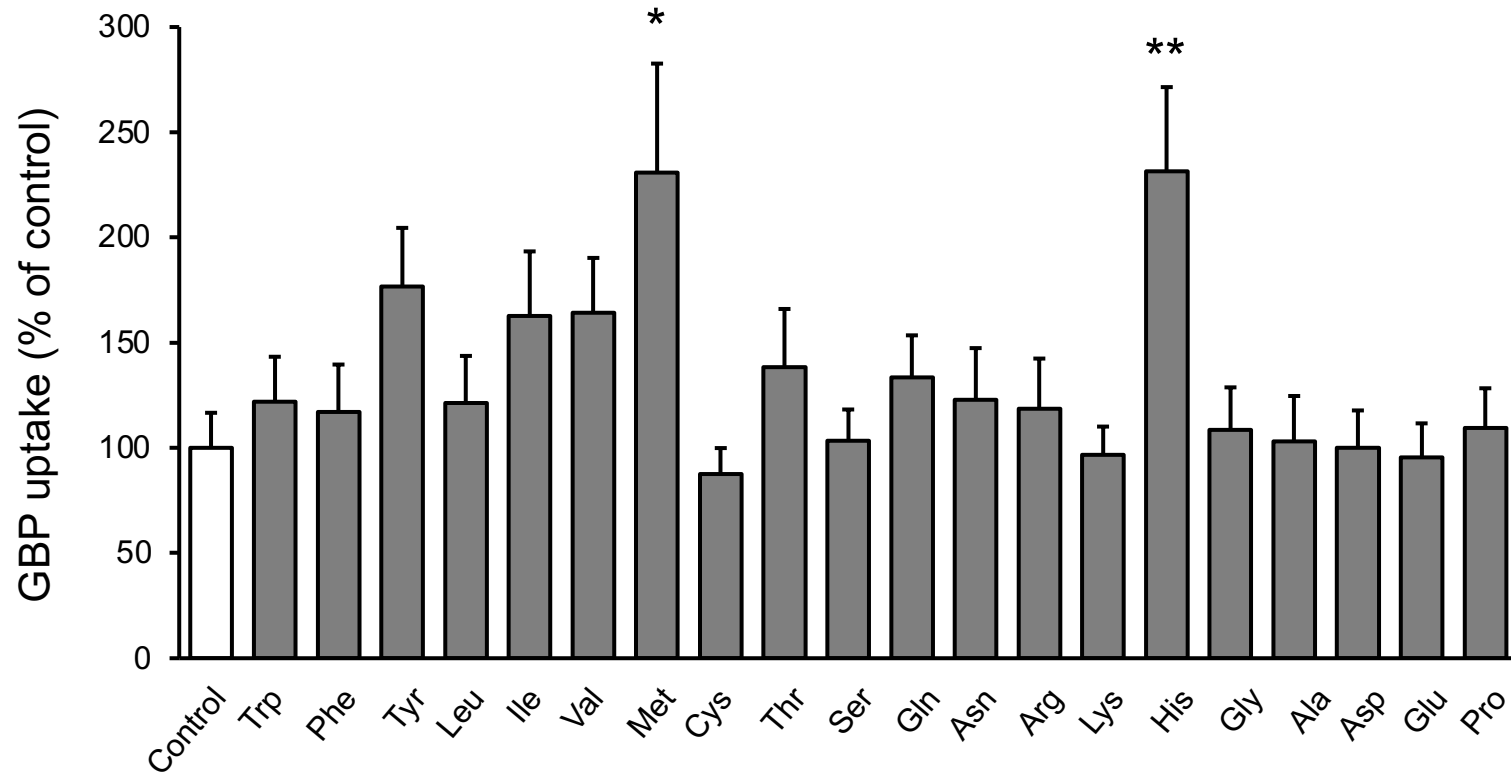
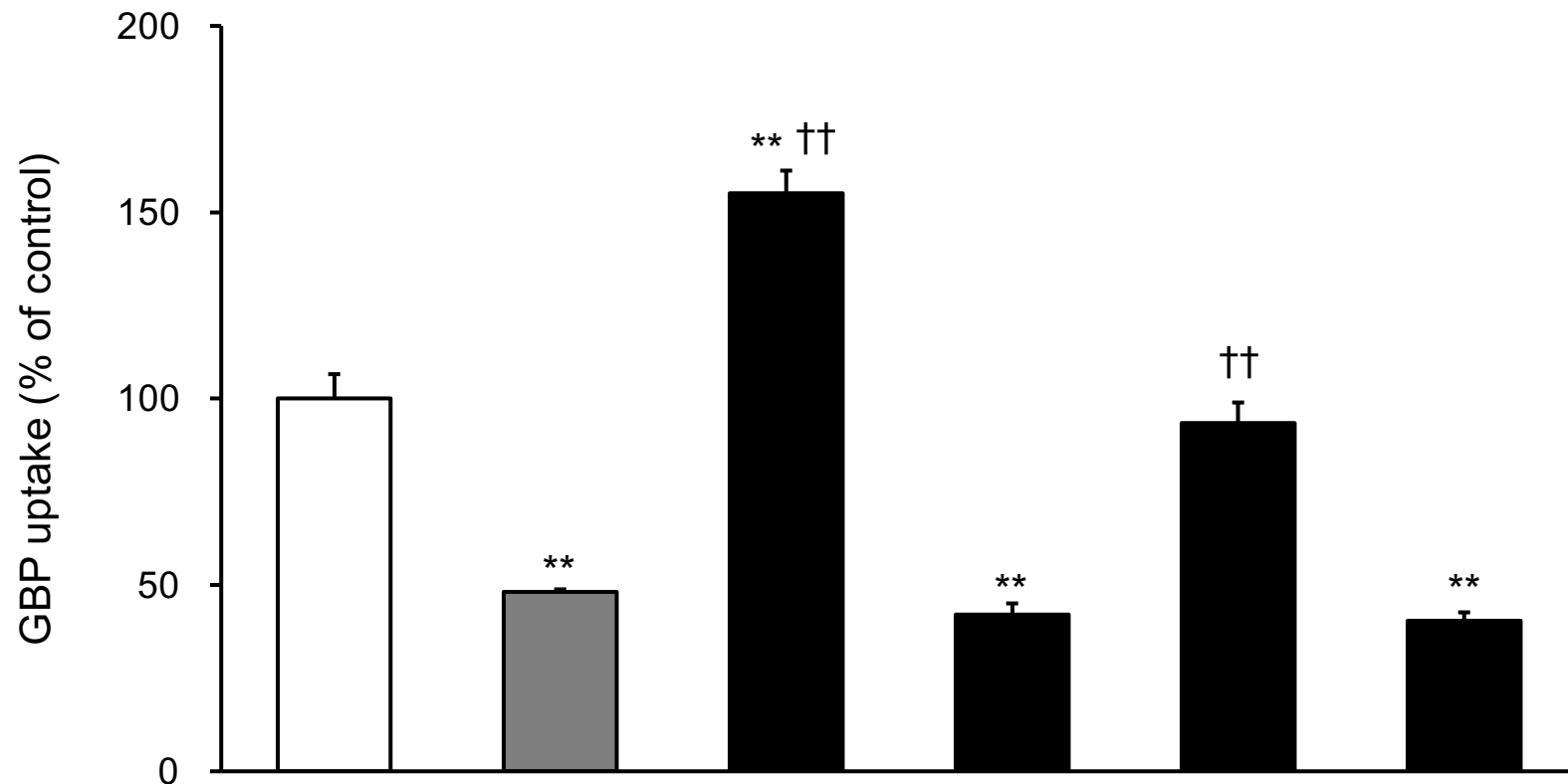




Figure 6



Forskolin (100  $\mu$ M)

-

+

+

+

+

+

Amino acids (1 mM)

-

-

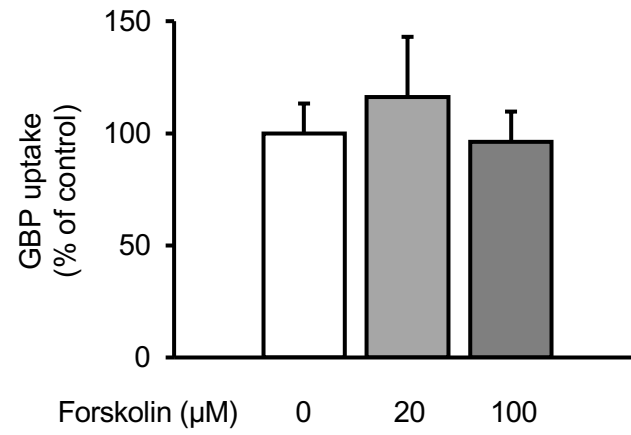
His

Lys

Met

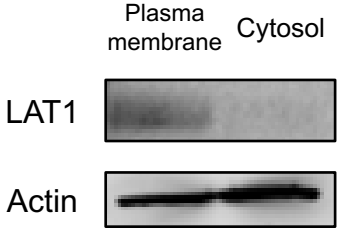
Cys

# Supplemental Fig. 1



# Supplemental Fig. 2

(A)



(B)

