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 μM or 100 μM 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 (1.0×10^5 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 (2.0×10^5 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 β-actin as a reference gene (Knerr et al. 2005).

2.5. Antiepileptic drug uptake

BeWo cells (1.0×10^5 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 μM of each antiepileptic drug. A concentration-dependent GBP uptake study was performed using drug concentrations ranging from 3.1 and 500 μM. 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 μM 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 μM and 100 μM 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 (1.0×10^5 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 μM GBP. After incubation, the buffer was removed and the cells were rinsed using ice-cold transport buffer.

2.7. Western blotting

BeWo cells (4.0×10^5 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 (2.0×10^6 cells/dish) were seeded on a 60.1 cm^2 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 (2.0×10^6 cells/dish) were seeded on a 60.1 cm^2 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 × *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 ± 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 μM) 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 μM forskolin) and 55% (100 μM 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 μM forskolin-treated groups. The values of K_m and V_{max} were 52.3 ± 10.2 μM and 3813.6 ± 927.8 pmol/mg protein/min, respectively, for the control and 29.1 ± 4.8 μM ($p = 0.10$, compared to the control) and 1260.1 ± 201.8 pmol/mg protein/min ($p < 0.05$, compared to the control), respectively, for 100 μM 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 (Delidaki 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 μM 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 μM forskolin) and 286% (100 μM forskolin), compared with the control (Figure 4A). Similarly, LAT1 whole protein expression was increased by 202% (20 μM forskolin) and 169% (100 μM 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 μM forskolin) and 219% (100 μM 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 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 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 ≈ 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 an Na^+-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.

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Conflicts of Interest

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References

Araújo JR, Correia-Branco A, Ramalho C, Gonçalves P, Pinho MJ, Keating E, Martel F. 2013. L-methionine placental uptake: characterization and modulation in gestational diabetes mellitus. Reprod Sci. 20(12):1492-1507.

Balthasar C, Stangl H, Widhalm R, Granitzer S, Hengstschläger M, Gundacker C. 2017. Methylmercury uptake into BeWo cells depends on LAT2-4F2hc, a system L amino acid transporter. Int J Mol Sci. 18(8):1730.

Bastida-Ruiz D, Van Hoesen K, Cohen M. 2016. The dark side of cell fusion. Int J Mol Sci. 17(5):638.

Bröer A, Rahimi F, Bröer S. 2016. Deletion of amino acid transporter ASCT2 (SLC1A5) reveals an essential role for transporters SNAT1 (SLC38A1) and SNAT2 (SLC38A2) to sustain glutaminolysis in cancer cells. J Biol Chem. 291(5):13194–13205.

Cole LA. 2010. Biological functions of hCG and hCG-related molecules. Reprod Biol Endocrinol. 8:102.

Cormerais Y, Massard PA, Vucetic M, Giuliano S, Tambutté E, Durivault J, Vial V, Endou H, Wempe MF, Parks SK, et al. 2018. The glutamine transporter ASCT2 (SLC1A5) promotes tumor growth independently of the amino acid transporter LAT1 (SLC7A5). J Biol Chem. 293(8):2877–2887.

Delidaki M, Gu M, Hein A, Vatish M, Grammatopoulos DK. 2011. Interplay of cAMP and MAPK pathways in hCG secretion and fusogenic gene expression in a trophoblast cell line. Mol Cell Endocrinol. 332(1-2):213–220.

Fujii H, Goel A, Bernard N, Pistelli A, Yates LM, Stephens S, Han JY, Matsui D, Etwell F, Einarson TR, et al. 2013. Pregnancy outcomes following gabapentin use: Results of a prospective comparative cohort study. Neurology. 80(17):1565–1570.

Furugen A, Ishiguro Y, Kobayashi M, Narumi K, Nishimura A, Hirano T, Iseki K. 2017. Involvement of l-type amino acid transporter 1 in the transport of gabapentin into human placental choriocarcinoma cells. Reprod Toxicol. 67:48–55.

Furugen A, Kobayashi M, Nishimura A, Takamura S, Narumi K, Yamada T, Iseki K. 2015. Quantification of new antiepileptic drugs by liquid chromatography/electrospray ionization tandem mass spectrometry and its application to cellular uptake experiment using human placental choriocarcinoma BeWo cells. J Chromatogr B Analyt Technol Biomed Life Sci.1002:228–233.

Guttuso T Jr, Messing S, Tu X, Mullin P, Shepherd R, Strittmatter C, Saha S, Thornburg LL. 2021. Effect of gabapentin on hyperemesis gravidarum: A double-blind, randomized controlled trial. Am J Obstet Gynecol MFM. 3(1):100273.

Goasdoué K, Miller SM, Colditz PB, Björkman ST. 2017. Review: The blood-brain barrier; protecting the developing fetal brain. Placenta. 54:111–116.

Hasegawa N, Furugen A, Ono K, Koishikawa M, Miyazawa Y, Nishimura A, Umazume T, Narumi K, Kobayashi M, Iseki K. 2020. Cellular uptake properties of lamotrigine in human placental cell lines: Investigation of involvement of organic cation transporters (SLC22A1-5). 2020. Drug Metab Pharmacokinet. 35(3):266–273.

Huang FD, Kung FL, Tseng YC, Chen MR, Chan HS, Lin CJ. 2009. Regulation of protein expression and function of octn2 in forskolin-induced syncytialization in BeWo Cells. Placenta. 30(2):187–194.

Kallol S, Moser-Haessig R, Ontsouka CE, Albrecht C. 2018. Comparative expression patterns of selected membrane transporters in differentiated BeWo and human primary trophoblast cells. Placenta. 72-73:48–52.

Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF 3rd. 1986. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology. 118(4):1567–1582.

Knerr I, Schubert SW, Wich C, Amann K, Aigner T, Vogler T, Jung R, Dötsch J, Rascher W, Hashemolhosseini S. 2005. Stimulation of GCMa and syncytin via cAMP mediated PKA signaling in human trophoblastic cells under normoxic and hypoxic conditions. FEBS Lett. 579(18):3991–3998.

Kudo Y, Boyd CA. 2002. Changes in expression and function of syncytin and its receptor, amino

acid transport system B (0) (ASCT2), in human placental choriocarcinoma BeWo cells during syncytialization. Placenta. 23(7):536–541.

Laskowska M, Leszczyńska-Gorzelak B, Oleszczuk J. 2001. Pregnancy in women with epilepsy. Gynecol Obstet Invest. 51(2):99–102.

Nicklin P, Bergman P, Zhang B, Triantafellow E, Wang H, Nyfeler B, Yang H, Hild M, Kung C, Wilson C, et al. 2009. Bidirectional transport of amino acids regulates mTOR and autophagy. Cell. 136(3):521–134.

Ohman I, Vitols S, Tomson T. 2000. Lamotrigine in pregnancy: Pharmacokinetics during delivery, in the neonate, and during lactation. Epilepsia. 41(6):709–713.

Ohman I, Vitols S, Luef G, Söderfeldt B, Tomson T. 2002. Topiramate kinetics during delivery, lactation, and in the neonate: Preliminary observations. Epilepsia. 43(10):1157–1160.

Ohman I, Vitols S, Tomson T. 2005. Pharmacokinetics of gabapentin during delivery, in the neonatal period, and lactation: Does a fetal accumulation occur during pregnancy? Epilepsia. 46(10):1621–1624.

Patorno E, Hernandez-Diaz S, Huybrechts KF, Desai RJ, Cohen JM, Mogun H, Bateman BT. 2020. Gabapentin in pregnancy and the risk of adverse neonatal and maternal outcomes: A population-based cohort study nested in the US Medicaid Analytic eXtract dataset. PLOS Med. 17(9):e1003322.

Prasad PD, Wang H, Huang W, Kekuda R, Rajan DP, Leibach FH, Ganapathy V. 1999. Human LAT1, a subunit of system L amino acid transporter: molecular cloning and transport function. Biochem Biophys Res Commun. 255(2):283-288.

Rothbauer M, Patel N, Gondola H, Siwetz M, Huppertz B, Ertl P. 2017. A comparative study of five physiological key parameters between four different human trophoblast-derived cell lines. Sci Rep. 7(1):5892.

Scalise M, Galluccio M, Console L, Pochini L, Indiveri C. 2018. The Human SLC7A5 (LAT1): The intriguing histidine/large neutral amino acid transporter and its relevance to human health. Front Chem. 6:243.

Sooranna SR, Burston D, Ramsay B, Steer PJ. 1994. Free amino acid concentrations in human first and third trimester placental villi. Placenta. 15(7):747-51.

Tetro N, Moushaev S, Rubinchik-Stern M, Eyal S. 2018. The placental barrier: The gate and the fate in drug distribution. Pharm Res. 35(4):71.

Tomson T, Palm R, Källén K, Ben-Menachem E, Söderfeldt B, Danielsson B, Johansson R, Luef G, Ohman I. 2007. Pharmacokinetics of levetiracetam during pregnancy, delivery, in the neonatal period, and lactation. Epilepsia. 48(6):1111–1116.

Vajda FJ, O'Brien T, Lander C, Graham J, Eadie M. 2014. The efficacy of the newer antiepileptic drugs in controlling seizures in pregnancy. Epilepsia. 55(8):1229-34.

Wang Q, Beaumont KA, Otte NJ, Font J, Bailey CG, van Geldermalsen M, Sharp DM, Tiffen JC, Ryan RM, Jormakka M, et al. 2014. Targeting glutamine transport to suppress melanoma cell growth. Int J Cancer. 135(5):1060-71.

Wempe MF, Rice PJ, Lightner JW, Jutabha P, Hayashi M, Anzai N, Wakui S, Kusuhara H, Sugiyama Y, Endou H. 2012. Metabolism and pharmacokinetic studies of JPH203, an L-amino acid transporter 1 (LAT1) selective compound. Drug Metab Pharmacokinet. 27(1):155-161.

Yanagida O, Kanai Y, Chairoungdua A, Kim DK, Segawa H, Nii T, Cha SH, Matsuo H, Fukushima J, Fukasawa Y, et al. 2001. Human L-type amino acid transporter 1 (LAT1): Characterization of function and expression in tumor cell lines. Biochim Biophys Acta. 1514(2):291–302.

Zaugg J, Huang X, Ziegler F, Rubin M, Graff J, Müller J, Moser-Hässig R, Powell T, Gertsch J, Altmann KH, et al. 2020. Small molecule inhibitors provide insights into the relevance of LAT1 and LAT2 in materno-foetal amino acid transport. J Cell Mol Med. 24(21):12681–12693.

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 μM or 100 μM) 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 μM or 100 μM) or DMSO (control). After forskolin treatment, cells were incubated in transport buffer containing 100 μM 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 μM forskolin, BeWo cells were incubated with a transport buffer containing 3.1-500 μM 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 μM) or DMSO (control). After forskolin treatment, cells were incubated in transport buffer containing 100 μM of GBP in the presence or absence of 10 mM BCH (A) or 10 μM 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 μM or 100 μM) 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 μM 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 μM) 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 μM 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 μM or 100 μM) or DMSO (control) for 14.5 min and subsequently incubated with 100 μM 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 μM or 100 μM) 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)						*p*-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 μM) 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 (*ERVW-1*)	Forward	5'-GGTACATGGCACCTCTAGCCCCTAC -3'	191
	Reverse	5'-CAGGGATTGAAACATATGGCCTGAAGTTC -3'	
LAT1 (*SLC7A5*)	Forward	5'-TCATCATCCGGCCTTCATCG -3'	159
	Reverse	5'-TCACGCTGTAGCAGTTCACG -3'	
4F2hc (*SLC3A2*)	Forward	5'-TTGGCTGAGTGGCAAAATATCA -3'	104
	Reverse	5'-GATTCGAGTAGGCTCAGGATCTG -3'	
β-actin (*ACTB*)	Forward	5'-TGGCACCCAGCACAATGAA -3'	186
	Reverse	5'-CTAAGTCATAGTCCGCCTAGAAGCA -3'	

Figure 1

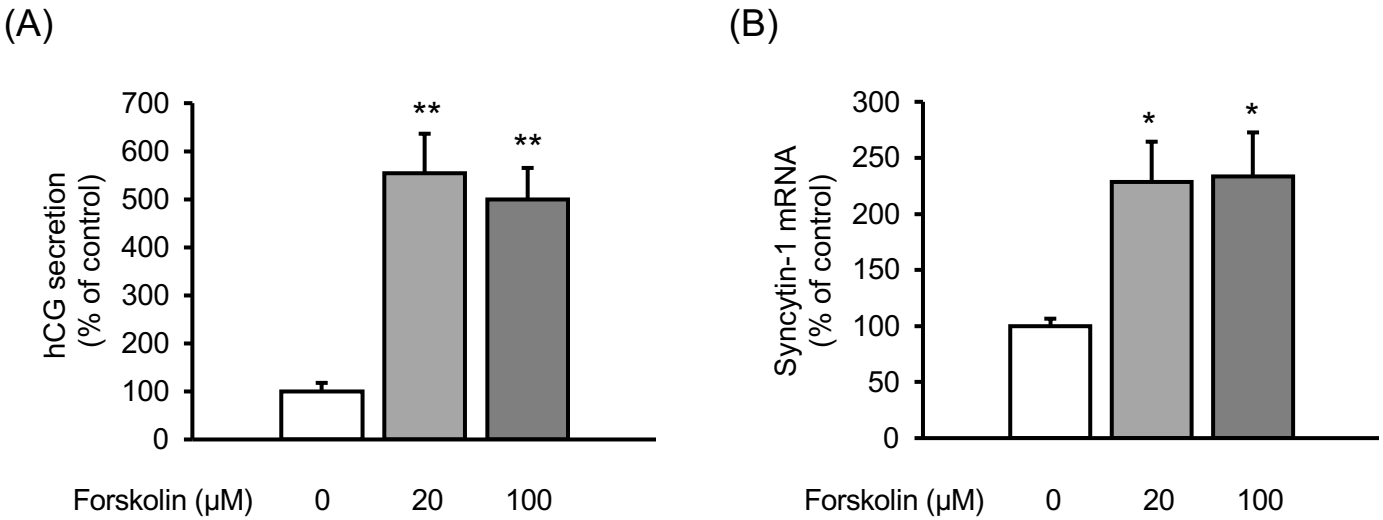

(A)
hCG secretion
(% of control)
700
600
500
400
300
200
100
0
**
**
Forskolin (μM)
0
20
100
(B)
Syncytin-1 mRNA
(% of control)
300
250
200
150
100
50
0
*
*
Forskolin (μM)
0
20
100

Figure 2

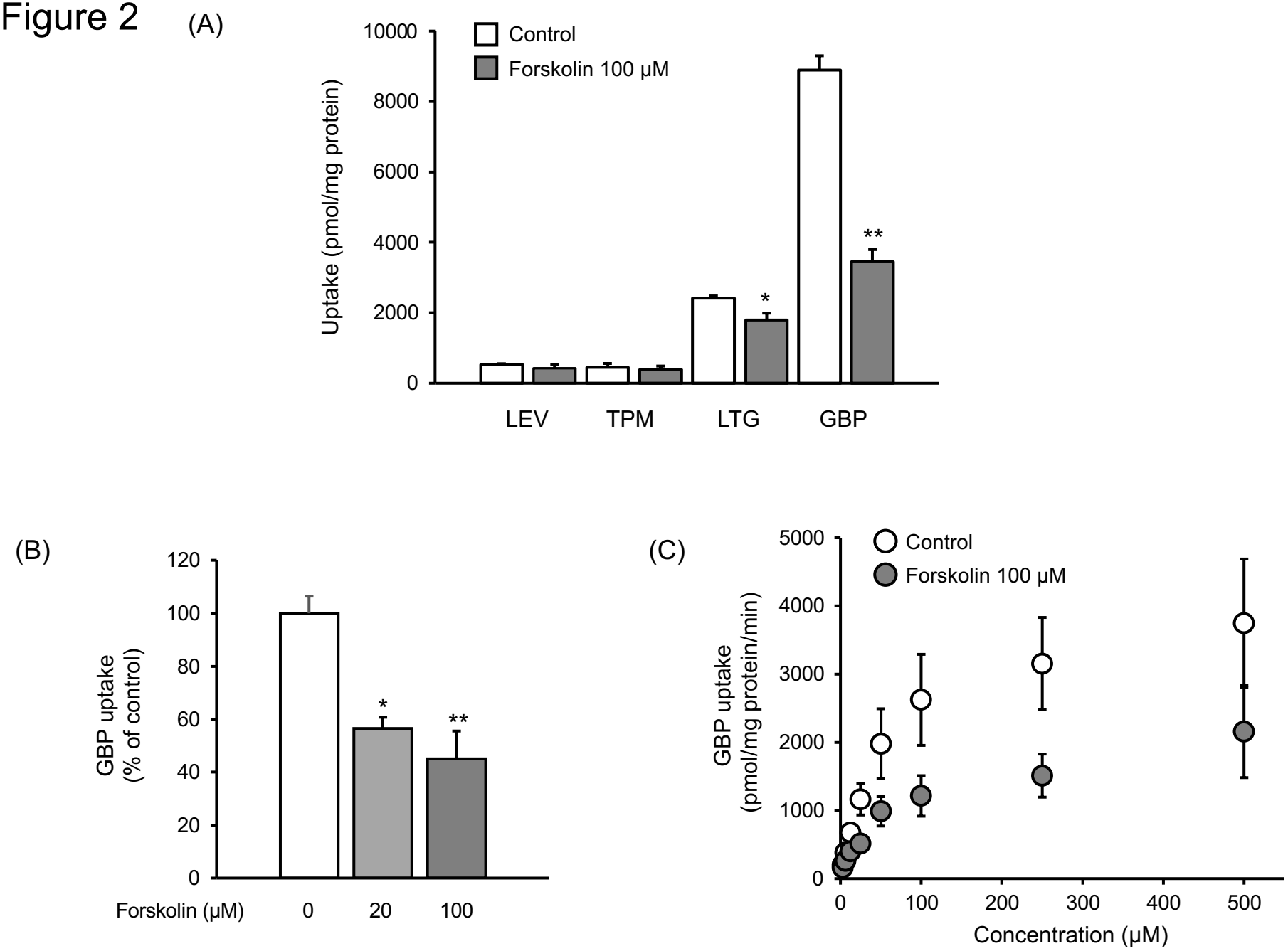

(A)
Control
Forskolin 100 μM
Uptake (pmol/mg protein)
10000
8000
6000
4000
2000
0
*
**
LEV
TPM
LTG
GBP
(B)
GBP uptake
(% of control)
120
100
80
60
40
20
0
*
**
Forskolin (μM)
0
20
100
(C)
Control
Forskolin 100 μM
GBP uptake
(pmol/mg protein/min)
5000
4000
3000
2000
1000
0
0
100
200
300
400
500
Concentration (μM)

Figure 3

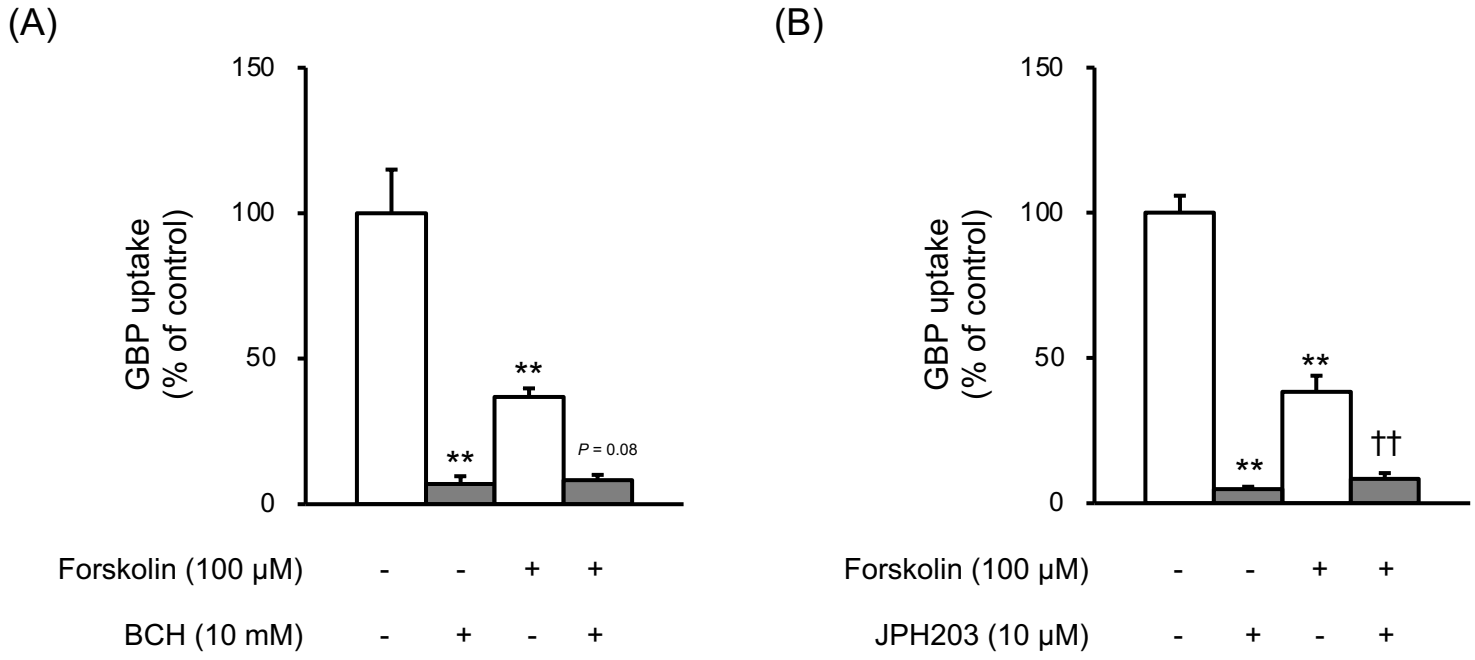

(A)
150
100
50
0
GBP uptake
(% of control)
**
**
P = 0.08
Forskolin (100 μM) - - + +
BCH (10 mM) - + - +
(B)
150
100
50
0
GBP uptake
(% of control)
**
**
††
Forskolin (100 μM) - - + +
JPH203 (10 μM) - + - +

Figure 4

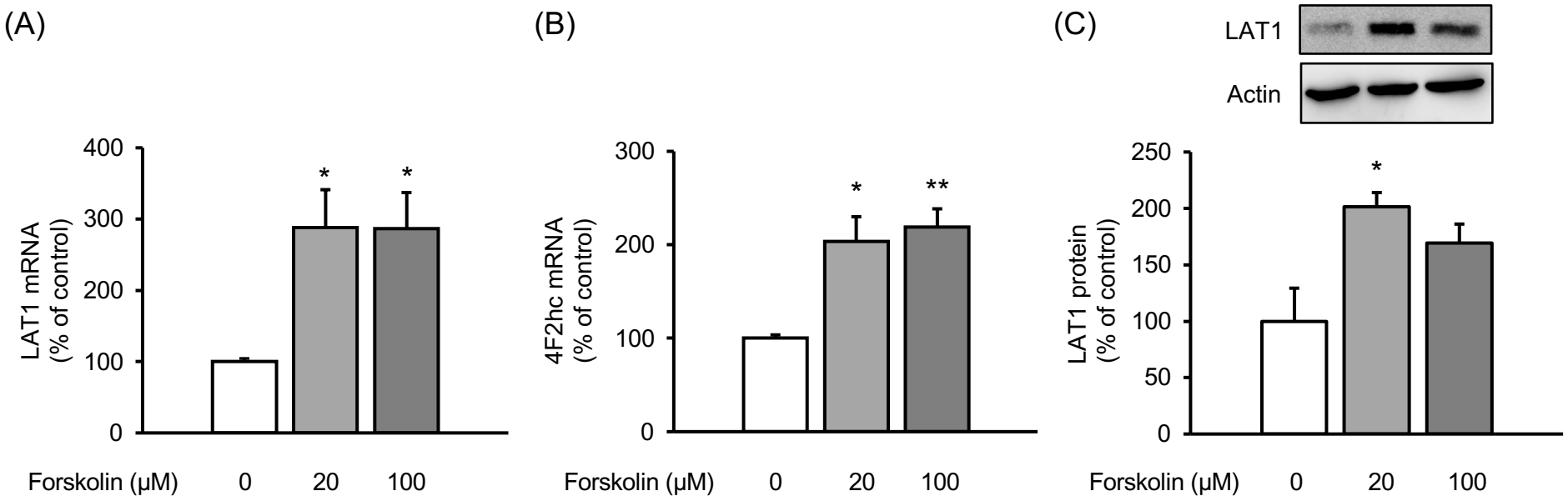

(A)
LAT1 mRNA
(% of control)
400
300
200
100
0
*
*
Forskolin (μM)
0
20
100
(B)
4F2hc mRNA
(% of control)
300
200
100
0
*
**
Forskolin (μM)
0
20
100
(C)
LAT1
Actin
LAT1 protein
(% of control)
250
200
150
100
50
0
*
Forskolin (μM)
0
20
100

Figure 5

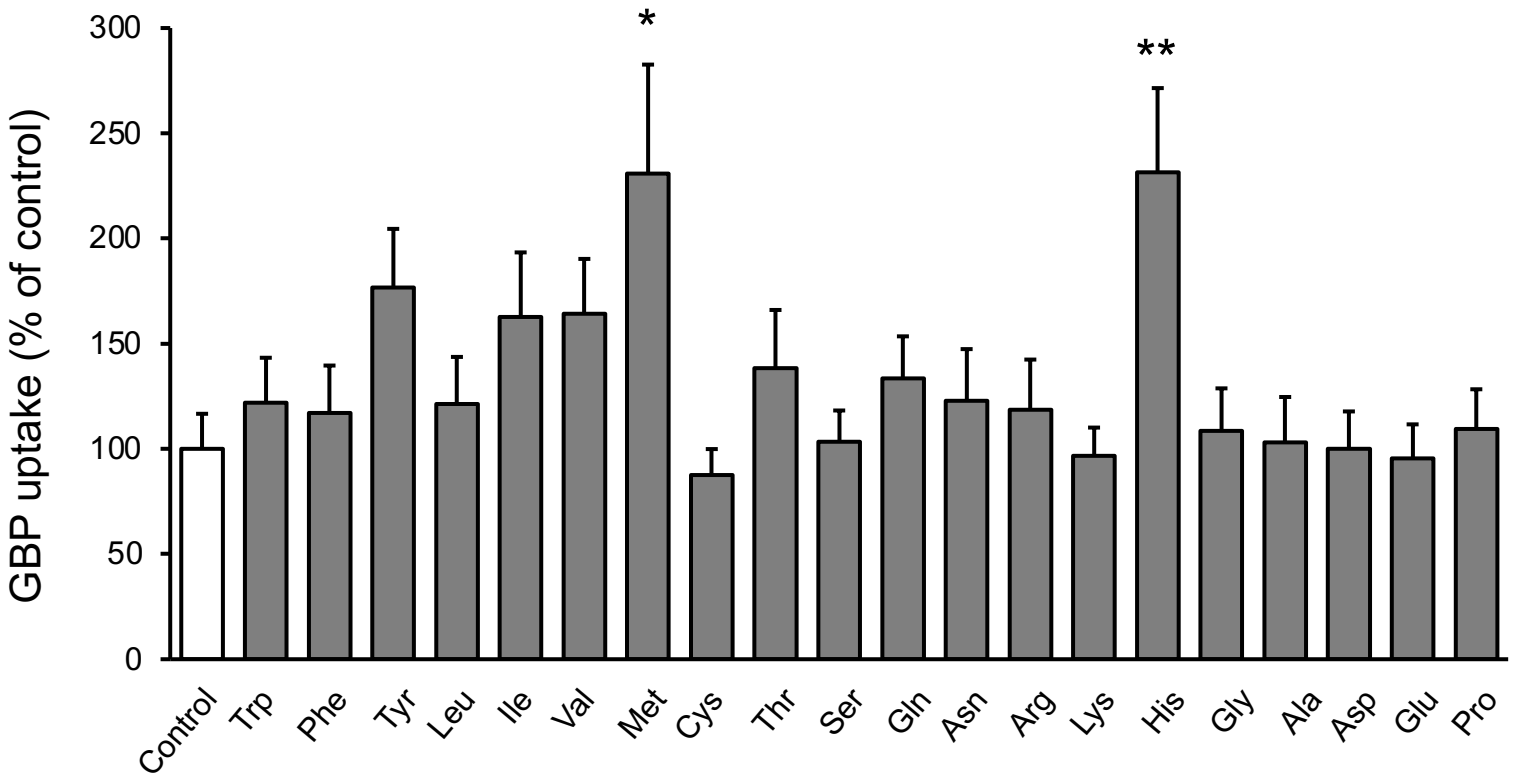
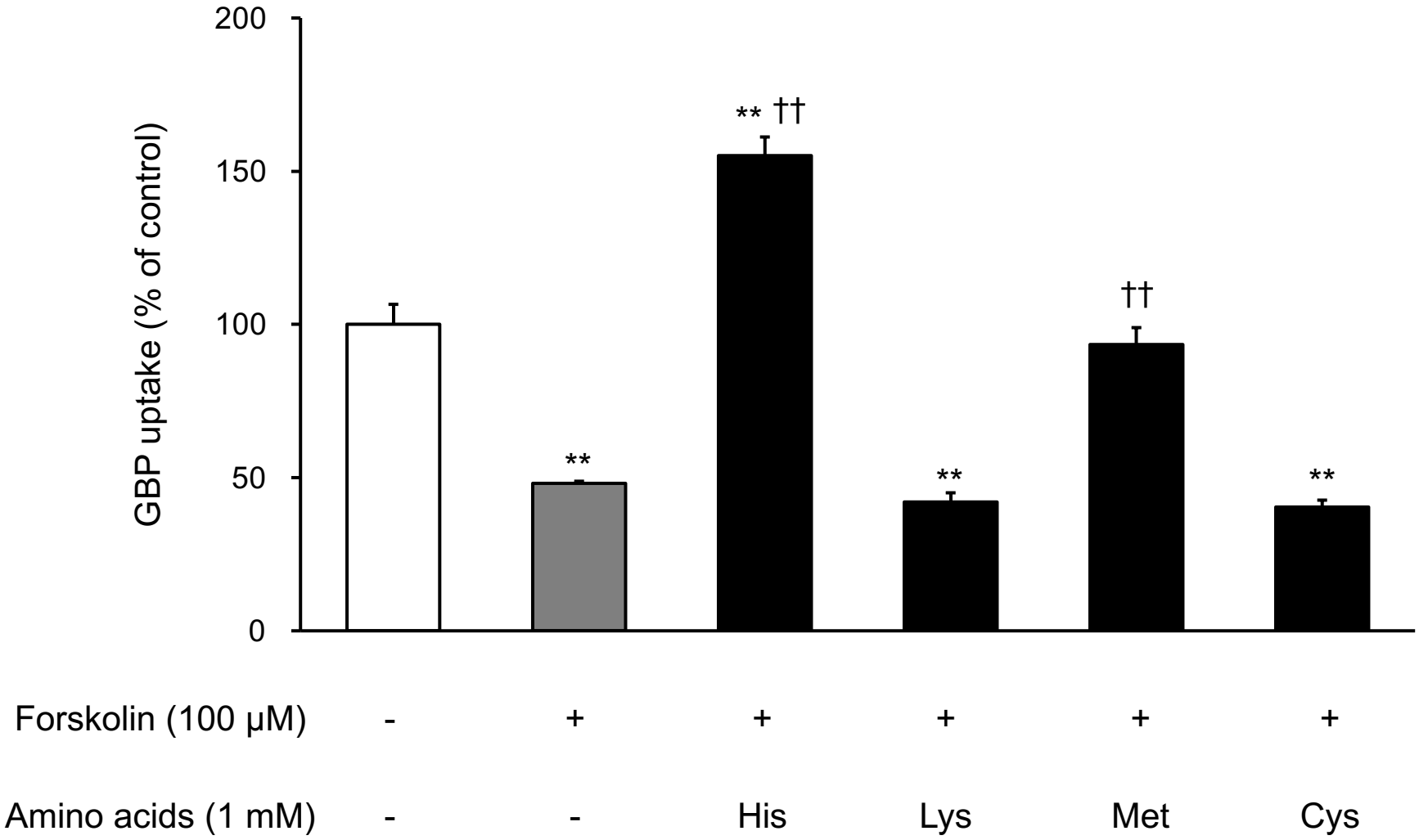
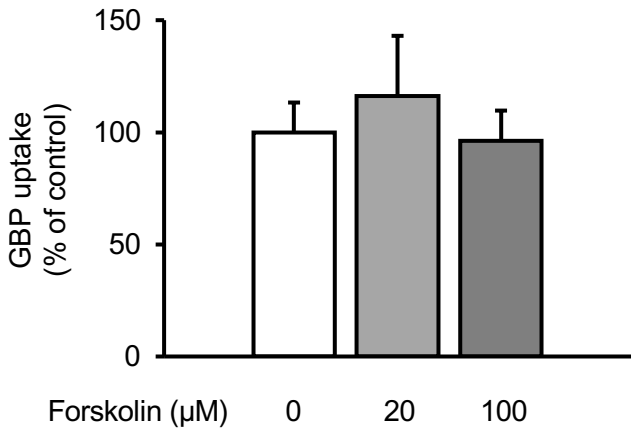

GBP uptake (% of control)
300
250
200
150
100
50
0
*
**
Control
Trp
Phe
Tyr
Leu
Ile
Val
Met
Cys
Thr
Ser
Gln
Asn
Arg
Lys
His
Gly
Ala
Asp
Glu
Pro

Figure 6

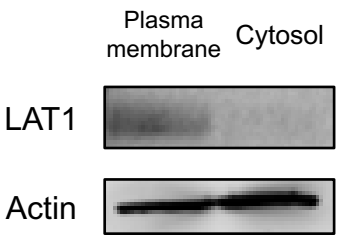

GBP uptake (% of control)
200
150
100
50
0
** ††
**
**
††
**
Forskolin (100 μM)
-
+
+
+
+
+
Amino acids (1 mM)
-
-
His
Lys
Met
Cys

Supplemental Fig. 1



Supplemental Fig. 2

(A)



(B)