



Title	Involvement of L-type amino acid transporter 1 in the transport of gabapentin into human placental choriocarcinoma cells.
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Abstract

Gabapentin (GBP) is a widely used antiepileptic drug, with potential for use in the treatment of epilepsy in pregnant women. Although studies have examined GBP transport mechanisms across the blood-brain barrier, kidney, and intestine, the mechanism in the placenta has not been fully elucidated. We previously reported that GBP accumulates at high concentrations in human placental choriocarcinoma BeWo cells. The purpose of this study was to examine the transport mechanism of GBP in placental choriocarcinoma cells (BeWo and JEG-3), and to identify the carrier involved. High concentrations of intracellular GBP accumulations were also found in JEG-3 cells. A kinetic analysis showed that a single carrier system was involved in the uptake of GBP. Furthermore, substrates for L-type amino acid transporter (LAT) and siRNAs targeted to LAT1 significantly decreased GBP uptake. Our observations from this study suggest that LAT1 is the main contributor to GBP transport in placental choriocarcinoma cells.

Keywords: gabapentin (GBP); placenta; L-type amino acid transporter (LAT); organic cation/carnitine transporter (OCTN); BeWo cells; JEG-3 cells

Highlights

- Gabapentin (GBP) transport in placental choriocarcinoma cells is carrier-mediated.
- L-type amino acid transporter (LAT1) is mainly involved in GBP transport in choriocarcinoma cells.
- LAT substrates, amino acids and thyroid hormones, inhibit GBP transport.

Involvement of L-type amino acid transporter 1 in the transport of gabapentin into human placental choriocarcinoma cells

Ayako Furugen^a, Yuri Ishiguro^a, Masaki Kobayashi^b, Katsuya Narumi^a, Ayako Nishimura^b, Takeshi Hirano^c, Ken Iseki^{ab*}

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

^bDepartment of Pharmacy, Hokkaido University Hospital, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060-8648, Japan

^cHealth Sciences University of Hokkaido, 1757 Kanazawa, Tobetsu-cho, Ishikari-gun, Hokkaido 061-0293, Japan

*¹Correspondence to: Ken Iseki, Ph.D. Laboratory of Clinical Pharmaceutics & Therapeutics,

Abbreviations: BCH, 2-Amino-2-norbornanecarboxylic acid; CBZ, carbamazepine; CLB, clobazam; CZP, clonazepam; GBP, gabapentin; LAT, L-type amino acid transporter; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LEV, levetiracetam; LTG, lamotrigine; NZP, nitrazepam; OCTN, organic cation/carnitine transporter; PB, phenobarbital; PHT, phenytoin; T₃, 3,3',5-triiodo-L-thyronine; T₄, L-thyroxine; TEA, tetraethylammonium; TPM, topiramate; VPA, valproic acid; ZNS, zonisamide

Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12-jo,

Nishi-6-chome, Kita-ku, Sapporo 060-0812, Japan

Phone/Fax: +81-11-706-3770; E-mail: ken-i@pharm.hokudai.ac.jp

1. Introduction

Epilepsy is a neurological condition that has been reported in 0.3-0.7% of all pregnant women [1]. Because epileptic seizures during pregnancy pose a risk to both the mother and the fetus, most pregnant women experiencing seizures are prescribed antiepileptic drugs [2]. However, exposure to certain antiepileptic drugs during pregnancy is associated with increased health risks to the fetus. For example, the risk of major congenital malformations increases with the use of certain earlier generation antiepileptic drugs such as valproic acid (VPA), phenobarbital (PB), and carbamazepine (CBZ) [2]. Furthermore, several studies have indicated that VPA is associated with increased risk of neurodevelopmental delay and autism spectrum disorder [3, 4].

Gabapentin [1-(Aminomethyl) cyclohexaneacetic acid, GBP] is a widely used antiepileptic drug. It is also used in the management of neuropathic pain. Protein binding of GBP is low (< 3 %), and GBP is mostly excreted by the kidney in its unchanged form [5]. Newer antiepileptic drugs, such as lamotrigine (LTG), levetiracetam (LEV), topiramate (TPM), and gabapentin (GBP) are being increasingly used, even during pregnancy [6]. Although several epidemiological studies have addressed the risks of new antiepileptic drugs on the fetus, the safety and efficacy of these drugs during pregnancy have not been fully established. Fujii *et al.* [7] conducted a prospective cohort study and reported that GBP administration during pregnancy does not appear to increase the risk for major malformations. However, the study indicated the possibility of increased risk for low birth weight and preterm birth when GBP was administered. Recently, a case report described neonatal

20 GBP withdrawal syndrome after prolonged *in utero* exposure [8]. Therefore, information on the
21 transport mechanisms of antiepileptic drugs in placental cell models might contribute to estimate the
22 adverse effect of these drugs on the fetus. However, little information is available on the transport
23 mechanism of GBP in placental cell models.

24 Ohman *et al.* [9] reported that umbilical-to-maternal GBP plasma concentration ratios
25 ranged from 1.3 to 2.1 (mean, 1.7), implying that the drug is actively transported across the
26 near-term placenta. GBP is a zwitterion and the distribution coefficient ($\text{Log}D$, pH 7.4) has
27 previously been reported to be -1.2 [10]. Therefore, a carrier-mediated mechanism might be
28 involved in its transport across the plasma membrane. We previously reported that the accumulation
29 of GBP in human placental choriocarcinoma BeWo cells occurred in higher concentrations than in
30 other new-generation antiepileptic drugs such as LTG, LEV, and TPM [11]. Furthermore, the
31 transport of GBP in BeWo cells showed saturation at high concentrations, suggesting that the
32 transfer of GBP to choriocarcinoma cells is carrier-mediated.

33 Membrane transporters play a role in the pharmacokinetic and physiological processes in
34 several organs, including the placenta. Over 400 membrane transporters have been identified and
35 classified as ATP-binding cassettes (ABC) and solute carriers (SLC). While taking into
36 consideration individual variation, it is crucial to examine the role of drug transporters in the
37 placenta in order to prevent potential drug-drug interactions. In brain endothelial cells, GBP is
38 transported by L-type amino acid transporter 1 (LAT1, *SLC7A5*) [10]. The organic cation/carnitine

39 transporter (OCTN1, *SLC22A4*) contributes to the urinary excretion of GBP, with its genetic variant
40 L503F resulting in decreased GBP transport [12]. Larsen *et al.* [13] suggest that in the intestine, a
41 carrier-mediated system, which is inhibited by 2-Amino-2-norbornanecarboxylic acid (BCH),
42 contributes to the transport of GBP in a rat model and in Caco-2 cells. System L transporter LAT1
43 catalyzes the sodium-independent transport of physiologically important substrates such as amino
44 acids [14] and thyroid hormones [15], and is highly expressed in the placenta [16]. OCTN1 is an
45 important transporter for various xenobiotics, and endogenous substrates such as L-carnitine, and is
46 expressed in the placenta [17, 18]. However, there is limited literature on studies investigating the
47 involvement of these transporters in the uptake of GBP in placental cell lines.

48 In the present study, we investigated the uptake mechanism of GBP in human placental
49 choriocarcinoma cells (BeWo and JEG-3) and the involvement of putative transporters in the
50 transport process. The cell lines, BeWo and JEG-3, have been widely used to examine the transport
51 mechanisms of xenobiotic in the placenta [19].

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56 **2. Materials and methods**

57 **2.1 Chemicals**

58 Gabapentin (GBP), lamotrigine (LTG), levetiracetam (LEV), and topiramate (TPM) were
59 purchased from Tokyo Chemical Industry (Tokyo, Japan). 2-Amino-2-norbornanecarboxylic acid
60 (BCH), L-carnitine, L-thyroxine (T₄), clobazam (CLB), cimetidine, quinidine, and amino acids were
61 purchased from Sigma-Aldrich (St. Louis, MO). 3, 3', 5-Triiodo-L-thyronine (T₃) was purchased
62 from Nacalai Tesque (Kyoto, Japan). Other chemicals were purchased from Wako (Tokyo, Japan).

63

64 **2.2 Cell culture**

65 BeWo cells were obtained from the Riken Cell Bank (Saitama, Japan). BeWo cells were
66 cultured in Ham's F-12K (Kaighn's modification) medium (Wako, Tokyo, Japan), supplemented
67 with 15 % fetal bovine serum and 1 % penicillin-streptomycin (Sigma Aldrich, St. Louis, MO), at
68 37 °C under 5 % CO₂. JEG-3 cells were purchased from DS Pharma Co. Ltd. (Osaka, Japan). JEG-3
69 cells were cultured in the Eagle's minimum essential medium (Wako), supplemented with 10 % fetal
70 bovine serum, 1 % non-essential amino acids, 1 mM sodium pyruvate, and 1 %
71 penicillin-streptomycin.

72

73 **2.3 Uptake experiment**

74 BeWo cells and JEG-3 cells were seeded on 24-well collagen-coated plastic plates. After

75 the cells had grown to confluence, they were used for the uptake experiment. Once the growth
76 medium was removed, cells were washed with a transport buffer and pre-incubated at 37 °C with
77 0.5 mL of transport buffer. The transport buffer consisted of Hank's balanced salt solution (HBSS)
78 with 25 mM HEPES, maintained at a pH of 7.4. Uptake was initiated by adding the transport buffer
79 containing the study drugs. To investigate the accumulation levels of GBP, LTG, LEV, and TPM,
80 cells were incubated with 50 μ M of each antiepileptic drug. The concentration of 50 μ M is close to
81 that of therapeutic levels of these drugs in blood. In plasma, reference concentration ranges are
82 between 10 and 120 μ M for GBP, 10 and 60 μ M for LTG, 70 and 270 μ M for LEV, and 15 and 60
83 μ M for TPM [20]. For the time-dependent study, 5 μ M of GBP was added to the transport buffer.
84 The concentration-dependent study was conducted by adding GBP at concentrations ranging
85 between 10 to 500 μ M. The effects of various inhibitors on the GBP uptake were investigated by
86 incubating the cells with transport buffer containing 100 μ M of GBP in the presence or absence of
87 inhibitors. To examine the concentration-dependent inhibition by BCH, the uptake of 10 μ M GBP
88 by cells was measured in the presence of increasing concentrations of BCH (~10 mM). The cells
89 were incubated for the indicated time at 37 °C. After incubation, cells were immediately rinsed with
90 ice-cold transport buffer. The cells were lysed with 200 μ L of 1 N NaOH and the lysate was
91 neutralized with HCl. The concentration of GBP was determined using liquid
92 chromatography/electrospray ionization tandem mass spectrometry (LC/MS/MS) as previously
93 described [11]. The uptake amount was normalized to the level of protein. The protein concentration

94 was determined using a Pierce[®] BCA Protein Assay Kit (Thermo Scientific, Rockford, IL), in
95 accordance with the manufacturer's instructions.

96

97 **2.4 Estimation of intracellular water volumes of BeWo and JEG-3 cells**

98 To investigate intracellular concentration of GBP, intracellular water volumes of BeWo
99 and JEG-3 cells were estimated using modified methods described by Rottenberg *et al.* [21] and
100 Shiraya *et al.* [22]. BeWo cells and JEG-3 cells were seeded on 24-well collagen-coated plastic
101 plates. After removal of growth medium, cells were washed with a transport buffer and
102 pre-incubated with 0.5 mL of buffer. The buffer was removed and 0.5 mL of buffer containing
103 [³H]-H₂O (1 μCi/mL) (PerkinElmer, Boston, MA), and [¹⁴C]inulin-carboxyl (0.5 μCi/mL)
104 (American Radiolabeled Chemicals, Inc. St. Louis, MO) was added to cells. The cells were
105 incubated in the buffer for 10 min. The extracellular buffer was collected, and cells were solubilized
106 with 1 % sodium dodecyl sulfate (SDS)/0.2 N NaOH. The samples were mixed with 3 mL of
107 scintillation cocktail (PerkinElmer) to measure radioactivity using a liquid scintillation counter.

108 The intracellular water volume was calculated using the following equation:

109 Intracellular cell volume = $V_s \times ({}^3\text{H}_c / {}^3\text{H}_s - {}^{14}\text{C}_c / {}^{14}\text{C}_s)$

110 where V_s is the volume of extracellular buffer, ${}^3\text{H}_c$ is the ³H counts in cells, ${}^3\text{H}_s$ is the ³H counts in
111 supernatant, ${}^{14}\text{C}_c$ is the ¹⁴C counts in cells, and ${}^{14}\text{C}_s$ is the ¹⁴C counts in supernatant.

112 The intracellular volume was expressed relative to protein.

113

114 **2.5 Transepithelial transport of GBP across JEG-3 cells**

115 JEG-3 cells were seeded (1×10^5 cells/cm²) in a 24-well TranswellTM (Pore Size, 0.4
116 μ m) (Corning, Kennebunk, ME) and grown for eight days. After removal of growth medium, cells
117 were washed with transport buffer and pre-incubated at 37 °C with transport buffer (apical side, 0.1
118 mL; basolateral side, 0.6 mL). After removal of incubation buffer, transport buffer containing GBP
119 (50 μ M) in the absence or presence of BCH (1 mM) was added to apical side or basolateral side.
120 The monolayers were incubated for 30 min at 37 °C. After incubation, an aliquot was collected from
121 the basolateral side or the apical side. The concentration of GBP was quantified using LC/MS/MS.

122 The apparent permeability coefficient (P_{app}) was calculated using the following equation:

123
$$P_{app} \text{ (cm/s)} = dQ/dt \times 1/(A \times C_0)$$

124 where dQ/dt is the linear appearance rate of mass in the receiver solution, A is the filter/cell surface
125 area (0.33 cm²), and C_0 is the initial donor concentration of substrate (50 μ M).

126

127 **2.6 LAT1 knockdown by small interfering RNA (siRNA)**

128 Three siRNAs of LAT1 (Oligo ID: HSS112004, HSS112005, and HSS188571) and a
129 negative control (NC) (StealthTM RNAi Negative Control medium GC Duplex) were purchased
130 from Thermo Fisher Scientific (Waltham, MA). Transfection (reverse-transfection) of siRNAs into
131 the cells was performed using LipofectamineTM RNAiMAX (Invitrogen), in accordance with the

132 manufacturer's instructions. The final RNAi duplex concentration was 10 nM. The cells were
133 incubated for 72 h at 37 °C under 5 % CO₂ and used for experiments.

134

135 **2.7 Western blotting**

136 For Western blotting, cells were lysed in ice-cold RIPA buffer (Cell Signaling Technology,
137 Beverly, MA) supplemented with 1 mM PMSF. The suspension was allowed to stand for 5 min on
138 ice and was sonicated briefly. The suspension was then centrifuged at 14,000 × *g* for 10 min at 4 °C.
139 The supernatant was used for Western blotting. The protein concentration was determined using a
140 Pierce[®] BCA Protein Assay Kit. Five micrograms of protein extract samples were denatured at
141 100 °C for 3 min in sample buffer containing 50 mM Tris-HCl, 2 % SDS, 5 % 2-mercaptoethanol,
142 10 % glycerol, 0.002 % BPB, and 3.6 M urea. Following the completion of SDS-PAGE (4.5 %
143 stacking and 10 % SDS polyacrylamide gels), the proteins were transferred electrophoretically onto
144 nitrocellulose membranes (Trans-Blot; Bio-Rad Laboratories, Richmond, CA). The membranes
145 were blocked with TBS containing 0.05 % Tween 20 (TBS/T) and 5 % non-fat dry milk for 1 h at
146 room temperature. After being washed with TBS/T, the membranes were incubated with primary
147 antibodies overnight. The primary antibodies used were rabbit anti-LAT1 polyclonal antibody
148 (1:1000, Cell Signaling Technology, #5347) and mouse anti-actin monoclonal antibody (1:500,
149 Clone C4/MAB1501, Chemicon, Temecula, CA). The bound proteins were detected using
150 horseradish peroxidase-conjugated secondary antibodies (1:4000, Santa Cruz Biotechnology, Santa

151 Cruz, CA) and visualized using enhanced chemiluminescence (Amersham Biosciences Corp.,
152 Piscataway, NJ).

153

154 **2.8 Reverse transcription polymerase chain reaction (RT-PCR) analysis**

155 Total RNA was prepared from cells using ISOGEN (Nippon Gene, Japan). Single-strand
156 cDNA was made from 1 µg of total RNA by reverse transcription using ReverTraAce (TOYOBO,
157 Japan). PCR was performed using Hot Star Taq PCR (QIAGEN) with specific primers through 35
158 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 15 s. The sense and antisense primer
159 sequences for hOCTN1 were 5' -CTG CTA TTG CGA ACC CTG CC-3' and, 5' -CAG CAT GAC
160 CAG ACC AAT GGA TAA G-3', respectively (product size: 129 bp) [23]. For hOCTN2, the sense
161 sequence was 5' -CAG CCA TCC TCA CCT TGT TTC-3' and, antisense sequence was 5' -TGT
162 GGG CCT TTC TTG ACC ATC-3' (product size: 146 bp) [23]. The sense sequence for hGAPDH
163 was 5'-AAG GTC ATC CCT GAG CTG AA-3' and, antisense sequence was 5'-TTC TAG ACG
164 GCA GGT CAG GT-3' (product size: 96 bp). The PCR products were subjected to electrophoresis
165 on 2 % agarose gel and then visualized by ethidium bromide staining.

166

167 **2.9 Statistical analysis and kinetic parameters**

168 All experiments were repeated at least three times. Data are presented as means ± S.E.
169 Student's *t*-test was used to determine the significance of differences between two group means.

170 Statistical significance among means of more than two groups was evaluated using Dunnett's test.

171 Statistical significance was defined as $p < 0.05$.

172 Nonlinear regression analysis was performed using Origin® (OriginLab Corporation, MA). Kinetic
173 parameters were calculated using the following equation:

174
$$v = V_{\max} \times s / (K_m + s)$$

175 where v is the uptake rate of compounds, s is the compound concentration, K_m is the
176 Michaelis–Menten constant, and $V_{\max}$ is the maximum uptake rate.

177 The IC_{50} value was determined by fitting the dose-response data in DoseResp equation
178 using Origin:

$$y = A1 + \frac{A2 - A1}{1 + 10^{(log x - p)}}$$

179 where y is the extent of inhibition (%), x is the logarithm of inhibitor concentration, $A1$ is the
180 bottom asymptote (fixed at 0 in Fig. 2C), $A2$ is the top asymptote, and p is the Hill slope. In Fig. 2C,
181 p was calculated to be 1.1 ± 0.1 .

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188 3. Results

189 3.1 Uptake mechanism of GBP in choriocarcinoma JEG-3 cell line

190 We previously reported that GBP accumulates in BeWo cells in higher concentrations than
191 other antiepileptic drugs, such as LTG, LEV, and TPM [11]. In the present study, the accumulation
192 levels of these drugs in JEG-3 cells were investigated. As shown in Table 1, after incubation with 50
193 μM of the antiepileptic drugs for 30 min at 37 °C, the cellular accumulation was as follows: GBP >
194 LTG > LEV $\approx$ TPM. The tendency for these drugs to accumulate in JEG-3 cells was consistent with
195 the results in BeWo cells.

196 Subsequently, uptake mechanism of GBP in JEG-3 cells was investigated. Figure 1A shows
197 the time-dependent uptake of GBP by JEG-3 cells following incubation with 5 μM GBP. The uptake
198 of GBP occurs in a linear fashion for the first 10 min; hence, the initial uptake was evaluated at 5
199 min. The transport of GBP in JEG-3 cells exhibited saturation at high concentrations (Fig. 1B). The
200 uptake of GBP at 4 °C was lower than that at 37 °C. Eadie–Hofstee plots (*inset* in Fig. 1B) showed a
201 single straight line. The kinetic parameters, K_m and $V_{\max}$ are calculated and summarized in Table 2.

202

203 3.2 Effects of OCTN and LAT inhibitors on GBP uptake

204 Previous reports suggest that OCTN1 and LAT1 may contribute to the transport of GBP in
205 other tissues. To elucidate the involvement of these transporters in the placenta, the effects of
206 various inhibitors on GBP uptake in BeWo cells was investigated. L-carnitine, tetraethylammonium

207 (TEA), quinidine, and cimetidine are OCTN inhibitors, while BCH is a LAT inhibitor. As shown in
208 Fig. 2 (A), OCTN inhibitors (1 mM) had no significant effect on GBP uptake in BeWo cells;
209 however, BCH (1 mM) decreased GBP uptake (Fig. 2(B)). Furthermore, BCH inhibited the uptake
210 of GBP in a concentration-dependent manner. The IC_{50} value was calculated as 0.113 ± 0.02 mM.
211 These results suggest GBP uptake in BeWo cells is mediated by a BCH-sensitive system.

212

213 **3.3 Effects of LAT1 knockdown on GBP uptake**

214 To evaluate the specific role of LAT1, the effect of LAT1 knockdown by siRNA was
215 investigated. In this study, three different siRNA constructs targeting the LAT1 gene were used. In
216 BeWo cells, all siRNAs significantly reduced LAT1 expression (approximately 50–80 % reduction)
217 at 72 h after transfection (Fig. 3A). In JEG-3 cells, siRNAs significantly reduced LAT1 expression
218 (approximately 35–80 % reduction) (Fig. 3C). LAT1 knockdown resulted in a reduction of GBP
219 uptake in both BeWo (Fig. 3B) and JEG-3 cells (Fig. 3D).

220

221 **3.4 Amino acids and thyroid hormones inhibit GBP uptake in choriocarcinoma cells**

222 Neutral amino acids and thyroid hormones, which are important for fetal development, are
223 substrates of LAT1. The effects of amino acids and thyroid hormones on the uptake of GBP were
224 investigated. Aromatic amino acids (Trp, Phe, Tyr), branched-chain amino acids (Leu, Ile, Val),
225 sulfur-containing amino acids (Met, Cys), and His strongly inhibited GBP uptake in BeWo cells (Fig.

226 4A). Thr, Ser, Gln, and Asn also significantly inhibited GBP uptake. When compared to the control,
227 thyroid hormones T₃ and T₄ inhibited GBP uptake by 35 and 64 %, respectively (Fig. 4B). The
228 inhibitory effect of T₃ was higher than that of T₄.

229

230 **3.5 Effects of antiepileptic drugs on GBP uptake in choriocarcinoma cells**

231 To assess the possibility of drug-drug interactions in the placenta, the effects of
232 antiepileptic drugs on GBP uptake were investigated. As shown in Table 3, the co-presence of
233 antiepileptic drugs, LEV, TPM, LTG, CBZ, phenytoin (PHT), phenobarbital (PB), VPA, zonisamide
234 (ZNS), nitrazepam (NZP), clobazam (CLB), and clonazepam (CZP) had no significant effect on
235 GBP uptake by BeWo cells.

236

237 **3.6 Transepithelial transport of GBP in JEG-3 cells**

238 To investigate the transepithelial transport of GBP, JEG-3 cells were seeded on Transwell.
239 The apparent permeability coefficient (P_{app}) of apical to basolateral GBP transport was 5.62 ± 0.64
240 ($\times 10^{-6}$ cm/s) (n=3), and that of basolateral to apical GBP transport was 4.96 ± 0.76 ($\times 10^{-6}$ cm/s)
241 (n=3).

242 The apical to basolateral transport of GBP was not significantly different from the basolateral to
243 apical transport. The apical to basolateral transport was reduced by the coexistence of BCH ($74 \pm$
244 8 % compared to the control) (n=3, $p < 0.05$). The basolateral to apical transport remained

245 unchanged in the co-presence of BCH (98 ± 3 % compared to the control) (n=3).

246 **4. Discussion**

247 Since antiepileptic drugs must cross the blood-brain barrier to exert their pharmacological
248 effects, ideally the substrate should be delivered to the brain, while keeping mother-to-fetus passage
249 across the placenta to a minimum [24]. Therefore, evaluating the involvement of transporters in the
250 transport process of antiepileptic drugs across the placenta may lead to the development of better
251 and safer antiepileptic drugs for pregnant women. GBP is an approved drug for the treatment of
252 epilepsy. GBP is also used for neuropathic pain and restless legs syndrome [25, 26]. Information
253 regarding the safety and risk of GBP administration during pregnancy is required in order for
254 pregnant patients to be fully informed. Active placental transport of GBP was suggested by Ohman
255 *et al.* [9] because of the high umbilical-to-maternal GBP plasma concentration ratio (mean, 1.7).
256 Placenta expresses a variety of influx and efflux transporters. However, little information is
257 currently available on the detailed mechanisms underlying the transport of GBP in placental cell
258 model. In the present study, we investigated the transport mechanism of GBP in choriocarcinoma
259 cell lines, BeWo and JEG-3.

260 The cellular accumulation of antiepileptic drugs in JEG-3 cells after incubation, occurred as
261 follows $\text{GBP} > \text{LTG} > \text{LEV} \approx \text{TPM}$ (Table 1). This tendency was consistent with BeWo cells as we
262 previously reported [11]. The extent of intracellular accumulation in the choriocarcinoma cells did
263 not correlate with the hydrophobicity of each compound. The uptake mechanism of GBP was

investigated in the present study, as the drug is present in high concentrations in choriocarcinoma cells, despite its hydrophilicity. Although the transport mechanisms of GBP in the blood-brain barrier [10], kidney [12], and intestine [13] have been reported, the mechanisms involved in the accumulation in placental cell model remain unclear. In brain endothelial cells, GBP has been shown to be transported by LAT1 [10]. The organic cation transporter (OCTN1, *SLC22A4*) contributes to the urinary excretion of GBP [12]. Reportedly, both LAT1 and OCTN1 are expressed in the placenta [16, 18], and this study investigated the involvement of these transporters in GBP transport.

Our previous study indicated that the transport of GBP in BeWo cells reached saturation at high concentrations, suggesting a carrier-mediated pathway [11]. The transport of GBP in JEG-3 cells followed saturation kinetics. Eadie-Hofstee plots showed single straight lines for both cell lines (BeWo, [11]; JEG-3, Fig. 1B), signifying a single carrier-mediated mechanism in the uptake of GBP. The K_m values were calculated as $105.4 \pm 6.4 \mu\text{M}$ for BeWo cells [11], and $123.7 \pm 22 \mu\text{M}$ for JEG-3 cells (Table 2). These values are similar to those obtained in LAT1-overexpressing HEK293 cells ($K_m = 217 \mu\text{M}$) [10]. The carrier has the potential to contribute to the transport of GBP, as the reference range for GBP plasma concentration varies approximately between 10–120 μM [20]. It has been reported that K_m value of OCTN1 for GBP is 417 μM [27].

BCH, an inhibitor of LAT, inhibited GBP transport in a concentration-dependent manner (Fig. 2B and 2C). The results indicate that the uptake of GBP in BeWo cells is mediated by LAT.

Knockdown studies revealed that LAT1 is involved in the transport of GBP in both BeWo and JEG-3 cells (Fig. 3). Furthermore, GBP transport was significantly inhibited by the biological substrates of LAT transporter, such as neutral amino acids and thyroid hormones. Amino acids, including Trp, Phe, Tyr, Leu, Ile, Val, Met, Cys, and His, strongly inhibited GBP uptake (Fig. 4A). The inhibitory effects of the amino acids were in accordance with the substrate selectivity of LAT1 [16]. The present study indicated that the inhibitory effect of T₃ was higher than that of T₄ (Fig. 4B). It has been reported that the affinity of LAT1 for T₃ is higher than that for T₄ (K_m values of 0.8 μ M for T₃, and 7.9 μ M for T₄) [28]. These results further suggest that LAT1 is the main contributor to the transport of GBP in choriocarcinoma cells.

The estimated value of the kinetic parameter V_{max} in BeWo cells was approximately twice as high as that obtained from JEG-3 cells (Table 2). Western blot analysis showed that the expression of LAT1 in BeWo cells was higher than its expression in JEG-3 cells (Supplemental Fig.1). These results suggest that the expression level of LAT1 affects the uptake capacity of BeWo and JEG-3 cells. It has been reported that the expression of LAT1 is higher in primary cytotrophoblasts isolated from human placenta than in differentiated syncytiotrophoblasts [29]. BeWo cells resemble undifferentiated cytotrophoblasts, and JEG-3 cells possess many of the biological and biochemical characteristics of syncytiotrophoblasts [19]. With respect to LAT1 expression, these cell lines may display a similar trend to that observed in primary cells.

RT-PCR analysis indicated that OCTN1 was expressed at mRNA level in both BeWo cells

302 and JEG-3 cells (Supplemental Fig. 2). However, typical inhibitors of OCTN1, including
303 L-carnitine, TEA, quinidine, and cimetidine, had no significant effect on GBP uptake (Fig. 2A). It
304 has been reported that L-carnitine, TEA, quinidine, and cimetidine significantly decreased the
305 OCTN1-mediated [¹⁴C]TEA transport in HEK293 cells [30]. Although mRNA expression of
306 OCTN1 was observed, the function of OCTN1 in placental cell lines might be low. Previous studies
307 reported strong expression of OCTN1 in kidney, skeletal muscle, bone marrow, and trachea [18].
308 Weaker expression of OCTN1 was observed in placenta [18]. On the other hand, strong expression
309 of OCTN2 was observed in placenta [18]. Furthermore, several groups have reported the functional
310 expression of the OCTN2 in BeWo cells [31, 32]. Therefore, the function of OCTN2 might be more
311 prominent than OCTN1 in placenta.

312 In the present study, we also investigated the transcellular transport of GBP using JEG-3
313 cells grown on Transwell. The apical to basolateral transport reduced by approximately 25 % by the
314 coexistence of BCH. On the other hand, the basolateral to apical transport remained unchanged in
315 the co-presence of BCH. This result may indicate that LAT1 expression in the basolateral membrane
316 in JEG-3 is low. Gaccioli *et al.* [33] reported that LAT1 protein is highly distributed in the
317 microvillus plasma membranes of the human term placenta, but it is not detected in the basolateral
318 membrane. Although it is not clear yet, passive diffusion or other mechanisms, may contribute to
319 basolateral to apical transport. It has been reported that umbilical cord-to-maternal plasma
320 concentration of GBP ratios ranged from 1.3 to 2.1 (mean, 1.7) [9]. However, the ratio of apical to

basolateral transport/basolateral to apical transport was approximately 1.1. In the present study, JEG-3 cells were grown on Transwell under normal culture conditions (Eagle's minimal essential medium). Ikeda *et al.* [34] suggested CS-C[®] medium condition was more appropriate for evaluating transcellular transport using JEG-3 cell line. Several cell lines, such as human intestinal Caco-2 cells and, Madin-Darby canine kidney MDCK cells, are widely used in transcellular transport and permeability studies. Although a fully validated system for choriocarcinoma cells is limited in comparison with these cell lines, several studies have used choriocarcinoma cells to investigate transcellular transport. BeWo b30, a clone of BeWo, was commonly used for transcellular transport studies [19]. Further investigations are required using BeWo b30 cells or JEG-3 cells grown under CS-C[®] medium condition.

To estimate intracellular concentration of GBP, intracellular water volumes of BeWo and JEG-3 cells were analyzed in the present study. The intracellular water volumes of BeWo and JEG-3 cells were calculated to be 5.12 ± 0.22 $\mu\text{L}/\text{mg}$ protein and 4.83 ± 0.82 $\mu\text{L}/\text{mg}$ protein, respectively. The concentrations of GBP in BeWo and JEG-3 cells after 60 min incubation with 5 μM of GBP were 3045 ± 328 pmol/mg protein for BeWo cells [11] and 2465 ± 188 pmol/mg protein for JEG-3 cells (Fig. 1A). Therefore, intracellular concentrations of GBP were estimated to be 595 ± 64 μM for BeWo cells and 510 ± 39 μM for JEG-3 cells. The results indicated that GBP accumulated in these cell lines. LAT1 is Na^+ -independent and obligatory exchanger [14]. It transports amino acid substrate into cells coupled with the efflux of another amino acid out of the cells. However, it has

340 been reported that LAT1 can actively uptake amino acid substrate by functionally coupling with
341 other amino acid transporter [35]. Leu transport by LAT1 is dependent on Gln that is actively
342 delivered into cells by Na⁺-dependent amino acid transporter ASCT2 (*SLC1A5*). LAT1 therefore
343 mediates the active influx of Leu into cells coupled to the efflux of intracellular Gln. Furthermore, it
344 has been reported that the intracellular affinities of LAT1 for amino acids are lower than the
345 extracellular affinities [36]. Therefore, GBP accumulation in placental choriocarcinoma cell line
346 may be attributed to the transport properties of LAT1. Future studies are required to investigate the
347 detailed mechanism of active transport of GBP in placental choriocarcinoma cell by LAT1.

348 Recently, it has been reported that several antiepileptic drugs alter the expression of
349 placental transporters, including that of LAT1 [37]. In the present study, we investigated whether
350 GBP uptake in choriocarcinoma cells is affected by the co-presence of other antiepileptic drugs.
351 Coexistence of antiepileptic drugs did not strongly affect GBP uptake in BeWo cells (Table 3).
352 These results suggest that these antiepileptic drugs do not directly affect LAT1 function and the
353 transport of GBP remains unchanged when other antiepileptic drugs are concurrently administered,
354 although the effect of long-term treatment is unclear. On the other hand, the present study indicated
355 that the biological substrates of LAT1, including thyroid hormones and several amino acids,
356 inhibited GBP transport in BeWo cells (Fig. 4). The results imply that plasma levels of these
357 substrates may affect the transport of GBP, while simultaneously; the transport of these biological
358 substrates may be inhibited by GBP. In previous reports, higher rates of preterm births and low birth

359 weights were documented in pregnant mothers using GBP [7]. Future studies should investigate the
360 possible association between fetal health risks and the transport mechanism of GBP.

361 It has been reported that the umbilical-to-maternal GBP plasma concentration ratio ranges
362 from 1.3 to 2.1, as found in a study with a small sample size of 6 women [9]. Single nucleotide
363 polymorphisms have also been known to occur in LAT1 (*SLC7A5*). It has been reported that an
364 *SLC7A5* polymorphism rs4240803 is associated with gastrointestinal toxicity of melphalan, which is
365 transported by LAT1 [38]. Future studies should investigate the effects of polymorphisms on the
366 variability of GBP transfer in the placenta.

367 In summary, the current study was undertaken to investigate the uptake mechanism of GBP,
368 an antiepileptic drug that accumulates in the choriocarcinoma cells, BeWo and JEG-3, in high
369 concentrations. Our results indicated that the transport of GBP in choriocarcinoma cells was
370 carrier-mediated and involved LAT1. Furthermore, GBP transport was modulated by the
371 co-presence of LAT biological substrates, such as large neutral amino acids and thyroid hormones.
372 To the best of our knowledge, this is the first study investigating the transport mechanism of GBP
373 and the contribution of transporters in choriocarcinoma cell lines, BeWo and JEG-3. Although these
374 cell lines have been widely used to examine the transport mechanism of xenobiotics in placenta,
375 these cell lines differ from the normal placental trophoblast. The extrapolation of findings observed
376 in choriocarcinoma cells to normal trophoblast must be made with caution. Further investigations
377 are required to elucidate the involvement of LAT1 in the transport of GBP in normal trophoblast.

378

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383

384 **Conflicts of Interest**

385 The authors report no conflicts of interest.

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513 **Figure captions**

514 **Figure 1.** Uptake mechanism of gabapentin (GBP) in JEG-3 cells. **(A)** Time dependence of GBP
515 uptake. JEG-3 cells were incubated with transport buffer containing 5 μ M of GBP at 37 °C. Each
516 point represents the mean $\pm$ S.E. of three independent experiments, each performed in triplicate. **(B)**
517 Concentration dependence of GBP uptake. JEG-3 cells were incubated with transport buffer
518 containing 10–500 μ M of GBP for 5 min at 37 °C (closed circles) or 4 °C (open circles). The GBP
519 uptake was obtained by subtracting the uptake at 4 °C from that at 37 °C (closed squares). Each
520 point represents the mean $\pm$ S.E. of four independent experiments, each performed in triplicate. The
521 inset shows an Eadie–Hofstee plot of GBP uptake.

522

523 **Figure 2.** Effects of OCTN inhibitors and LAT inhibitor on GBP transport in choriocarcinoma cells.
524 **(A)** BeWo cells were incubated with transport buffer containing 100 μ M of GBP in the presence or
525 absence of OCTN inhibitors (1 mM L-carnitine, 1 mM TEA, 1 mM quinidine, 1 mM cimetidine) for
526 5 min. Each column represents the mean $\pm$ S.E. of three independent experiments, each performed
527 in triplicate. **(B)** BeWo cells were incubated with transport buffer containing 100 μ M of GBP in the
528 presence or absence of 1 mM BCH for 5 min. Each column represents the mean $\pm$ S.E. of three
529 independent experiments, each performed in triplicate. **(C)** Concentration-dependent inhibition of
530 GBP uptake by BCH. BeWo cells were treated with transport buffer containing 10 μ M of GBP in
531 the presence or absence of BCH (0.01 – 10 mM) for 5 min. Each point represents the mean $\pm$ S.E. of

532 four independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$ when compared
533 to the control.

534

535 **Figure 3.** Effects of LAT1 siRNAs on GBP transport in choriocarcinoma cells. **(A and C)** Western
536 blot of BeWo cells (A) and JEG-3 cells (C) transfected with negative control siRNA, LAT1 siRNA 1
537 (HSS112004), LAT1 siRNA 2 (HSS112005), or LAT1 siRNA 3 (HSS188571). **(B and D)** Effects of
538 LAT1 siRNAs on GBP transport in BeWo cells (B) and JEG-3 cells (D). Cells were incubated with
539 transport buffer containing 100 μ M of GBP for 5 min. Each column represents the mean $\pm$ S.E. of
540 three independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$ when compared
541 to the control.

542

543 **Figure 4.** Effects of LAT biological substrate on GBP uptake in choriocarcinoma cells. BeWo cells
544 were incubated with transport buffer containing 100 μ M of GBP for 5 min in the presence or
545 absence of amino acids (1 mM) (A) or thyroid hormones (10 μ M) (B). Each column represents the
546 mean $\pm$ S.E. of three independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$
547 when compared to the control.

548

549 **Supplemental Figure 1.** Western blot examination of LAT1 expression in BeWo and JEG-3 cells.

550

551 **Supplemental Figure 2.** RT-PCR analysis of mRNA expression in BeWo and JEG-3 cells: GAPDH
552 was detected as a loading control. PCR was performed using specific primers as described in
553 materials and methods section [2.8].

Table 1. Accumulation concentrations of lamotrigine (LTG), levetiracetam (LEV), gabapentin (GBP), and topiramate (TPM) in choriocarcinoma cell lines.

	Accumulation (pmol/mg protein/30 min)	
	JEG-3	BeWo ^[11]
GBP	9410 ± 1191	14055 ± 2660
LTG	744 ± 81	1272 ± 81
LEV	454 ± 13	315 ± 31
TPM	443 ± 9	195 ± 9

JEG-3 cells were incubated with transport buffer containing 50 μ M of LTG, LEV, GBP, or TPM for 30 min at 37 °C. Data represents the mean $\pm$ S.E. of three independent experiments, each performed in triplicate.

Table 2. Summary of kinetic parameters for GBP uptake in choriocarcinoma cell lines.

Cell line	K_m (μM)	V_{max} (pmol/mg protein/min)	Reference
BeWo	105.4 ± 6.4	8153 ± 348	[11]
JEG-3	123.7 ± 22	4136 ± 522	Present study

Table 3. Effects of antiepileptic drugs on GBP uptake in BeWo cells.

Antiepileptic drug	Relative uptake (% of control)
LEV	99 ± 4
TPM	92 ± 2
LTG	92 ± 9
CBZ	88 ± 6
PHT	93 ± 6
PB	89 ± 7
VPA	83 ± 7
ZNS	93 ± 12
NZP	100 ± 9
CLB	84 ± 8
CZP	91 ± 5

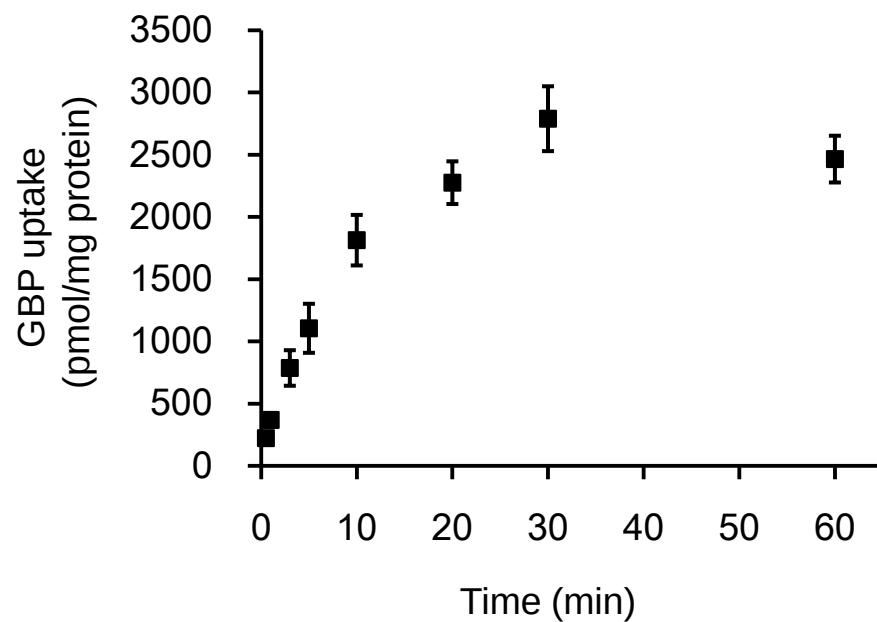
BeWo cells were incubated with transport buffer containing 100 μ M of GBP for 5 min in the presence or absence of 0.5 mM of the antiepileptic drugs, LEV (levetiracetam), TPM (topiramate), LTG (lamotrigine), CBZ (carbamazepine), PHT (phenytoin), PB (phenobarbital), VPA (valproic acid),

ZNS (zonisamide), NZP (nitrazepam), CLB (clobazam), and CZP (clonazepam). Each data represents the mean $\pm$ S.E. of three independent experiments, each performed in triplicate.

Figure 1

Fig. 1

(A)



(B)

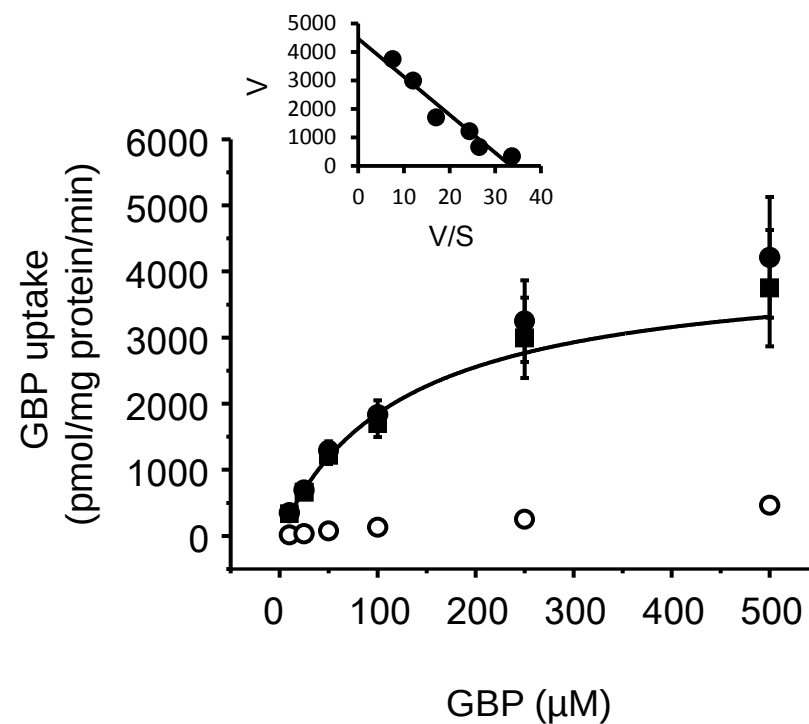
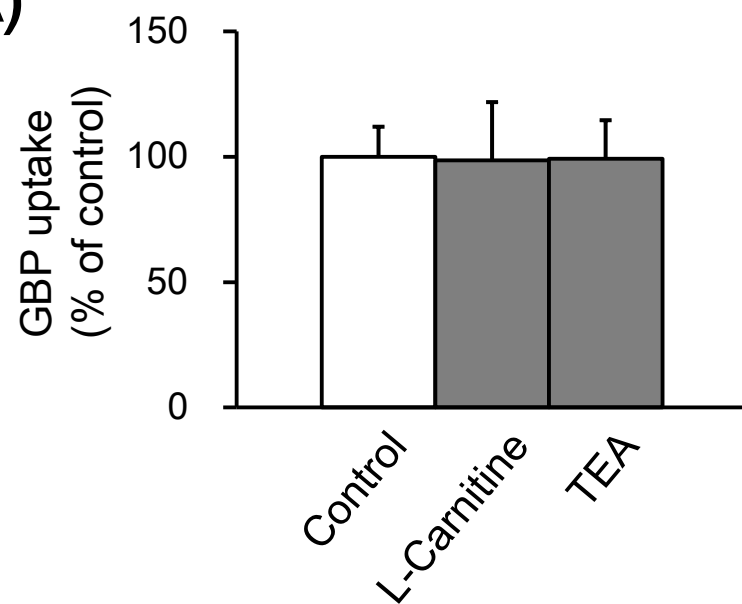
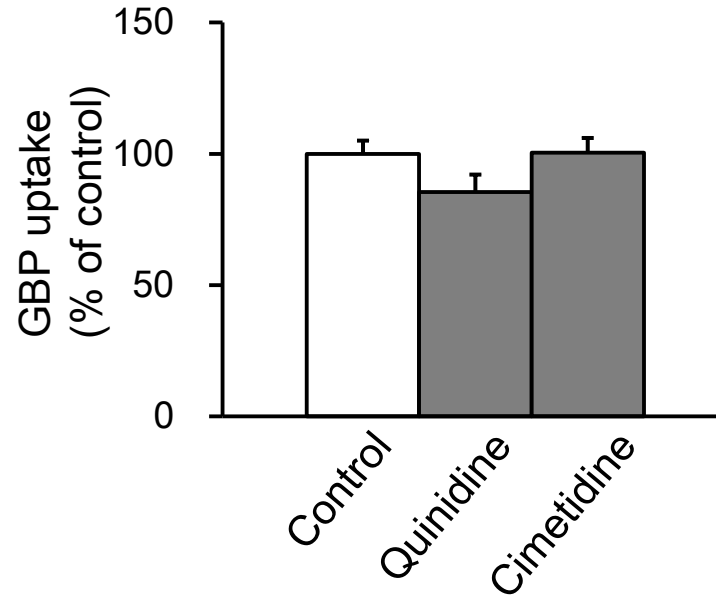


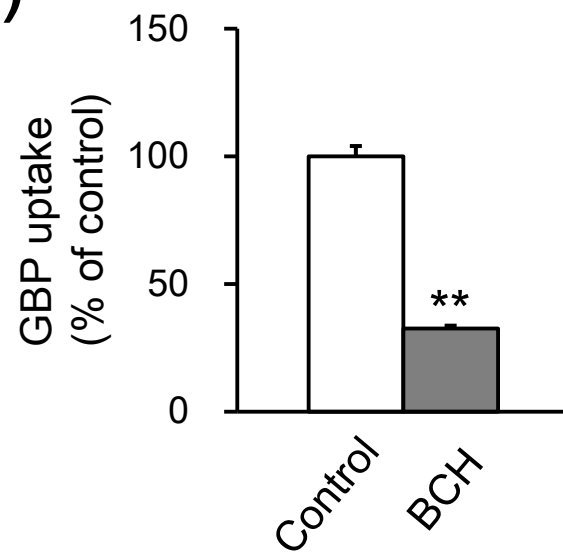
Figure 2

Fig. 2

(A)



(B)



(C)

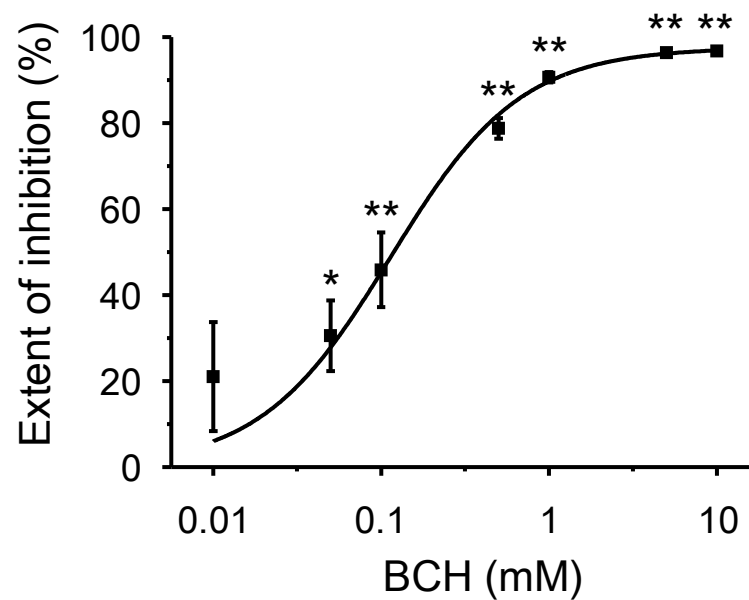


Figure 3
Fig. 3

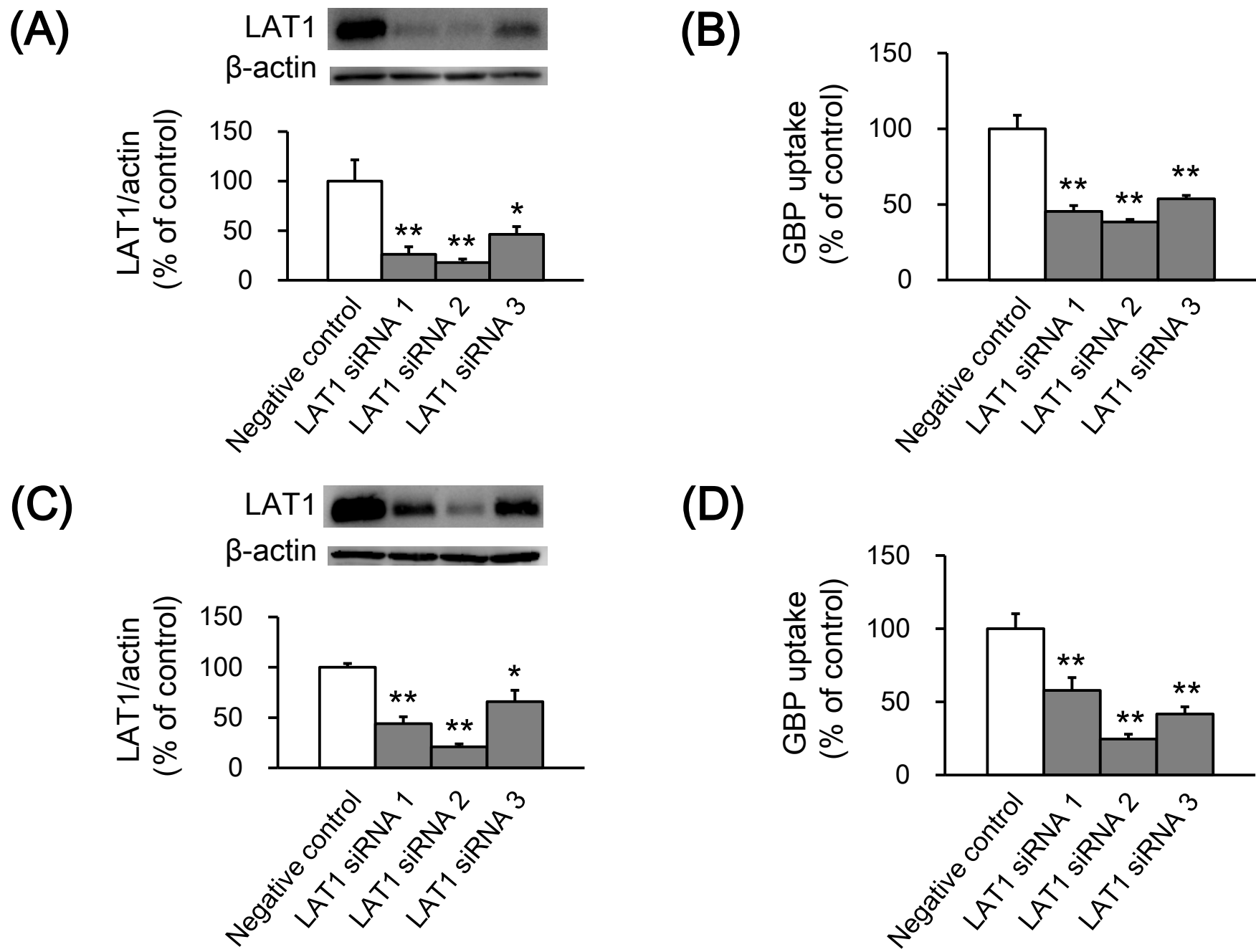
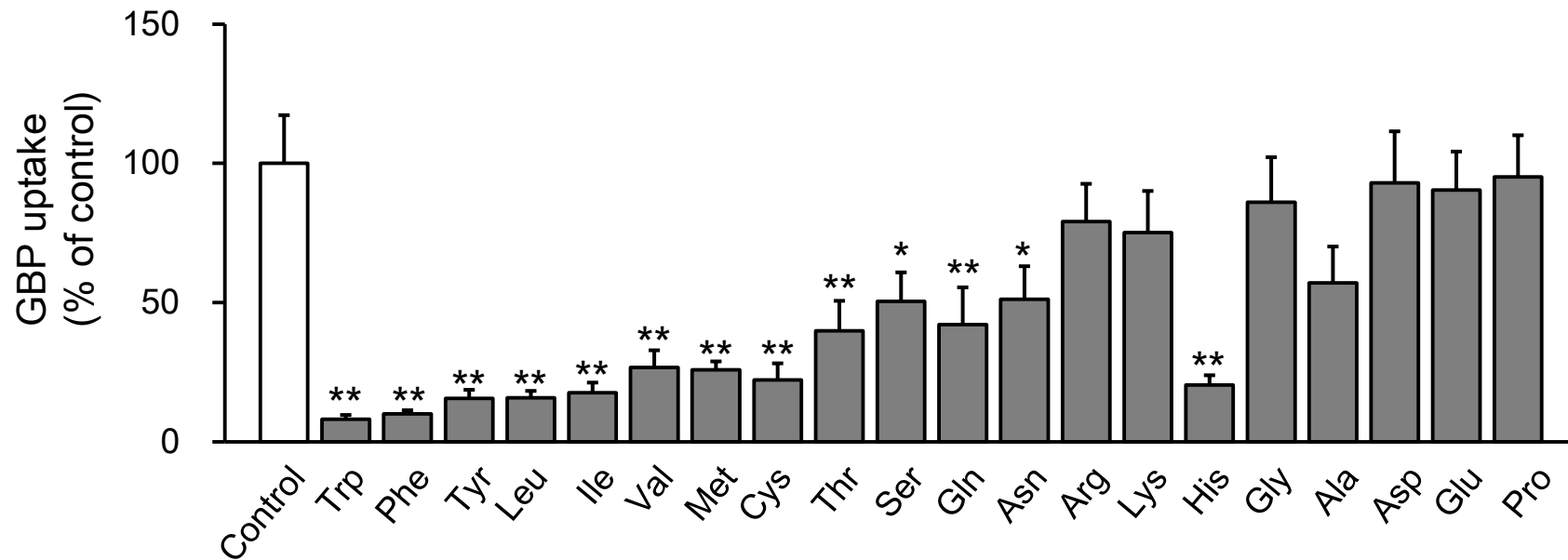
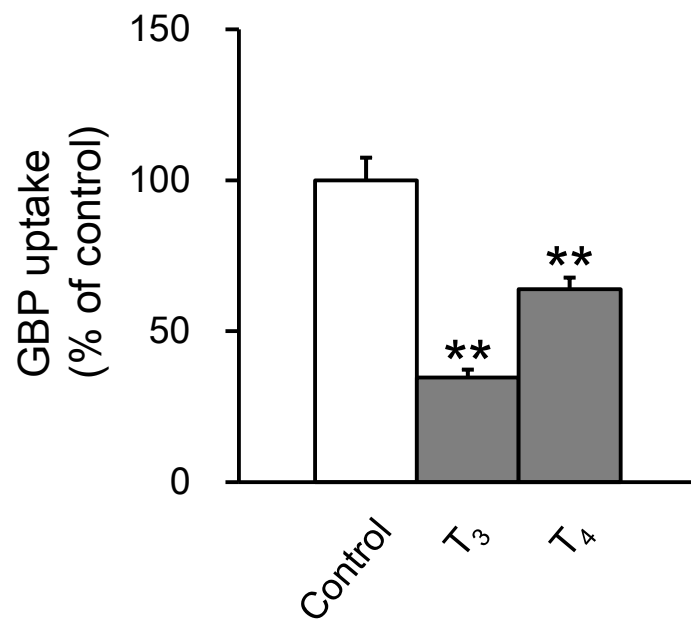


Figure 4
Fig. 4

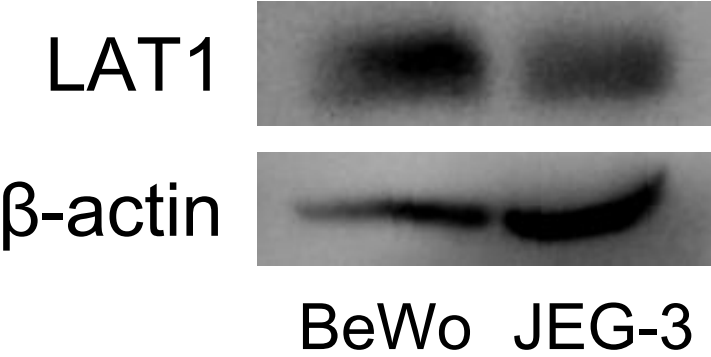
(A)



(B)

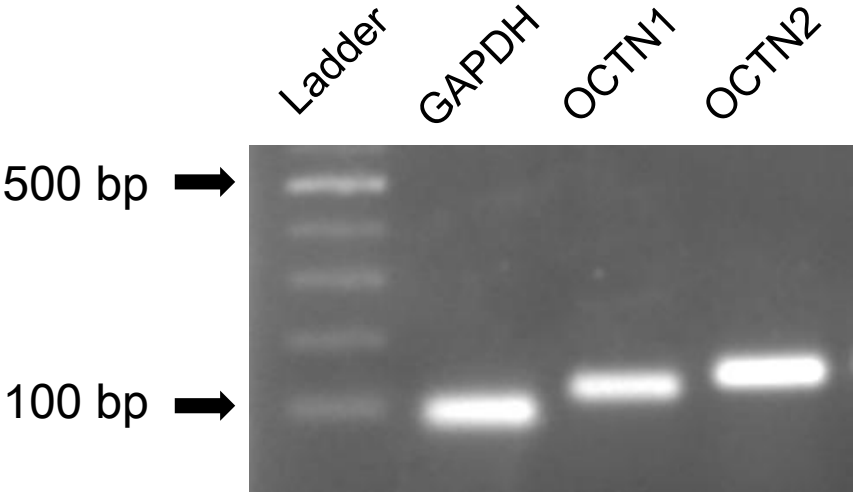


Supplemental Fig. 1



Supplemental Fig. 2

BeWo



JEG-3

