

# Branched Chain Amino Acids Promote ATP Production Via Translocation of Glucose Transporters

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**PURPOSE.** We have previously shown that maintenance of ATP levels is a promising strategy for preventing neuronal cell death, and that branched chain amino acids (BCAAs) enhanced cellular ATP levels in cultured cells and antagonized cell death. BCAAs attenuated photoreceptor degeneration and retinal ganglion cell death in rodent models of retinal degeneration or glaucoma. This study aimed to elucidate the mechanisms through which BCAAs enhance ATP production.

**METHODS.** Intracellular ATP concentration was measured in HeLa cells under glycolysis and citric acid cycle inhibited conditions. Next, glucose uptake was quantified in HeLa cells and in 661W retinal photoreceptor-derived cells under glycolysis inhibition, endoplasmic reticulum stress, and glucose transporters (GLUTs) inhibited conditions, by measuring the fluorescence of fluorescently labeled deoxy-glucose analog using flow cytometry. Then, the intracellular behavior of GLUT1 and GLUT3 were observed in HeLa or 661W cells transfected with enhanced green fluorescent protein-GLUTs.

**RESULTS.** BCAAs recovered intracellular ATP levels during glycolysis inhibition and during citric acid cycle inhibition. BCAAs significantly increased glucose uptake and recovered decreased glucose uptake induced by endoplasmic reticulum stress or glycolysis inhibition. However, BCAAs were unable to increase intracellular ATP levels or glucose uptake when GLUTs were inhibited. Fluorescence microscopy revealed that supplementation of BCAAs enhanced the translocation of GLUTs proteins to the plasma membrane over time.

**CONCLUSIONS.** BCAAs increase ATP production by promoting glucose uptake through promotion of glucose transporters translocation to the plasma membrane. These results may help expand the clinical application of BCAAs in retinal neurodegenerative diseases, such as glaucoma and retinal degeneration.

**Keywords:** branched chain amino acid, neuroprotection, ATP, glucose uptake, glucose transporters

Neuronal cell death causes incurable eye diseases, such as retinal degenerative diseases and glaucoma, which are major causes of blindness worldwide. Among retinal degenerative diseases, retinitis pigmentosa is the most common type of hereditary retinal degeneration, and is characterized by progressive damage to photoreceptors. Although various potential therapeutic strategies are currently being investigated<sup>1-5</sup> and several clinical trials are ongoing,<sup>6</sup> therapies that prevent photoreceptor cell death have not been established. For glaucoma, intraocular pressure reduction is the only established effective strategy to prevent retinal ganglion cell death; however, there are considerable cases in which visual impairment progresses even when the intraocular pressure is reduced.<sup>7</sup>

We have previously shown that the maintenance of intracellular ATP levels antagonizes cell death. Prevention of ATP consumption by inhibiting the ATPase activity of valosin-containing protein, the most abundant soluble ATPase within a living organism, prevented neuronal cell death in glaucoma,<sup>8</sup> retinal degeneration,<sup>9-11</sup> and retinal artery occlusion.<sup>12,13</sup>

Another way to maintain intracellular ATP levels is by enhancing ATP production. We have previously shown that supplementation with branched-chain amino acids (BCAAs) increased intracellular ATP levels and protected cultured cells against endoplasmic reticulum (ER) stress and mitochondrial respiratory chain inhibition stress.<sup>14,15</sup> Additionally, we have shown that supplementation with BCAAs attenuated photoreceptor cell death in mouse models of retinitis pigmentosa, and attenuated retinal ganglion cell death in a mouse model of glaucoma.<sup>14</sup>

BCAAs are important energy sources. Degraded BCAAs are used as energy sources in various proportions in the citric acid cycle, depending on the cell type or conditions.<sup>16-18</sup> BCAAs have also been reported to improve glucose metabolism in cirrhotic liver,<sup>19</sup> and promote glucose uptake in skeletal muscle in rodents.<sup>20-22</sup> However, the mechanisms through which BCAAs promote glucose metabolism have not been fully elucidated. Clinically, BCAAs have been used to treat patients with liver cirrhosis, and have been shown to reduce the incidence of complications.<sup>23-25</sup>



In the current study, we elucidated the mechanisms through which BCAAs enhance ATP production.

## METHODS

### Cell Culture and ATP Measurement

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L of glucose and 0.1% fetal bovine serum without amino acids (Wako Pure Chemical Industries, Ltd., Osaka, Japan, 048-33575). BCAAs (L-isoleucine: L-leucine: L-valine = 1:2:1.2, the same as LIVACT; Ajinomoto Co., Tokyo, Japan) were added to the medium (0.04, 4.0, or 40 mM, molecular weight was calculated as 126.829 g/mol from the respective molecular weights and the abundance ratio in the formulation).<sup>14</sup> Glycolysis inhibition was induced by a hexokinase inhibitor, lonidamine (1-(2,4-Dichlorobenzyl)-1H-indazole-3-carboxylic acid, 300  $\mu$ M, [used in a previous study at 150  $\mu$ M for HeLa cells,<sup>26</sup> the same concentration as shown in Supplementary Fig. S1A], Abcam, Cambridge, UK, ab142442); a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inhibitor, heptelidic acid (0.6  $\mu$ M [used in a previous study at 35  $\mu$ M for various cancer cell lines<sup>27</sup>; at this concentration, we observed excessive cell death in the current study], Abcam, ab144269); or a pyruvate kinase M2 (PKM2) inhibitor, shikonin (5,8-dihydroxy-2-[(1R)-1-hydroxy-4-methyl-3-penten-1-yl]-1,4-naphthalenedione, 3  $\mu$ M, [used in a previous study at 1 or 10  $\mu$ M for B16 cells],<sup>28</sup> Sigma-Aldrich, St. Louis, MO, USA, S7576). Citric acid cycle inhibition was induced by 5  $\mu$ M UK5099 ((E)-2-Cyano-3-(1-phenyl-1H-indol-3-yl) acrylic acid, (E)-2-Cyano-3-(1-phenyl-1H-indol-3-yl)-2-propenoic acid, [used in a previous study at 10  $\mu$ M for LNCaP cells],<sup>29</sup> Funakoshi Co., Ltd., Tokyo, Japan, AG-CR1-3691-M0005), a mitochondrial pyruvate carrier inhibitor.

To measure relative ATP levels, HeLa cells were cultured for 24 hours in medium with or without lonidamine, heptelidic acid, and BCAAs, or for 48 hours in medium with or without UK5099 and BCAAs.

The 661W cells<sup>30</sup> were kindly provided by Dr. Muayyad R. Al-Ubaidi (University of Houston, Houston, TX, USA). Glucose transporters (GLUT) inhibition was induced by WZB117 (Sigma; SML0621, an inhibitor of GLUT1, 3 and 4, used in a previous study at 0.7–60  $\mu$ M for erythrocytes).<sup>31</sup> The 661W cells were cultured in DMEM containing 4.5 g/L of glucose and 0.1% fetal bovine serum without amino acids and with or without 40 mM BCAAs for 2 hours, and then incubated with or without BCAAs and 100  $\mu$ M WZB117 for 2 hours before measurement of relative ATP levels.

Relative ATP levels in cultured cells were measured by luciferase activity using an intracellular ATP assay kit (Toyo B-net, Tokyo, Japan) with a Nivo Multimode Microplate Reader (PerkinElmer, Inc., Waltham, MA, USA). After trypsinization, live cell numbers were measured with a TC20 cell counter (Bio-Rad, Hercules, CA, USA).

### Flowcytometry Analysis

To quantify the glucose uptake, the fluorescence of fluorescently labeled deoxy-glucose analog (2-NBDG; Wako) was quantified using flow cytometry. Tunicamycin (Nacalai Tesque, Kyoto, Japan) was added to induce ER stress, 2-deoxy-D-glucose (2-DG, Nacalai Tesque, hexokinase inhibitor) was added to inhibit glycolysis.

HeLa cells were cultured in DMEM containing 4.5 g/L glucose and 0.1% fetal bovine serum without amino acids for 24 hours, with or without 40 mM BCAAs, with or without 7  $\mu$ g/mL tunicamycin, and with or without 75 mM 2-DG. The cells were then incubated in DMEM without glucose without amino acids containing 100  $\mu$ M 2-NBDG for 15 minutes, with or without 40 mM BCAAs, with or without 7  $\mu$ g/mL tunicamycin, and with or without 75 mM 2-DG before flow cytometry analysis.

HeLa cells and 661W cells were cultured in DMEM containing 4.5 g/L glucose and 0.1% fetal bovine serum without amino acids with or without BCAAs (0.04, 4.0, or 40 mM) for 24 hours, followed by culture with 100  $\mu$ M 2-NBDG for 15 minutes before flow cytometry analysis.

HeLa cells and 661W cells were incubated in DMEM containing 4.5 g/L glucose and 0.1% fetal bovine serum without amino acids with or without 40 mM BCAAs for 3 hours. Next, the cells were incubated in DMEM containing 4.5 g/L glucose with or without 40 mM BCAAs, with or without WZB117 (250 or 100  $\mu$ M for HeLa cells and 125  $\mu$ M for 661W cells) for 1 hour before incubation with 100  $\mu$ M 2-NBDG for 15 minutes.

The number of live cells and the fluorescence of 2-NBDG taken into the cells were quantified using FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA; Supplementary Fig. S2).

### Transfection of EGFP-GLUT1 or GLUT3 and Fluorescence Microscopy

Vector plasmids carrying enhanced green fluorescent protein (EGFP) fusion-GLUT1 or GLUT3 were prepared using the pEGFP-C2 vector (Clontech Laboratories, Mountain View, CA, USA; 6083-1). GLUT1 and GLUT3 cDNA was amplified from HeLa cells using the following primers: GLUT1, primer-F: CTCAAGCTTCGAATTCATGGAGC-CCAGCAGCAA, and primer-R: TAGATCCGGTGGATCTCACTTGGGAATCAGCC; GLUT3, primer-F: CTCAAGCTTCGAATTGATGGGGACACAGAAGGT, and primer-R: TAGATCCGGTGGATCTTAGACATTGGTGGTGGTC. The N-terminal of GLUT1 and GLUT3 was fused with EGFP. HeLa cells and 661W cells were transfected with the prepared vectors using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA). EGFP-GLUT1 or GLUT3 expression in HeLa or 661W cells was confirmed by Western blot analysis (Supplementary Fig. S3).

HeLa cells and 661W cells expressing EGFP-GLUT1 or GLUT3 were incubated in DMEM containing 4.5 g/L of glucose and 0.1% fetal bovine serum without amino acids with or without 80 mM BCAAs. Cells were imaged using a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan) at 0, 60, 120, and 180 minutes after incubation. The fluorescence intensities of EGFP-GLUTs were quantified using BZ-II Analyzer software (Keyence). The ratio of fluorescence intensities of the plasma membrane to the cytosol was calculated.

### Statistical Analysis

Data are presented as the mean  $\pm$  standard deviation. A Tukey honestly significant difference (HSD) test was used to compare parameters under multiple conditions in HeLa and 661W cells. The unpaired *t*-test was used to compare

glucose uptake in HeLa cells, with or without BCAAs. Statistical significance was set at  $P < 0.05$ .

## RESULTS

### BCAA Supplementation Raises ATP Levels in Cultured Cells During Glycolysis or Citric Acid Cycle Inhibition

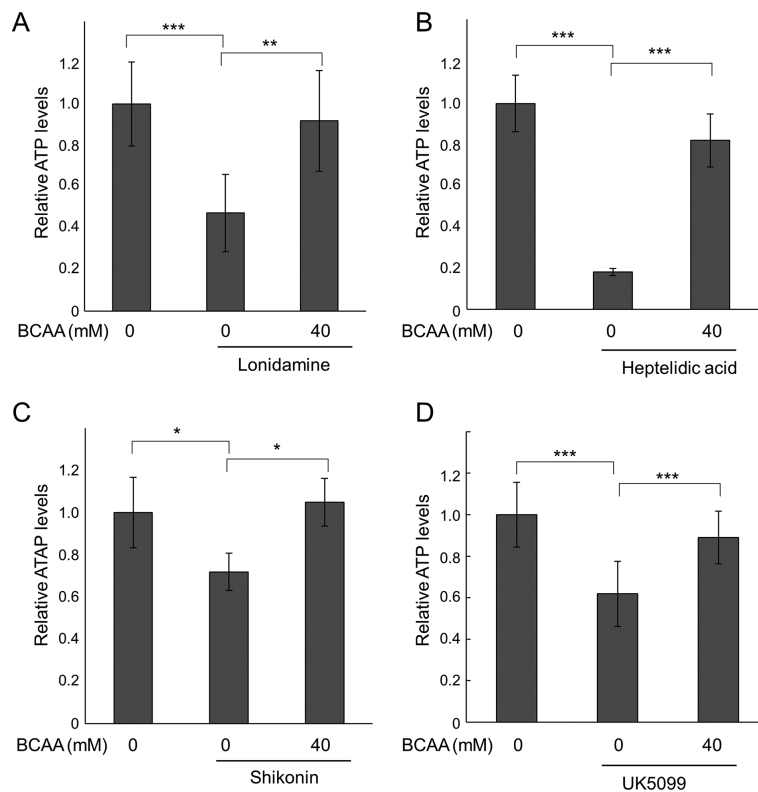
In HeLa cells, inhibition of glycolysis or citric acid cycle decreased intracellular ATP concentration ( $P = 0.002$ ,  $P < 0.0001$ ,  $P = 0.03$ , and  $P < 0.0001$ , lonidamine [glycolysis hexokinase inhibitor], heptelidic acid [glycolysis GAPDH inhibitor], Shikonin [glycolysis PKM2 inhibitor], and UK5099 [citric acid cycle mitochondrial pyruvate carrier inhibitor], respectively, Tukey HSD; Fig. 1). The addition of a formulation of BCAAs, L-isoleucine, L-leucine, and L-valine (1:2:1.2), recovered intracellular ATP levels during glycolysis inhibition ( $P = 0.006$ ,  $P < 0.0001$ , and  $P = 0.013$ , lonidamine, heptelidic acid, and Shikonin, respectively, Tukey HSD; see Figs. 1A–C, Supplementary Fig. S1), and during citric acid cycle inhibition ( $P = 0.002$ , Tukey HSD; see Fig. 1D). BCAAs at  $\geq 4$  mM recovered the decreased intracellular ATP levels and live cell numbers during glycolysis inhibition induced by lonidamine (Supplementary Fig. S4). This BCAA concentration also increased ATP levels to prevent cell death under ER stress, as observed in our previous study,<sup>14</sup> and was 10-fold higher than the human plasma concentration in the static state.<sup>32</sup>

Thus, BCAAs recovered the decrease in ATP levels induced by glycolysis inhibitors, which inhibit hexokinase, GAPDH, or PKM2, and by a citric acid cycle inhibitor, which inhibits mitochondrial pyruvate carrier activity. In our previous study, BCAAs also recovered ATP levels during mitochondrial respiratory chain inhibition.<sup>14</sup>

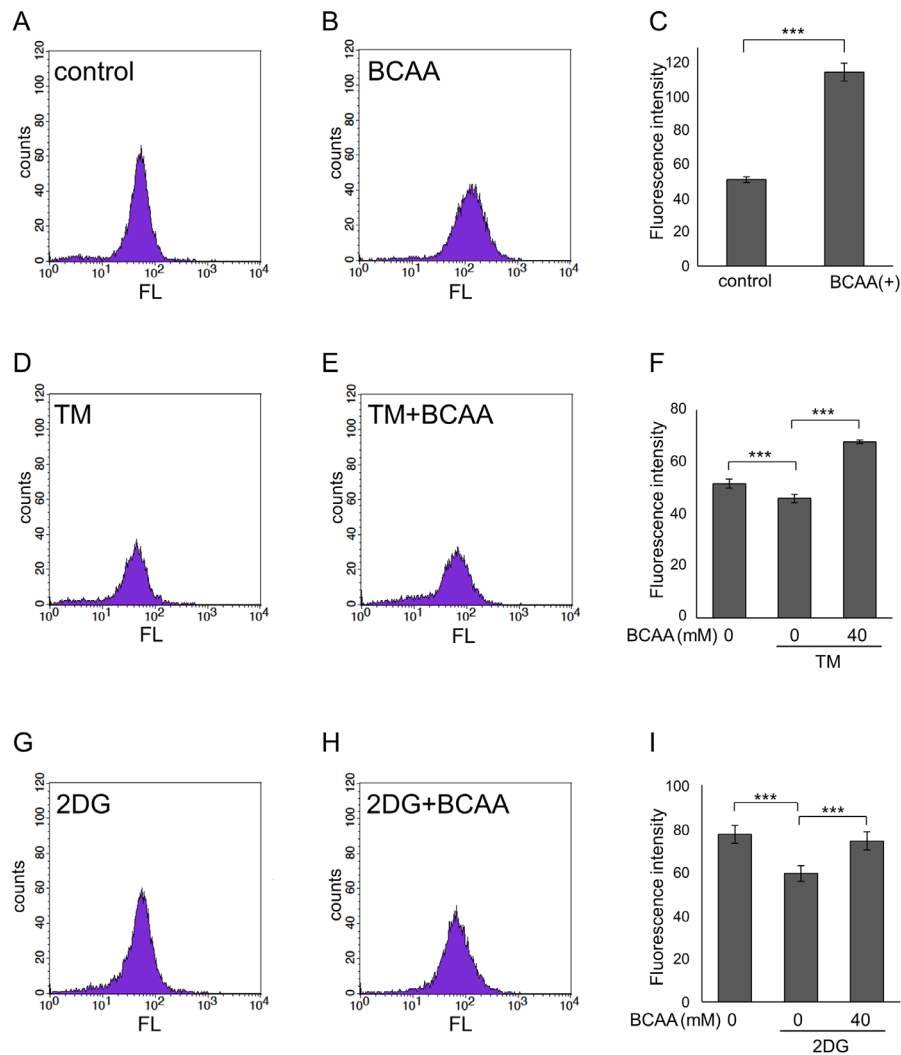
### BCAA Supplementation Promotes Glucose Uptake Thorough Glucose Transporter

Next, we investigated the effect of BCAAs on glucose uptake. Glucose uptake quantified by measuring the fluorescence of 2-NBDG using flow cytometry was significantly increased by addition of BCAAs in HeLa cells ( $P < 0.0001$ , unpaired  $t$ -test; Figs. 2A–C). BCAAs at  $\geq 4$  mM increased glucose uptake (Supplementary Fig. S5A). Glucose uptake was significantly decreased when ER stress was induced by tunicamycin ( $P = 0.001$ , Tukey HSD), and glucose uptake was recovered by BCAA supplementation ( $P < 0.0001$ , Tukey HSD; see Figs. 2D–F). Glucose uptake was also significantly decreased when glycolysis was inhibited by 2-DG ( $P < 0.0001$ , Tukey HSD), and glucose uptake was recovered by BCAA supplementation ( $P = 0.001$ , Tukey HSD; see Figs. 2G–I).

Glucose uptake by cells is mediated by glucose transporters (GLUTs).<sup>33</sup> GLUT1, GLUT3, and GLUT4 have been reported to be expressed in retinal cells.<sup>34–38</sup> For this reason, we studied the effect of BCAA supplementation on GLUT inhibition. The addition of WZB117, an inhibitor of GLUT1,



**FIGURE 1.** BCAAs recovered intracellular ATP decrease in cultured cells. HeLa cells were cultured in an amino acid-free medium containing 4.5 g/L of glucose with or without 40 mM Branched-chain amino acids (BCAAs), and with or without 300  $\mu$ M lonidamine (glycolysis hexokinase inhibitor, **A**,  $N = 6$ ), 0.6  $\mu$ M heptelidic acid (glycolysis glyceraldehyde 3-phosphate dehydrogenase [GAPDH] inhibitor, **B**,  $N = 4$ ), Shikonin (glycolysis pyruvate kinase M2 [PKM2] inhibitor, **C**, 3  $\mu$ M,  $N = 4$ ), or 5  $\mu$ M UK5099 (citric acid cycle inhibitor, **D**,  $N = 7$ ). After HeLa cells were cultured for 24 hours (**A–C**) or 48 hours (**D**), relative intracellular ATP levels were determined by luciferase activity. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.005$ , Tukey honestly significant difference (HSD). Bars represent standard deviation.



**FIGURE 2.** BCAAs promoted glucose uptake in HeLa cells. HeLa cells were cultured in an amino acid-free medium containing 4.5 g/L of glucose with or without 40 mM BCAAs (A–I) and with or without 7  $\mu$ g/mL tunicamycin (TM; endoplasmic reticulum stress inducer) (D–F) or 75 mM 2-deoxy-D-glucose (2-DG, glycolysis hexokinase inhibitor) (G–I) for 24 hours before addition of fluorescently labeled deoxy-glucose analog (100  $\mu$ M 2-NBDG). A, B, D, E, G, H Histograms showing distribution of cell counts for fluorescence intensities (FL) by flow cytometry analysis. C, F, I Geometric average of fluorescence intensities of live cells. C \*\*\* $P$  < 0.0001, unpaired  $t$ -test, F, I \*\*\* $P$  < 0.005, Tukey HSD,  $N$  = 4. Bars represent the standard deviation.

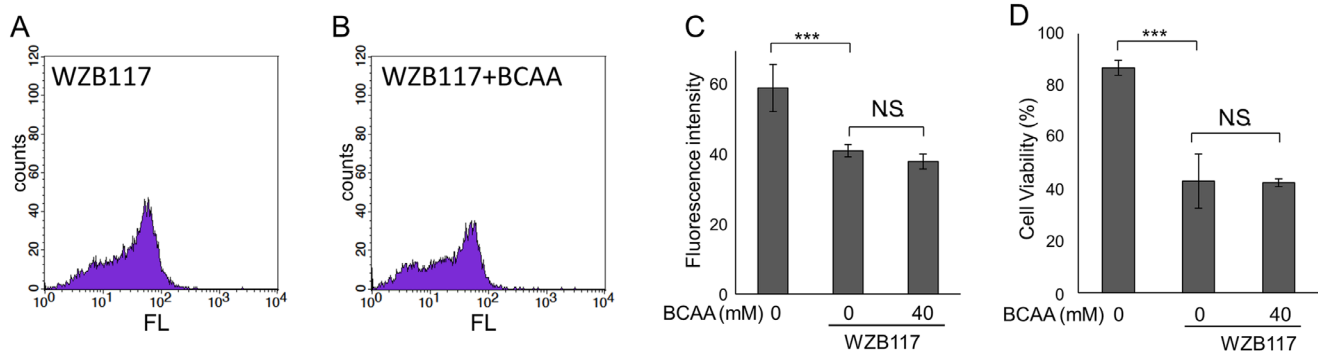
3, and 4, resulted in suppression of glucose uptake in HeLa cells ( $P$  < 0.001, Tukey HSD), and this was not recovered by BCAAs ( $P$  = 0.58, Tukey HSD; Figs. 3A–C, Supplementary Fig. S6A). Under these conditions, cell viability decreased ( $P$  < 0.0001, Tukey HSD), and did not recover by addition of BCAAs ( $P$  = 0.99, Tukey HSD; see Fig. 3D, Supplementary Fig. S6B).

Next, we evaluated glucose uptake in retinal photoreceptor-derived cells, 661W.<sup>30</sup> BCAAs also increased glucose uptake by 661W cells (see Supplementary Fig. S5B). Glucose uptake in 661W cells was decreased by the addition of WZB117 ( $P$  < 0.0001, Tukey HSD), and was not recovered by BCAAs supplementation ( $P$  = 0.57, Tukey HSD; Figs. 4A–C). Under these conditions, the intracellular ATP concentration also decreased ( $P$  = 0.004, Tukey HSD), and did not recover by addition of BCAAs ( $P$  = 0.997, Tukey HSD; see Fig. 4D). Similarly, cell viability decreased ( $P$  < 0.0001, Tukey HSD), and did not recover by the addition of BCAAs ( $P$  = 0.22, Tukey HSD; see Fig. 4E).

These results show that BCAA supplementation promotes glucose uptake through GLUTs and recovers intracellular ATP levels.

### BCAA Supplementation Promotes Translocation of Glucose Transporter to Cellular Surface Membrane

Next, we studied the effect of BCAAs on the expression of GLUTs. First, HeLa cells were cultured with or without BCAAs. Western blotting analysis of the whole cells showed no apparent differences in the expression of GLUT1 and GLUT3 among the various conditions (Supplementary Fig. S7A–C). Then, we evaluated GLUT1 expression on the plasma membrane under stress conditions. HeLa cells were cultured with or without BCAAs and with or without tunicamycin, and the plasma membrane fraction was extracted. The amount of GLUT1 protein in the plasma membrane

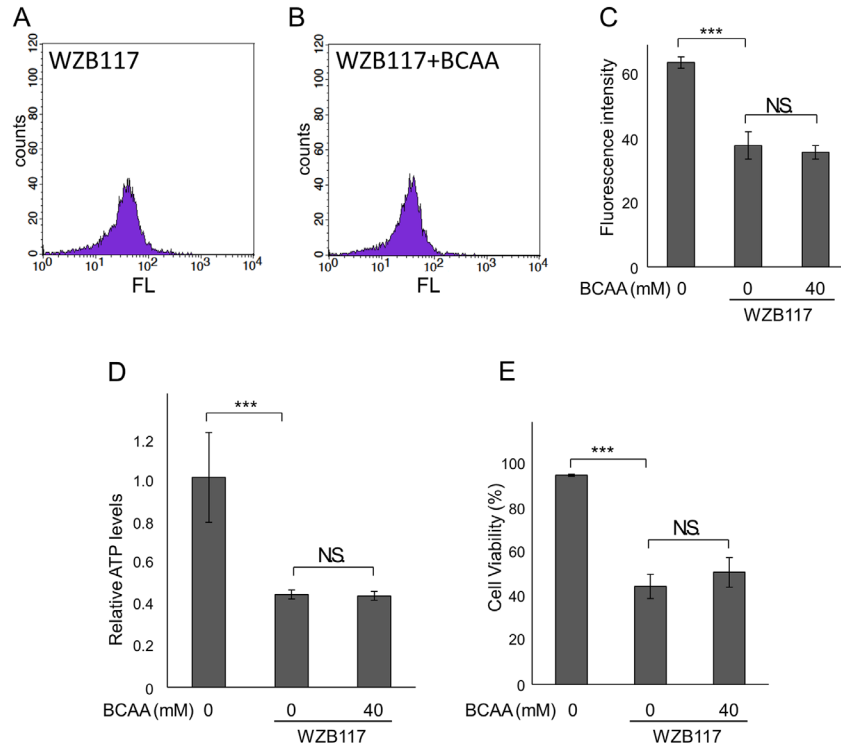


**FIGURE 3.** Inhibition of glucose transporters prevented BCAAs from promoting glucose uptake. HeLa cells were cultured in an amino acid-free medium containing 4.5 g/L of glucose with or without 40 mM BCAAs for 3 hours and then incubated with or without 250 μM WZB117 (inhibitor of glucose transporter [GLUT] 1, 3, and 4) for 1 hour before addition of fluorescently labeled deoxy-glucose analog (100 μM 2-NBDG). **A–C** Glucose uptake with or without BCAAs and with or without WZB117 in HeLa cells. **A, B** Histograms showing distributions of cell counts for fluorescence intensities (FL) by flowcytometry analysis. **C** Geometric average of fluorescence intensities of live cells. **D** Cell viability with or without BCAAs and with or without WZB117 in HeLa cells. **C, D** \*\*\* $P < 0.005$ , N.S. no significant difference, Tukey HSD,  $N = 4$  each. Bars represent standard deviation.

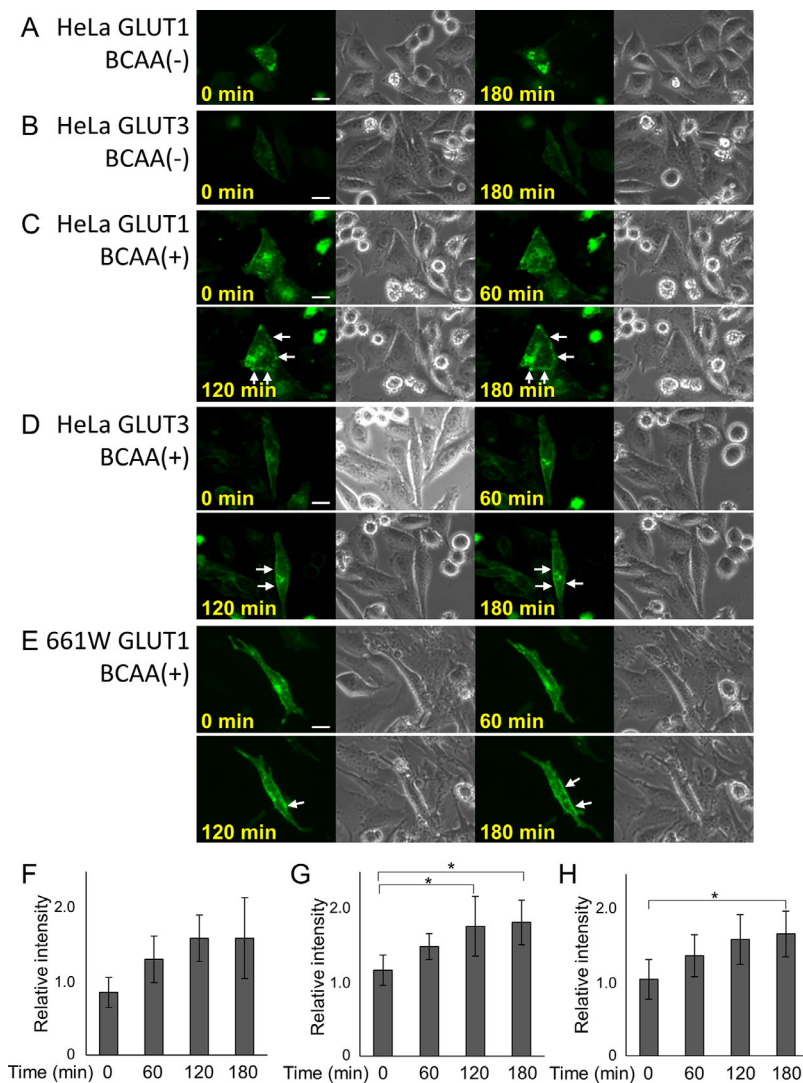
was less in tunicamycin-treated cells ( $P = 0.02$ , Tukey HSD) in comparison with non-treated cells (see Supplementary Figs. S7D–E).

To confirm whether the translocation of GLUTs to the plasma membrane is promoted by BCAAs, we transfected EGFP-GLUT1 or GLUT3 in HeLa or 661W cells, to observe the intracellular behavior of GLUT1 and GLUT3. Fluorescence

microscopy revealed that EGFP-GLUT proteins remained in the cytoplasm in cells cultured without BCAA supplementation, and these proteins were translocated to the plasma membrane over time, in the cells supplemented with BCAAs (Fig. 5,  $P = 0.052$ ,  $P = 0.026$ , and  $P = 0.046$ , 0 vs. 180 minutes, for GLUT1 in HeLa cells, GLUT3 in HeLa cells and GLUT1 in 661W cells, respectively).



**FIGURE 4.** Inhibition of glucose transporters prevented BCAAs from promoting glucose uptake in photoreceptor-derived 661W cells. Retinal photoreceptor-derived cells, 661W cells, were cultured in an amino acid-free medium containing 4.5 g/L of glucose with or without 40 mM BCAAs, and then incubated with or without WZB117. **A–C** Glucose uptake quantified after incubation with 100 μM 2-NBDG after incubation with or without BCAAs for 3 hours and then with or without WZB117 (125 μM) for 1 hour. **A, B** Histograms showing distributions of cell counts for fluorescence intensities (FL) by flowcytometry analysis. **C** Geometric average of fluorescence intensities of live cells. **D** Relative ATP level in live cells after incubation with or without BCAAs for 2 hours and then with or without 100 μM WZB117 for 2 hours. **E** Cell viability with or without BCAAs and with or without 125 μM WZB117. **C–E** \*\*\* $P < 0.005$ , N.S.: no significant difference, Tukey HSD, **C, E**  $N = 4$  and **D**  $N = 3$ . Bars represent the standard deviation.



**FIGURE 5.** BCAAs promoted glucose transporters (GLUT) translocation to the plasma membrane. HeLa cells and 661W cells expressing enhanced green fluorescent protein (EGFP)-GLUT1 or GLUT3 were incubated in an amino acid-free medium containing 4.5 g/L of glucose with or without BCAAs and imaged under fluorescence microscope at 0, 60, 120, and 180 minutes after incubation. **A, B** HeLa cells expressing EGFP-GLUT1 (**A**) or EGFP-GLUT3 (**B**) were incubated without BCAAs. **C, D, F, G** HeLa cells expressing EGFP-GLUT1 (**C, F**) or EGFP-GLUT3 (**D, G**) were incubated with 80 mM BCAAs. **E, H** 661W cells expressing EGFP-GLUT1 were incubated with 80 mM BCAAs. White arrows indicate EGFP-fusion GLUTs translocated to the plasma membrane (**C–E**). Scale: 20  $\mu\text{m}$ . **F, G, H** Fluorescence intensities of EGFP-GLUT1 (**F, H**) or GLUT3 (**G**) were quantified in HeLa cells (**F, G**) or 661W cells (**H**) at 0, 60, 120, and 180 minutes after incubation. Fluorescence intensities are shown as the ratio of that at the plasma membrane to that in the cytosol. \* $P < 0.05$ , Tukey HSD,  $N = 5$  each.

## DISCUSSION

In the current study, we showed that BCAAs prevented the decrease in intracellular ATP even when glycolysis or the citric acid cycle was inhibited. Moreover, we clearly showed that BCAAs enhanced the translocation of GLUTs to the plasma membrane, and promoted glucose uptake.

We previously showed that BCAAs prevented the decrease in intracellular ATP and antagonized cell death during mitochondrial respiratory chain inhibition stress or ER stress.<sup>14</sup> In the current study, we showed that BCAAs prevented decreases in intracellular ATP even when glycolysis or citric acid cycle was inhibited by a hexokinase inhibitor (lonidamine), a GAPDH inhibitor (heptelidic acid), a PKM2 inhibitor (Shikonin), or mitochondrial pyruvate carrier inhibitor (UK5099) as well (see Fig. 1). Fluores-

cence imaging of glucose uptake using a fluorescently labeled deoxy-glucose analog showed that BCAAs promoted glucose uptake (see Fig. 2). However, when hexokinase was inhibited by 2-DG, intracellular ATP levels further decreased following addition of BCAAs in our previous study,<sup>14</sup> whereas glucose uptake was enhanced by BCAAs in the presence of 2-DG (see Figs. 2G–I). This result is reasonable because enhanced uptake of 2-DG by BCAAs further strengthened the feedback inhibition of hexokinase by 2-DG-6 phosphate. In contrast, BCAAs did not antagonize the decrease in ATP nor cell death when GLUTs were inhibited (see Figs. 3, 4), likely because GLUTs were the target of ATP enhancement by BCAAs.

Furthermore, in the current study, fluorescence imaging of EGFP-GLUTs (GLUT1 or GLUT3) apparently showed that BCAAs enhanced the translocation of GLUTs to the

plasma membrane (see Fig. 5). Thus, BCAAs enhanced ATP production by promoting glucose uptake through promotion of GLUTs translocation to the plasma membrane, thereby preventing cell death.

The translocation of GLUTs to the plasma membrane has been shown to be induced by a variety of stimuli, including insulin,<sup>39,40</sup> platelet-derived growth factor,<sup>41</sup> and epidermal growth factor.<sup>42</sup> In addition, BCAAs have been suggested to promote GLUT translocation to the plasma membrane, and consequently promote glucose uptake in skeletal muscle.<sup>43,44</sup> Paradoxically, BCAAs have been reported to increase insulin resistance.<sup>45,46</sup> Thus, the influence of BCAAs on glucose uptake or GLUTs is not fully understood.<sup>17</sup> In the current study, we directly observed the intracellular behavior of GLUTs using transfected EGFP-GLUT1 or GLUT3. Fluorescent microscopy clearly revealed that BCAAs promoted the translocation of GLUTs to the plasma membrane in HeLa cells and in the photoreceptor-derived neuronal cells, 661W, consequently promoting glucose uptake, enhancing ATP production, and attenuating cell death.

We have previously shown that BCAAs attenuated photoreceptor degeneration in rodent models of retinal degeneration, and attenuated retinal ganglion cell death in a rodent model of glaucoma.<sup>14</sup> Although high plasma concentrations of BCAAs are associated with a high incidence of cardiovascular disease, BCAA concentrations are not associated with cardiovascular diseases when HbA1c and insulin resistance were adjusted.<sup>47</sup> Moreover, leucine has been reported to attenuate cardiac damage, and improve cardiac function and survival after acute myocardial infarction.<sup>48</sup> In contrast, energy depletion has been associated with neuronal cell death, including in retinal neurons.<sup>49–52</sup> Considering that BCAAs antagonize cell death in the heart and the retina, both of which are organs that require a large amount of energy,<sup>53–56</sup> BCAAs might have the potential to protect cells against cell death during high energy requirement conditions.

This study has several limitations. First, the influence of each BCAA on glucose uptake was not investigated separately. Leucine and isoleucine have been reported to promote glucose uptake in skeletal muscle,<sup>20</sup> and leucine is known to activate mammalian target of rapamycin (mTOR).<sup>57</sup> Excess leucine without other amino acids has been reported to have growth-inhibitory effects.<sup>58</sup> Because we aimed to clinically apply BCAAs for retinal neurodegenerative diseases, we studied the mechanisms of glucose uptake by formulation of BCAAs, leucine, isoleucine, and valine, which have already been used clinically in patients with liver cirrhosis.<sup>24</sup> Second, the possible effect of BCAAs on insulin resistance was not studied in the current study. It has been suggested that leucine and isoleucine induce the translocation of GLUT1 and GLUT4, which is insulin-sensitive GLUT, to the plasma membrane.<sup>43</sup> In the current study, we clearly showed that BCAAs promote the translocation of GLUTs to the plasma membrane, and consequently promote glucose uptake in cultured cells. Whereas insulin-sensitive GLUT4, which is mainly expressed in adipose tissue, skeletal muscle, and cardiomyocytes,<sup>35</sup> has been reported to be expressed in retinal neuronal cells,<sup>38</sup> GLUT1 has been reported to play the most important role in glucose uptake by cone photoreceptors.<sup>59</sup> On the contrary, stimulation of insulin/mTOR pathways has been reported to attenuate cone photoreceptor cell death.<sup>51,59</sup> The effect of insulin on glucose uptake in the presence of BCAAs requires further study.

In conclusion, BCAAs promote the translocation of GLUTs to the plasma membranes, and consequently increase intracellular ATP concentration by enhancing glucose uptake into the cells. BCAAs have the potential to be a novel therapeutic strategy for retinal neurodegenerative diseases by preventing decrease in intracellular ATP levels.

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