PERSPECTIVE

Adenosine triphosphate maintenance by branched chain amino acids as a novel neuroprotective strategy for retinal neurodegenerative diseases

Retinal neuronal cell death is caused in many incurable eye diseases such as retinitis pigmentosa (RP) and glaucoma, which are leading causes of adult blindness. In RP, progressive loss of photoreceptor cells leads to visual disturbance. No established treatments are available to date for this condition, although potential treatments, including regenerative medicine, gene therapy, and neurotropic factor therapy are being investigated. In glaucoma, retinal ganglion cell death leads to visual disturbance. Although intraocular pressure reduction is the only established treatment to slow the loss of visual function in glaucoma, there are a fair number of cases where visual impairment progresses despite intraocular pressure being maintained within the normal range. Therapeutic strategies to prevent neuronal cell death are being pursued.

Energy depletion has been associated with neuronal cell death (Lin and Beal, 2006). Retinal cells demand a large amount of energy, and energy depletion has been shown to be associated with neuronal cell death in the retina (Thomas et al., 2000; Punzo et al., 2009). We recently reported that maintenance of intracellular adenosine triphosphate (ATP) by naphthalene derivatives, Kyoto University Substances, which modulate the ATPase activity of valosin-containing protein, an abundant intracellular soluble ATPase, prevents neuronal cell death (Ikeda et al., 2014; Hasegawa et al., 2016a; Nakano et al., 2016; Hata et al., 2017). Prevention of neuronal cell death by maintaining intracellular ATP levels via enhancement of ATP production might be another useful therapeutic strategy.

Branched chain amino acids promote glucose uptake and protect cells under stress conditions: Branched chain amino acids (BCAAs) are amino acids that have aliphatic side-chains with branches, including leucine (Leu), isoleucine (Ile), and valine (Val).

BCAAs have been used to treat patients with liver cirrhosis. Animal studies have shown that BCAAs improve glucose metabolism in rats with liver cirrhosis and glucose uptake in rat skeletal muscle (Nishitani et al., 2004). Hence, we investigated whether BCAA supplementation may prevent cell death by enhancing glucose uptake and ATP production.

When BCAAs (40 mM, L-Ile:L-Leu:L-Val = 1:2:1.2, marketed as LIVACT®) were added to HeLa cells under an amino acid-free and tunicamycin induced endoplasmic reticulum (ER) stress condition, intracellular ATP levels were increased and cell death was attenuated (**Figure 1A–C**), while addition of glucose alone (2 or 4.5 g/L) increased neither the ATP levels nor the live cell numbers (**Figure 1A–C**) (Hasegawa et al., 2018). Moreover, glucose uptake was significantly promoted by BCAAs (Hasegawa et al., 2018). In addition, BCAAs maintained intracellular ATP levels and protected cells from cell death when HeLa cells were cultured with antimycin, a specific inhibitor of mitochondrial respiratory chain complex III (Hasegawa et al., 2018). In contrast, BCAAs did not maintain the intracellular ATP nor protect cells when glycolysis was inhibited by 2-deoxy-D-glucose (Hasegawa et al., 2018). These results indicate that BCAAs enhance glucose uptake and glycolytic ATP production. Maintenance of ATP levels and cell protection by BCAAs were also effective in a photoreceptor-derived cell line, 661W cells (Al-Ubaidi et al., 2008; Hasegawa et al., 2018).

To further elucidate mechanisms underlying cell protection by BCAAs, we examined C/EBP-homologous protein (CHOP) protein, an ER stress marker by western blot analysis. The CHOP protein under stress was suppressed by BCAAs (**Figure 1D**) (Hasegawa et al., 2018).

These results indicate that BCAAs promote cell survival through suppression of intracellular ATP depletion and ER stress (**Figure 1E**).

Branched chain amino acids as a promising new therapeutic strategy for retinal neurodegenerative diseases: Since BCAAs protect cells, including photoreceptor derived cell lines, under stress conditions, they might prove to be a new therapeutic strategy for prevention of neuronal cell death in neurodegenerative diseases. We investigated the potential neuroprotective effect of BCAAs using mouse models of RP and glaucoma (Hasegawa et al., 2018).

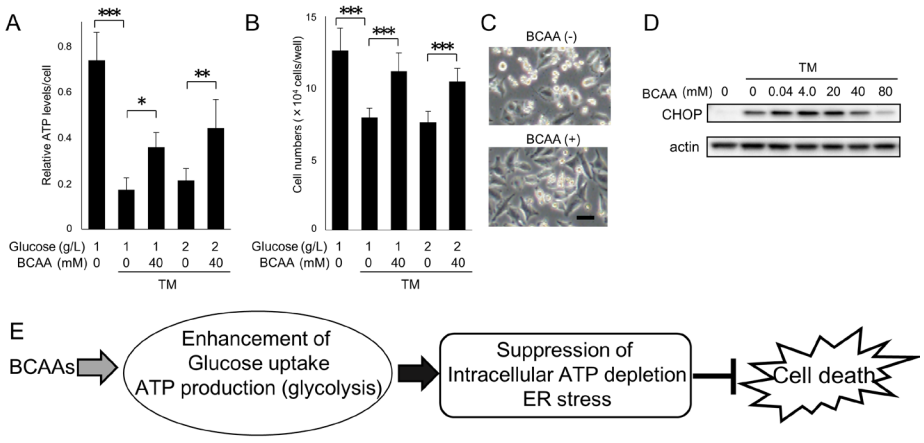
As a model of RP, we examined *rd10* mice, which have a missense mutation in the *Pde6b* gene. Administration of BCAAs was started at 7 days of age and retinal thickness was examined using spectral domain-optical coherence tomography (Hasegawa et al., 2018). The thinning of the entire retina and the photoreceptor layer (composed of the outer nuclear layer, photoreceptor myoid and ellipsoid zones, and outer segment layer), was significantly attenuated by administration of BCAAs (Hasegawa et al., 2018). Immunohistochemical analyses of the 37-day-old *rd10* mice retinae showed that extinction of the cone photopigments, M-opsin and S-opsin, was prevented in BCAA treated mice (Hasegawa et al., 2018). To examine retinal function, we performed scotopic and photopic electroretinography. The a-wave of the scotopic electroretinogram, which reflects photoreceptor function in the

dark, and the b-wave of the photopic electroretinogram, which measures retinal function in the light, were significantly larger in the BCAA-administered mice (Hasegawa et al., 2018). These results show that BCAAs attenuated photoreceptor cell death morphologically and functionally in a mouse model of RP.

To investigate whether BCAAs can protect photoreceptors after the onset of disease, we examined later-stage *rd12* mice, another RP mouse model with a nonsense mutation in the *Rpe65* gene. Administration of BCAAs was started at 13 months of age (**Figure 2A**) (Hasegawa et al., 2018), when the disease had already progressed (Hasegawa et al., 2016b). After 6 months of BCAA administration, the thinning of the entire retina and the photoreceptor layer was significantly attenuated in BCAA-administered mice (Hasegawa et al., 2018). Photopic electroretinography revealed that the b-wave amplitude was significantly larger in the BCAA-administered mice (**Figure 2B** and **C**) (Hasegawa et al., 2018). These results show that BCAAs retarded disease progression even when the administration was started in later disease stages.

We then examined whether BCAAs can protect retinal ganglion cells in glaucoma, using glutamate-aspartate transporter (GLAST) knockout mice as a glaucoma model (Harada et al., 2007). Administration of BCAAs was started at 1 month of age (Hasegawa et al., 2018). The retinal ganglion cells counted on retinal flat-mounts were significantly higher in the BCAA-administered mice (Hasegawa et al., 2018).

To elucidate the mechanisms of neuronal cell protection by BCAAs in animal models, we examined the CHOP protein. Western blot analysis of *rd12* mice retinae and immunohistochemical analyses of *rd10* mice and



(A–C) HeLa cells were cultured under an amino acid deficit and tunicamycin (TM) (3 μg/mL) for 16 hours with different concentrations of glucose (1 and 2 g/L), with or without BCAAs (40 mM). (A) Relative ATP levels determined by luciferase activity. (B) Live cell numbers counted after trypsinization. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Tukey honestly significant difference (HSD) test. $n = 6$. (C) Representative photographs of HeLa cells cultured with 4.5 g/L of glucose, with or without BCAAs (40 mM). Scale bar: 20 μm. (D) HeLa cells cultured with tunicamycin (3 μg/mL) for 6 hours with or without BCAAs. C/EBP homologous protein (CHOP) was analyzed by western blot analysis. Actin served as a loading control. (E) Schema of the mechanisms of neuroprotective effects of BCAAs.

Figure 1 Branched chain amino acids (BCAAs) suppress adenosine triphosphate (ATP) depletion and endoplasmic reticulum (ER) stress in tunicamycin-treated cells and work against cell death [based on Hasegawa et al. (2018)].

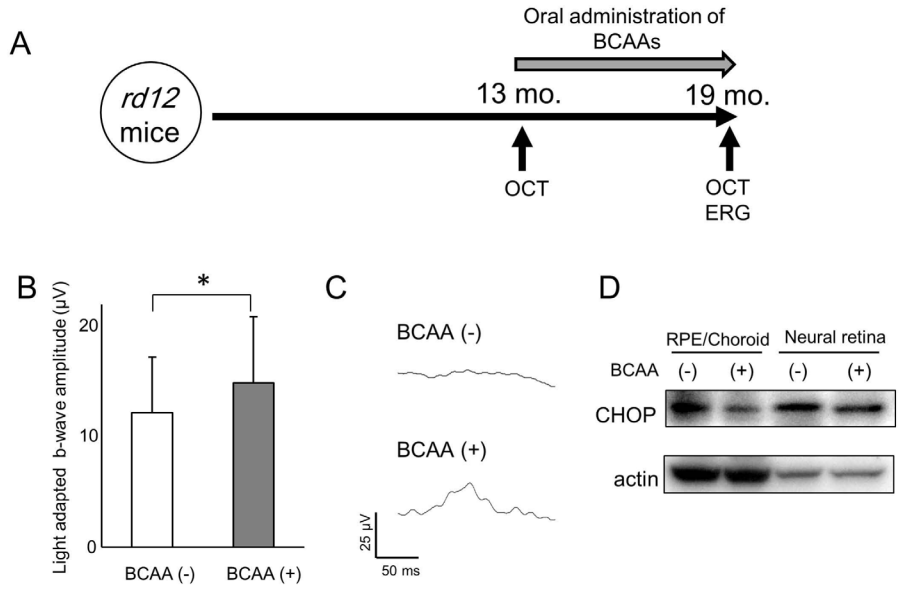


Figure 2 Branched chain amino acids (BCAAs) supplementation after the onset of the disease suppressed ER stress and worked against disease progression in later stage *rd12* mice [based on Hasegawa et al. (2018)].

(A) Schema of the experimental schedule with *rd12* mice. (B, C) Light-adapted ERG at a stimulus intensity of 10 cds/m^2 in 19-month (mo.)-old *rd12* mice with or without 6 months of oral BCAA administration. (B) Comparison of b-wave amplitudes. *$P < 0.05$, student's *t*-test. (C) Typical ERG records. (D) C/EBP homologous protein (CHOP) was analyzed with western blot analysis. Neural retinas and the combination of retinal pigment epithelium (RPE), choroid, and sclera (RPE/choroid) from 19-month-old *rd12* mice were separately analyzed. Actin served as a loading control. OCT: Optical coherence tomography; ERG: electroretinography.

GLAST (+/–) mice showed that CHOP expression was reduced in BCAA-administered mice (**Figure 2D**) (Hasegawa et al., 2018). These results indicate that reduction in ER stress may underlie BCAA-induced neuronal cell protection in animal models of RP and glaucoma.

We are currently preparing for an investigator-initiated clinical trial to test the safety and efficacy of BCAAs in RP patients.

Statistical analyses were performed using SPSS Statistics version 21.0 (IBM SPSS Inc., Chicago, IL, USA). Variables among cells or mice with or without BCAA therapy were compared with the Turkey honestly significant difference (HSD) test or Student's *t*-test.

Conclusion: We showed that BCAA retarded cell death through maintenance of intracellular ATP levels and suppression of ER stress. BCAAs worked against neuronal cell death in mouse models of RP and glaucoma. Our findings may provide a new, widely available, therapeutic strategy for neurodegenerative diseases such as RP and glaucoma.

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Kyoto University has applied for patents related to this study (PCT/JP2016/053914, 2016-023044), with TH and HOI listed as inventors.

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