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The role of glutamine in neurogenesis promoted by the green tea amino acid theanine in neural progenitor cells for brain health

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ABSTRACT

The green tea amino acid theanine is abundant in green tea rather than black and oolong teas, which are all made of the identical tea plant "Chanoki" (Camellia sinensis). Theanine has a molecular structure close to glutamine (GLN) compared to glutamic acid (Glu), in terms of the absence of a free carboxylic acid moiety from the gamma carbon position. Theanine efficiently inhibits [^{3}H]GLN uptake without affecting [^{3}H]Glu uptake in rat brain synaptosomes. In contrast to GLN, however, theanine markedly stimulates the abilities to replicate and to commit to a neuronal lineage following prolonged exposure in cultured neural progenitor cells (NPCs) prepared from embryonic and adult rodent brains. Upregulation of transcript expression is found for one of the GLN transporter isoforms, *Slc38a1*, besides the promotion of both proliferation and neuronal commitment along with acceleration of the phosphorylation of mechanistic target of rapamycin (mTOR) and relevant downstream proteins, in murine NPCs cultured with theanine. Stable overexpression of *Slc38a1* similarly facilitates both cellular replication and neuronal commitment in pluripotent embryonic carcinoma P19 cells. In P19 cells with stable overexpression of *Slc38a1*, marked phosphorylation is seen for mTOR and downstream proteins in a manner insensitive to further additional phosphorylation by theanine. Taken together, theanine would exhibit a novel pharmacological property to up-regulate *Slc38a1* expression for activation of the intracellular mTOR signaling pathway required for neurogenesis after sustained exposure in undifferentiated NPCs in the brain. In this review, a novel neurogenic property of the green tea amino acid theanine is summarized for embryonic and adult neurogenesis with a focus on the endogenous amino acid GLN on the basis of our accumulating evidence to date.

1. Introduction

Theanine (=L-γ-glutamylethylamide) is an amino acid ingredient supposed to be more abundant in green tea than in black and oolong teas, although these tea beverages are all made of the identical tea plant "Chanoki" (*Camellia sinensis*). Production processes are different from each other with the individual tea beverages (Fig. 1). For example, all enzymatic activities are stopped by steaming for Japanese green tea and by roasting for Chinese green tea respectively, immediately after the harvest of tea leaves. By contrast, tea leaves are left as they are toward the exhibition of all enzymatic activities, partially for oolong tea and totally for black tea. During the latter enzymatic process of oxidation called "fermentation" along with withering, existing theanine is converted to glutamic acid (Glu) and ethylamine in tea leaves (Ekborg-Ott et al., 1997). Moreover, amino acids are supposed to be subject to oxidization by catechin o-quinones and subsequent Strecker degradation for new aroma components during black tea manufacture (Ekborg-Ott et al., 1997).

Theanine is first identified as an amide constituent of green tea in Japan in 1949 (Sakato, 1949), while high-quality green teas, such as

Abbreviations: ADHD, attention deficit hyperactivity disorder; bHLH, basic helix-loop-helix; BrdU, 5-bromo-2′-deoxyuridine; DG, dentate gyrus; EAA, essential amino acids; ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; GLN, glutamine; GLNase, glutaminase; GLNT, glutamine transporter; Glu, glutamic acid; MCI, mild cognitive impairment; MeAIB, N-methylaminoisobutyric acid; mTOR, mechanistic target of rapamycin; mTORC, mechanistic target of rapamycin complex; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NPCs, neural progenitor cells; NSCs, neural stem cells; Slc38a1, solute carrier 38a1; SNAT, sodium-coupled neutral amino acid transporter

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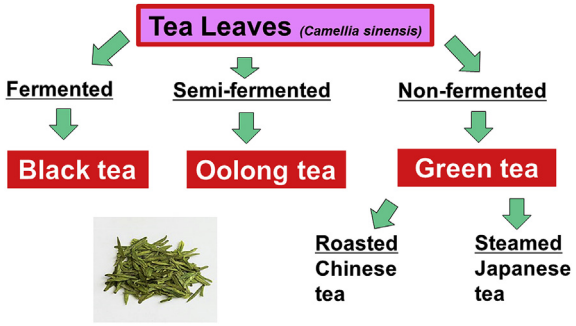

Fig. 1. Production Procedures for Tea Beverages. The tea plant *Camellia sinensis* has been used for the production of different tea beverages. Tea leaves are subjected to steaming for Japanese green tea and to roasting for Chinese green tea to terminate all enzymatic activities immediately after cultivation, whereas tea leaves are left as they are partially for oolong tea and totally for black tea after being picked up. During this fermentation period after cultivation, theanine is converted to a variety of polyphenols such as catechins. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

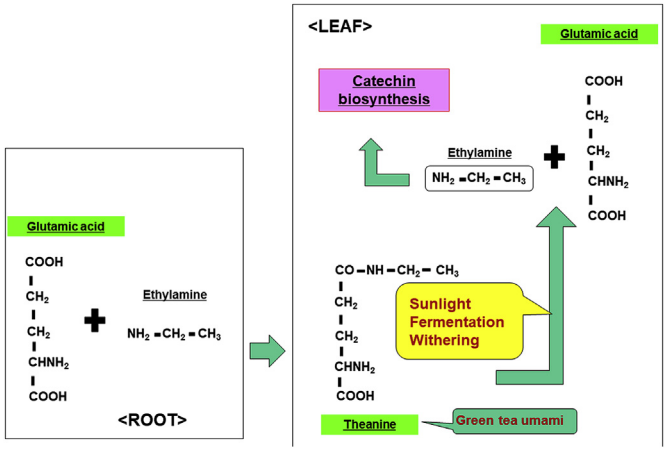

Fig. 2. Theanine Biosynthesis. Theanine is synthesized from Glu and ethylamine in association with nitrogen supply in the roots and subsequently translocated to the leaves. In the leaves, theanine is degraded to both Glu and ethylamine in response to sunlight, fermentation and withering, followed by usage of ethylamine for the biosynthesis of different catechin polyphenols.

Gyokuro and Matcha, are known to contain much more theanine than normal-grade green teas. To produce Gyokuro, for instance, tea leaves are protected from sunlight during the last 2–4 weeks before harvest to increase the content of theanine with mild sweetness. Cultivation under shade deteriorates the hydration of theanine for the biosynthesis of flavonoids such as catechins (Ikegaya et al., 1984; Ashihara, 2015; Unno et al., 2018). Theanine is synthesized from Glu and ethylamine in a manner dependent on nitrogen supply absorbed from the roots for subsequent translocation to the leaves (Fig. 2). Ethylamine is a common constituent produced by the enzymatic decarboxylation of alanine in many plants (Ekborg-Ott et al., 1997). In addition, mature leaves contain less caffeine than the bud and young leaves in *Camellia* plants. Matcha is thus rich in both theanine and caffeine together with a lower content of catechins than normal-grade green teas (Unno et al., 2018). Nevertheless, the reason why actual measured values of theanine do not always coincide well with the aforementioned theoretical abundance in green tea leaves (Ekborg-Ott et al., 1997; Syu et al., 2008; Unno et al., 2018) is not fully clarified so far. The possibility that the grade of green tea leaves used for measurement is fluctuating between studies is not ruled out.

On the basis of the apparent structural similarity to the excitatory neurotransmitter Glu and the inhibitory neurotransmitter γ-aminobutyric acid (GABA), theanine has been believed to play a role in neurotransmissions mediated by Glu and GABA in brain neurons for years. However, our precise structural evaluation has indicated that theanine has a chemical structure close to glutamine (GLN) rather than Glu and GABA in terms of the absence of a free carboxylic acid from the gamma carbon position (Kakuda et al., 2008; Yoneda, 2017). In this review, we will focus on pharmacological properties of the green tea amino acid theanine in relation to signals mediated by GLN in undifferentiated neural progenitor cells (NPCs), in place of the modulation of glutamatergic and GABAergic neurotransmissions in neuronal network within mature neurons.

2. Green tea for human brain health

2.1. Green tea for cognition improvement

In almost one thousand elderly Japanese people at an age over 70 years old in the Tsurugaya district, consumption of green tea is inversely correlated with the incidence of cognition impairment (Kuriyama et al., 2006). In a double-blinded, randomized controlled study on elderly nursing home residents with cognitive dysfunction, however, no significant improvement is seen in their cognitive functions assessed every 3 months during consumption of green tea powder for 12 consecutive months (Ide et al., 2016). In a recent cohort study, daily intake of green tea leads to almost four times less incidence of cognitive declines, such as dementia and mild cognitive impairment (MCI), in elderly residents in the Noto peninsula compared to daily coffee or black tea drinkers when studied for 5 consecutive years (Noguchi-Shinohara et al., 2014). Similarly, daily oral intake of capsules containing powdered high-quality green tea results in significant alleviation of the cognition ability scores assessed by double-blinded physicians in healthy elderly people compared to those with powdered normal-grade green tea capsules for 7 to 12 consecutive months (Kataoka et al., 2009; Kakuda, 2011). However, no significant difference is found in the cognition ability between elderly people given high-quality and normal-grade green tea capsules during the initial 6 months. High-quality green tea is shown to contain much more theanine than normal-grade green tea as mentioned above. Even high-quality green teas, such as “Gyokuro” and “Matcha”, contain theanine by at most 3% (Goto et al., 1996). This amount is considerably higher than the content in other tea beverages, such as black tea and oolong tea, which are all made of the identical tea plant with different production procedures as described above. The amount of theanine taken is estimated to be close to 20 mg after the ingestion of 1 g of powdered green tea (Matcha) usually used for the traditional tea ceremony in Japan.

Most tea beverages contain caffeine along with a variety of polyphenols such as catechins to an extent much more than theanine, in contrast, while even normal-grade green tea has caffeine by 3–5% and catechins by 15%, respectively. In previous studies (Levites et al., 2001; Mandel et al., 2004; Mercer et al., 2005), several polyphenols including catechin and (−)-epigallocatechin-3-gallate are shown to be neuroprotective against neuronal damage caused by different neurotoxins *in vitro* and *in vivo*. However, these *in vivo* neuroprotective properties are almost derived from experiments using intraperitoneal and intravenous administrations rather than oral ingestion. In contrast, a prevailing view is that most polyphenols are not readily absorbed from the intestinal mucosa to the circulating blood stream after oral intake (Olthof et al., 2001; Manach et al., 2004; Kim et al., 2016). Accordingly, participation of polyphenols in the aforementioned beneficial effectiveness of oral green tea ingestion for cognition impairment seems to be at least re-elucidated by *in vivo* experiments using oral administration in future studies. These previous findings thus argue in favor of an idea that theanine at least in part mediates the property of green tea ingestion beneficial for cognition improvement in elderly people besides

polyphenols and caffeine which are both similarly abundant in coffee and other tea beverages.

2.2. Theanine for sleep improvement

In healthy young people, theanine elicits protection against a variety of responses to psychological and physiological stressors (Kimura et al., 2007). In human adults subjected to physical and psychological stress tasks, oral intake of theanine more effectively ameliorates increases in both anxiety and blood pressure than the intake of the other green tea ingredient caffeine (Yoto et al., 2012). In patients suffering from major depressive disorders, several beneficial improvements are seen in depressive symptoms, anxiety, sleep disturbance and cognitive impairments when they are orally given theanine for 8 weeks (Hidese et al., 2017). In boys with attention deficit hyperactivity disorder (ADHD), daily oral intake of theanine for 6 weeks improves the quality of sleep (Lyon et al., 2011). Similar mitigation is seen in ADHD-relevant sleep disorders with respect to sleep efficiency rather than total sleeping time (Barrett et al., 2013). The quality of sleep is improved through a mechanism relevant to anxiolysis and relaxation by theanine even at a dose of 2 g/kg body weight/day in humans without remarkable adverse side effects (Rao et al., 2015). Oral intake of theanine is thought to improve the sleep quality not by sedation but through anxiolysis without inducing daytime drowsiness (Rao et al., 2015).

2.3. Theanine for mood improvement

In a clinical study with double-blinded and randomized protocols for evaluation of the efficacy and tolerability of the psychiatric treatment of patients with chronic schizophrenia and schizoaffective disorders, oral intake of theanine leads to promotion of ongoing antipsychotic effectiveness in a manner associated with reduced anxiety when given at a dose of 400 mg/day for 8 weeks (Ritsner et al., 2011). A possible positive correlation is seen between serum levels of brain-derived neurotrophic factor and the effectiveness of theanine in schizophrenic and schizoaffective patients with oral administration for 8 weeks (Miodownik et al., 2011). Extrapolated therapeutic benefits of theanine are discussed in other psychiatric disorders as well. These include anxiety disorders, panic disorder, obsessive compulsive disorder and bipolar disorder (Lardner, 2014). In an open clinical study on patients suffering from major depressive disorder, oral intake of theanine at 250 mg/day for 8 weeks promotes the therapeutic efficacy of the current medication for depressive symptoms, anxiety, sleep disturbance and cognitive impairment as described above (Hidese et al., 2017).

2.4. Theanine for cognition improvement

In a cell model of Alzheimer's disease, theanine is shown to protect neurotoxicity mediated by Glu as an antagonist at the N-methyl-D-aspartate receptor subtype of Glu receptors (Zhao and Zhao, 2012). However, the affinity is considerably lower for each neurotoxic ionotropic Glu receptor subtype as an antagonist effective in preventing neuronal cell death by Glu than well-known antagonists (Kakuda et al., 2002). Oral consumption of theanine at 5–6 mg/kg results in alleviation of learning impairment, in addition to mitigating shortened lifespan, cerebral atrophy, behavioral depression and oxidative DNA damage, in mice with chronic psychiatric social stress (Unno et al., 2011). In mice defective of the gene klotho identified as a suppressor of ageing (Kuro-o et al., 1997; Kurosu et al., 2005), cognition impairment is seen in a manner relevant to oxidative stress (Nagai et al., 2003). In our recent study using these klotho-null mice, significant improvement is found in memory impairment after oral intake of theanine at 4 mg/kg in drinking water for 24 consecutive days (Nguyen et al., 2019). It thus appears that oral intake of theanine would improve cognition and/or memory impairment in different experimental animals. However, the underlying mechanisms are still not clarified to date.

3. Neural stem cells

3.1. Theanine on neurogenesis

Neural stem cells (NSCs) are defined as a primitive cell endowed to renew for replication and to commit differentiation to several daughter cell lineages in the brain (Gage et al., 1995; Suhonen et al., 1996; Doetsch et al., 1999; Johansson et al., 1999; Temple and Alvarez-Buylla, 1999). These include neurons, astrocytes and oligodendrocytes. Neural progenitor cells (NPCs) are composed of a mixture of progenitors for neurons, astrocytes and oligodendrocytes as well as NSCs. Round spheres called neurospheres are progressively formed by clustered proliferating cells during the culture with several growth factors of NPCs isolated from embryonic rat (Yoneyama et al., 2007) and mouse (Fukui et al., 2008) neocortex and adult mouse hippocampus (Kitayama et al., 2003, 2004), under non-adherent conditions. These neurospheres feature the property to commit differentiation to neuronal and astroglial lineages as seen in NSCs. Sustained exposure to theanine promotes the growth of neurospheres, in addition to increasing 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reducing activity determined as an index of the proliferative activity, in a concentration-dependent manner at 1–100 μM in undifferentiated NPCs of embryonic rat neocortex (Takarada et al., 2016a). In these neurospheres with prior exposure to theanine, marked promotion is seen in spontaneous and induced commitment to a neuronal lineage with deteriorated astroglial specification. Prior sustained exposure to theanine more than doubles transcript expression of the GLN transporter (GLNT) isoform, solute carrier 38a1 (Slc38a1), in undifferentiated NPCs from embryonic rat and mouse neocortex (Takarada et al., 2016a), in addition to promoting cell growth and neuronal commitment as described above (Takarada et al., 2016b). In newborn rats with chronic oral administration of theanine in drinking water during pregnancy *in vivo*, object recognition memory is significantly facilitated along with accelerated 5-bromo-2′-deoxyuridine (BrdU) accumulation in the hippocampal dentate granule cell layer (Takeda et al., 2011).

Hippocampal NPCs are isolated from young adult mice with predominant overexpression of green fluorescent protein (GFP) in cells expressing nestin (Mignone et al., 2004). In hippocampal sections dissected from these adult *Nestin*-GFP transgenic mice, GFP fluorescence predominates in the subgranular zone, which is well known to be enriched of NSCs expressing nestin, below the granular cell layer stained with Hoechst33342 (Takarada et al., 2016b). Prolonged culture with theanine significantly increases the size of neurospheres formed during the culture of GFP-expressing NPCs prepared from the hippocampus of adult *Nestin*-GFP transgenic mice (Takarada et al., 2016b). These results give support for the proposal that theanine promotes proliferation for self-replication during adult neurogenesis following chronic exposure.

3.2. Theanine for stress disorders

In adult mice, the fatal traumatic stress water immersion restrain stress (Yoneda et al., 1983) is shown to induce different long-term bidirectional behavioral abnormalities, such as flashback, numbing and hyper-arousal, which are all seen in human patients with posttraumatic stress disorder (Tamaki et al., 2008). A transient decrease of BrdU accumulation is found in cells localized in the hippocampal dentate gyrus (DG) in adult mice with the aforementioned severe traumatic stress, along with bidirectional behavioral abnormalities relevant to numbing and hyper-arousal (Tamaki et al., 2008). Theanine is effective in preventing the transient decrease in the number of total and clustered cells incorporating BrdU in the DG when given orally for 5 consecutive days even after the fatal stress (Takarada et al., 2015). The bidirectional abnormal behaviors are improved by a daily intraperitoneal injection of several antidepressants shown to be effective in human patients (Tamaki et al., 2008), whereas these effective antidepressants are all known to stimulate both proliferation and neuronal differentiation in

NPCs during adult neurogenesis (Sairanen et al., 2005; Li et al., 2009, 2012). Theanine would be thus endowed to promote the neurogenesis to produce new mature neurons via neuroblasts toward possible orchestration of new intact neuronal network, in addition to modulating a variety of features of prevailing mature neurons, in adult brain.

4. Analogy to glutamine

4.1. Similarities between theanine and glutamine

Glutamic acid has been thought to play a dual role as an endogenous excitotoxic neurotoxin (Szatkowski and Attwell, 1994) and as an excitatory neurotransmitter in the brain. In contrast, GLN is a principal precursor required for the synthesis of Glu sequestered in a neurotransmitter pool within the Glu/GLN cycle after the incorporation into glutamatergic neurons in the brain (Schousboe et al., 1979). In contrast to acidic Glu, theanine easily enters the brain through the blood brain barrier in conscious rats (Yokogoshi et al., 1998). Alleviation is usually seen against excessive excitation by caffeine of brain excitability as revealed by electroencephalography in rats after an intravenous injection of theanine (Kakuda et al., 2000a). Theanine is also shown to suppress elevations of systemic blood pressure and brain 5-hydroxyindoles levels in spontaneous hypertensive rats (Yokogoshi et al., 1995). Theanine protects hippocampal CA1 pyramidal neurons from delayed neuronal cell death in gerbils with bilateral forebrain global ischemia following a prior intracerebroventricular injection (Kakuda et al., 2000b). However, theanine is a weak displacer of ligand binding to different neurotoxic ionotropic Glu receptor subtypes in rat cortical synaptic membranes (Kakuda et al., 2002). Although theanine has been thought to modulate both neurotransmission and neurotoxicity mediated by Glu in the literature (Di et al., 2000; Ota et al., 2015), the close structural similarity gives rise to an idea that GLN would be at least in part responsible for particular pharmacological properties of theanine, besides Glu, in the brain.

4.2. Glutamate/glutamine cycle

The prevailing idea is that Glu is released into synaptic clefts upon stimulation from glutamatergic neurons, and taken up by excitatory amino acid transporters for the synthesis of GLN in adjacent astrocytes and subsequent efflux to extracellular spaces in a manner dependent on GLNTs such as sodium-coupled neutral amino acid transporter (SNAT)3 and SNAT5 (Butterworth, 2014). Extracellular GLN is then taken up through the GLNT, SNAT1, which is expressed by neurons to fuel the neurotransmitter pool of Glu at glutamatergic nerve terminals as the Glu/GLN cycle (Fig. 3). In glutamatergic synapses in the central nervous system, GLN is thought to be a main substrate for GLNT to provide a precursor for the synthesis of the neurotransmitter Glu (Schousboe et al., 1979; Broer, 2002; Dolinska et al., 2004). In neurons, GLN is hydrolyzed to Glu by the catalytic action of phosphate-dependent glutaminase (GLNase) (Schousboe et al., 1979) for the condensation into synaptic vesicles by vesicular glutamate transporters expressed at vesicular surfaces preparing for subsequent exocytotic release into synaptic clefts. However, theanine is not a good substrate for this phosphate-dependent GLNase enriched in the brain, but metabolized by phosphate-independent GLNase responsible for the degradation of GLN in the kidney (Tsuge et al., 2003). The localization of functionally active GLNase in astrocytes is still under argument (Butterworth, 2014).

4.3. [^{3}H]Theanine accumulation

Extracellular GLN is taken up into intracellular spaces in rat brain slices (Balcar and Johnston, 1975), with different transporter isoforms responsible for the active transmembrane migration. At least three distinct sodium-dependent transporters are known to mediate GLN transport across plasma membranes in the brain; (1) the system A

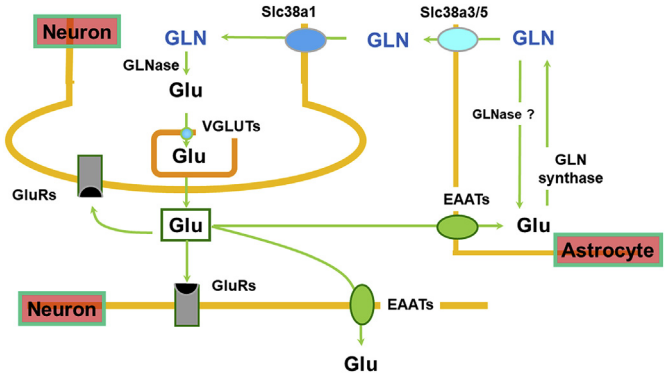

Fig. 3. Proposed Glu/GLN Cycle at Glutamatergic Synapse. In glutamatergic neurons, GLN is converted to Glu by the catalytic action of GLNase for subsequent sequestration by vesicular glutamate transporters (VGLUTs) in synaptic vesicles as a neurotransmitter compartment. In response to neuronal stimuli, Glu is released from the neurotransmitter pool into synaptic clefts to activate Glu receptors (GluRs) located at presynaptic and postsynaptic neurons. Glutamic acid is then taken up by excitatory amino acid transporters (EAAs) from synaptic clefts into adjacent astrocytes for the synthesis of GLN by GLN synthase and subsequent efflux mediated by GLNT isoforms such as Slc38a3 and Slc38a5 to extracellular spaces. Extracellular GLN is then taken up through another isoform of GLNT, Slc38a1, to fuel the neurotransmitter pool of Glu at glutamatergic nerve terminals as the Glu/GLN cycle.

(Reimer et al., 2000; Sugawara et al., 2000; Varoqui et al., 2000); (2) the system ASC (Broer et al., 1999; Utsunomiya-Tate et al., 1996) and (3) the system N (Broer et al., 2002; Chaudhry et al., 1999). Furthermore, GLN is taken up through the sodium-independent system L transporter comprised of heteromeric assemblies between different subunits (Broer, 2002; Chillaron et al., 2001; Wagner et al., 2001). Although these four transporter isoforms are able to carry GLN across plasma membranes, SNAT1(=ATA1/SAT1/GlnT) classified as a member of the system A family is believed to be exclusively expressed by neurons with relatively high affinity for GLN in the brain (Nagaraja and Brookes, 1996, Albers et al., 2001, Chaudhry et al., 2002).

In contrast to undetectable binding activity, [^{3}H]theanine is highly accumulated in a temperature-dependent manner in rat brain synaptosomes, with a saturation isotherm of at least two components with Km values of 42.3 μM and 1.88 mM, and Vmax values of 3.92 and 104.2 nmol/h/mg protein, respectively (Kakuda et al., 2008). [^{3}H]Theanine accumulation is selectively inhibited by GLN, whereas [^{3}H]GLN accumulation is sensitive to the inhibition by theanine with similar pharmacological profiles. The mutual inhibition profile supports the assumption that extracellular theanine could be a determinant of extracellular and intracellular levels of endogenous GLN under the delicate control by GlnT expressed in cells adjacent to glutamatergic synapses in a particular pathological situation. In fact, a higher level of GLN is found in the cerebrospinal fluid of patients with Alzheimer's disease than in normal subjects (D'Aniello et al., 2005). Taken together, theanine would suppress glutamatergic neurotransmission through a mechanism relevant to the inhibition of GLN incorporation across plasma membranes into neurons toward neuroprotection against delayed neuronal cell death mediated by excessive extracellular Glu after ischemia. A significant decrease is seen in the extracellular levels of endogenous Glu in culture medium from rat cortical neurons cultured with theanine (Kakuda et al., 2008). Indeed, inhibition of GLN transport depletes neurotransmitter pools of GABA as well as Glu in guinea-pig cortical slices and human BE(2)-C neuroblastoma cells (Rae et al., 2003). Theanine could suppress spontaneous and/or exocytotic release of Glu supplied by the Glu/GLN cycle through a mechanism associated with the inhibition of GLN incorporation into glutamatergic neurons in a particular situation.

4.4. Slc38a1 isoform

Sustained exposure is required for the selective upregulation by theanine of transcript expression of the GLNT Slc38a1 in undifferentiated NPCs prepared from embryonic rat and mouse neocortex (Takarada et al., 2016a). The GLNT, SNAT1, is a member of the system A neutral amino acid transporter superfamily first identified at the molecular level as Slc38a1. Of different transporter isoforms cloned to date, Slc38a1 is a membrane carrier with the highest affinity for GLN via the sodium/amino acid cotransport mechanism (Mackenzie et al., 2003). A typical feature is the selective inhibition by the amino acid analog N-methylaminoisobutyric acid (MeAIB) of the GLN transport mediated by Slc38a1 (Christensen, 1990; McGivan and Pastor-Anglada, 1994). Constitutive expression is not detected for *Slc38a1* mRNA in cultured astrocytes, with marked expression in cultured cortical neurons and cerebellar granular cells (Dolinska et al., 2004). In these cultured astrocytes, in fact, [^{3}H]GLN is accumulated in a manner insensitive to the inhibition by several system A substrates including MeAIB, proline and glycine. The system A transporter Slc38a1 thus seems to mediate predominantly the transmembrane GLN transport in neurons. Recent studies give support for the constitutive expression of *Slc38a1* mRNA in cultured astrocytes together with [^{3}H]GLN uptake and efflux activities insensitive to MeAIB (Heckel et al., 2003; Deitmer et al., 2003). Double labeling immunohistochemistry demonstrates the co-localization of immunoreactivities for Slc38a1 and an astroglial marker protein in the cerebral cortex of adult rat and human brains (Melone et al., 2004). We have also found constitutive expression of *Slc38a1* mRNA along with MeAIB-sensitive [^{3}H]GLN uptake in cultured rat cortical astrocytes (Ogura et al., 2006). However, both transient (Ogura et al., 2007) and stable (Ogura et al., 2011) overexpression of *Slc38a1* invariably exacerbates the vulnerability to the oxidative cytotoxicity in cultured astrocytes. Transmembrane GLN transport would be thus mediated by Slc38a1 belonging to the system A superfamily, in addition to different transporter isoforms classified as the system ASC, system N, and system L families, which are all capable of transporting GLN across plasma membranes even in astrocytes as mentioned in a previous review article (Yoneda, 2017). One possible but hitherto unidentified speculation is that Slc38a1 would be expressed with different roles between neurons and astrocytes which are both derived from identical NSCs enriched of Slc38a1.

5. Pluripotent P19 cells

5.1. Similar promotion by theanine

Mouse embryonal carcinoma P19 cells feature a phenotype similar to that of cells of the primitive ectoderm together with an ability to commit differentiation to a neural phenotype in the presence of retinoic acid and to a muscular phenotype in the presence of dimethylsulfoxide, respectively (McBurney and Rogers, 1982; Jones-Villeneuve et al., 1982). P19 cells rapidly grow to form clusters expressing several early embryonic markers without limitation during culture. Culture with retinoic acid leads to the formation of round spheres composed of clustered proliferating cells along with an ability to commit differentiation to neuronal and astroglial cells as seen with NPCs. In undifferentiated P19 cells with sustained exposure to theanine (Ogura et al., 2012), a marked increase is seen in their proliferative activity, followed by increased number of cells immunoreactive for a neuronal marker protein with a concomitant decrease in the number of cells immunoreactive for an astroglial marker protein after spontaneous differentiation, in line with our studies using NPCs prepared from embryonic rat and mouse neocortex.

5.2. Theanine on Slc38a1 gene expression

To analyze the usefulness as a cell model for evaluating underlying mechanisms, pluripotent P19 cells were further used for determination of the promoter activity of *Slc38a1* gene expression using luciferase reporter plasmids with different lengths from −2959 bp to −768 bp upstream (Takarada et al., 2016a). Luciferase activity is more than doubled in P19 cells with a full-length luciferase reporter plasmid of the *Slc38a1* promoter after sustained exposure to theanine, while theanine fails to stimulate the luciferase activity in cells with deleted promoter regions. The deletion plasmid analysis gives support to the requirement of the promoter region between −1626 and −768 bp for the upregulation of *Slc38a1* expression in pluripotent P19 cells with sustained exposure to theanine (Takarada et al., 2016a). *In silico* analysis reveals the presence of several responsive elements at the sensitive upstream promotor region from −198 bp to −26 bp of *Slc38a1* gene (Ogura et al., 2007). These include activator protein-1 and cyclic AMP responsive element binding protein, which are both shown to induce a drastic stimulation of the promotor activity of *Slc38a1* gene in C6 glioma cells (Ogura et al., 2007).

5.3. Stable overexpression of Slc38a1

In line with the upregulation by theanine of *Slc38a1* expression as described above, stable overexpression of *Slc38a1* similarly promotes proliferation for self-renewal along with facilitated neuronal commitment in undifferentiated P19 cells (Ogura et al., 2012). In these stable Slc38a1 transfectants before differentiation, moreover, drastic upregulation is seen in transcript expression for different basic helix-loop-helix (bHLH) transcription factors (Ogura et al., 2012), which are responsible for the positive and negative regulation of the properties of undifferentiated NSCs (Bertrand et al., 2002). In undifferentiated P19 cells, constitutive expression is indeed seen with transcripts for several bHLH factors. Neuronal commitment is highly stimulated by the activator type of bHLH factors including Mash1, Math3 and NeuroD1, for example, whereas the repressor type such as Hes1 and Hes5 is endowed to maintain primitive features for suppressed differentiation in progenitors (Fig. 4). Notch signals mediate a cell-cell interaction to maintain dividing cells in a fashion dependent on the repressor type bHLH genes such as *Hes1* and *Hes5*, which prevents the stimulation of the activator type neuronal bHLH factors including Mash1 (Kageyama and Ohtsuka, 1999). Both Hes1 and Hes5 are essential Notch effectors in negatively regulating mammalian neuronal differentiation (Ohtsuka et al., 1999). The drastic upregulation of both activator and repressor types of bHLH genes would be thus involved in molecular mechanisms underlying the promotion of both proliferation and neuronal commitment in pluripotent P19 cells with stable overexpression of *Slc38a1*.

However, theanine is highly effective in drastically stimulating the

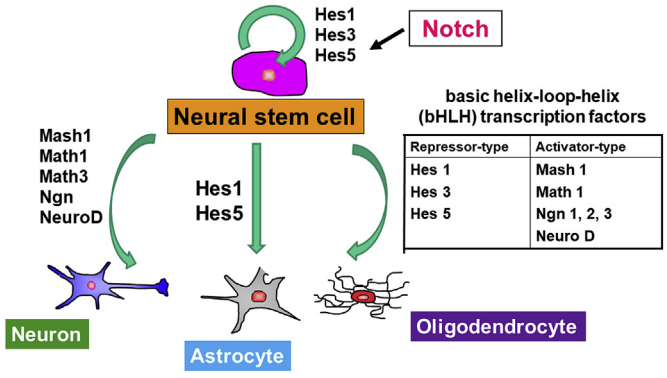

Fig. 4. Typical Transcription Factors with a bHLH Structure in NPCs. The bHLH transcription factors are all responsible for positive and negative regulations of the primitive features of undifferentiated NPCs. The repressor type is able to maintain the primitive properties to proliferate for self-replication with suppressed differentiation, whereas the activator type is endowed to promote commitment to progeny cell lineages along with suppressed self-renewal.

promoter activity of the activator type *NeuroD1* after sustained exposure in control P19 cells with an empty vector, but fails to promote additionally all activities already stimulated for proliferation, neuronal commitment and *NeuroD1* gene expression in stable *Slc38a1* transfectants (Ogura et al., 2012). The activator type bHLH factor NeuroD1 is entirely necessary for the survival and neuronal commitment of NSCs during adult neurogenesis (Kuwabara et al., 2009; Gao et al., 2009), while *NeuroD1* is identified as a gene highly related to the terminal differentiation to neurons during postnatal and adult neurogenesis (Boutin et al., 2010). Overexpression of *NeuroD1* is shown to result in a rapid appearance of cells featuring morphological and molecular properties of mature neurons *in vivo*, while NeuroD1 *shRNA* drastically suppresses the terminal neuronal differentiation. Taking into consideration the complete absence of the additivity, theanine could promote both proliferation and neuronal commitment through a mechanism common to the promotion mediated by stable *Slc38a1* overexpression in pluripotent P19 cells before differentiation.

6. Intracellular mTOR signaling

6.1. Phosphorylation signaling cascade

Mechanistic target of rapamycin (mTOR) is a serine/threonine protein kinase with a molecular weight of 289 kilodalton encoded by a single gene localized at chromosome 1p36.2 (Jhanwar-Uniyal et al., 2017). This kinase is classified to the phosphoinositide 3-kinase-related kinase family with homology throughout all eukaryotes (Russell et al., 2011; Laplante and Sabatini, 2012). Mammalian cells are shown to have two functionally distinct mTOR complexes such as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). In addition to other relevant proteins, mTORC1 is composed of mTOR, rapamycin-sensitive adaptor protein of mTOR (Raptor) and LST8, while mTORC2 is orchestrated by rapamycin-insensitive companion of mTOR (Rictor), LST8 and Sin1 (Loewith et al., 2002). Evidence is accumulating for the regulation by mTORC1 of protein translation through activation of p70 S6 kinase (p70S6K) and inhibition of eukaryotic initiation factor 4E binding protein (Sabatini, 2006), along with promotion of RNA translation via S6 ribosomal protein (Volarevic and Thomas, 2001). Activation of mTORC1 is brought about by nutrients, amino acids and growth factors, while mTORC2 has been implicated to be responsible for cytoskeletal organization and cell survival through Akt (=protein kinase B) (Jacinto et al., 2004). The prevailing view is that mTOR is highly responsible for the integration of extracellular growth signals with nutrients through the two distinct multiprotein complexes, mTORC1 and mTORC2, toward the regulation of cell proliferation, migration, growth, autophagy, metabolism and survival.

6.2. Requirement for GLN in mTOR signaling

Extracellular GLN is a prerequisite for the activation of mTOR signaling pathway as an obligatory component in Jarkat cells (Fumarola et al., 2005). Intracellular GLN levels are a crucial determinant for the activation of mTORC1 signaling pathway in association with several membrane transporters for this amino acid in cultured HeLa cells (Nicklin et al., 2009). Several independent lines of evidence indicate the importance of mTORC1 signaling pathway as an intracellular downstream signal of extracellular essential amino acids (EAAs) in the literature (Russell et al., 2011). Increased intracellular GLN levels are shown to facilitate the influx of extracellular EAAs such as leucine in exchange for the efflux of intracellular GLN by particular membrane transporters, followed by consequential activation of the mTOR1 signaling pathway toward accelerated cell growth and proliferation (Nicklin et al., 2009). In our previous studies using P19 cells with stable overexpression of *Slc38a1*, more than doubled intracellular GLN levels are found together with the promotion of both cell growth and neuronal commitment, which occurs in a manner associated with upregulation of bHLH gene expression (Ogura et al., 2012) and facilitated phosphorylation of mTOR and downstream proteins (Takarada et al., 2016b) in the absence of theanine. Upregulation of Hes5 and Pax6 expression is thought to participate in mechanisms underlying the control by mTOR signals of the primitive progenitor features in P19 cells (Endo et al., 2009). However, extracellular GLN inhibits the activation of the mTOR signaling by arginine and leucine in rat intestinal epithelial cells (Nakajo et al., 2005).

6.3. Theanine on mTORC1 signaling

The failure by theanine in stable Slc38a1 transfectants argues in favor of a common mechanism between sustained exposure to theanine and stable overexpression of *Slc38a1* with respect to the marked promotion of both cellular proliferation and neuronal commitment activities in P19 cells. An attempt is made to determine whether intracellular mTORC1 signaling at least in part participates in mechanisms underlying the promotion of cell growth and neural commitment in cultured NPCs exposed to theanine. In undifferentiated murine NPCs cultured with theanine for a relatively long period (Takarada et al., 2016b), drastically stimulated phosphorylation is seen in mTOR and downstream proteins such as p70S6K and S6, which are all key proteins for the mTORC1 kinase pathway responsive to different amino acid signals in the cytoplasm. In P19 cells with stable overexpression of Slc38a1, similarly marked phosphorylation is found for mTOR, p70S6K and S6 without alteration of the endogenous level of p70S6K in the absence of theanine. In control mock transfectants of P19 cells, theanine highly accelerates the phosphorylation of mTOR, p70S6K and S6 in a concentration-dependent manner as seen in murine NPCs, but fails to promote additionally the phosphorylation of these mTOR signaling molecules in stable *Slc38a1* transfectants. Taken together, both sustained exposure to theanine and stable Slc38a1 overexpression could similarly stimulate cell growth and neuronal commitment through a common mechanism relevant to upregulation of particular bHLH factors toward the promotion of mTORC1 signaling pathway in undifferentiated NPCs.

Nevertheless, the molecular mechanism by which theanine stimulates the phosphorylation of mTOR and downstream proteins in place of GLN in undifferentiated NPCs is not well clarified so far. Theanine could be incorporated to the cytoplasm through particular membrane GLNT isoforms other than Slc38a1 together with GLN, but differentially affect the mTOR kinase signaling in a peculiar manner. Theanine would be more efficient than GLN in promoting the mTOR signaling pathway in NPCs in a particular situation. For instance, GLN would be metabolized through intracellular glutaminolysis processes to produce α-ketoglutarate, which in turn facilitates the intracellular mTOR kinase signaling in association with the EAA leucine (Duran et al., 2012). The possibility that theanine may modulate the mTOR signaling activity through a mechanism completely irrelevant to GLN in the cytoplasm is thus still conceivable so far.

7. Theanine hypothesis

Taking into consideration the unique expression profile of bHLH factors in primitive undifferentiated progenitors rather than differentiated progeny lineages, theanine seems to promote embryonic, perinatal, developing and adult neurogenesis through mechanisms associated with upregulation of *Slc38a1* expression leading to increased intracellular GLN levels. Increased intracellular GLN levels would then facilitate the influx of extracellular EAAs required for the activation of intracellular mTORC1 signaling to the transactivation of activator and repressor types of bHLH factors after an exchange process carried by the Slc7a5/8 isoforms other than Slc38a1 isoform. In this scheme, theanine has two predominant action sites needed for the promotion of neurogenesis in NSCs. The first aspect is preferential upregulation by theanine of *Slc38a1* expression in NSCs, while the second point is

predominant transactivation by theanine of bHLH factors after stimulation of mTORC1 signals in NSCs as summarized in the Graphic Abstract. Although Slc38a1 isoform is highly localized in the brain (Yoneda, 2017), the mechanism underlying the activation by theanine of *Slc38a1* promoter in undifferentiated NSCs still remains to be elucidated in future studies. The possibility that Slc38a1 isoform is upregulated as a compensation to the prolonged inhibition of the influx of extracellular GLN during exposure to the transport competitor theanine for a long term is not ruled out so far.

In addition to a possible role as a modulator of embryonic and adult neurogenesis in NPCs as described above, theanine could also improve cognition impairment through a mechanism relevant to the inhibition of GLN uptake mediated by Slc38a1 necessary for fueling the neurotransmitter pool of Glu as the Glu/GLN cycle at glutamatergic synapses within mature neurons in the brain (Walton and Dodd, 2007; Butterworth, 2014). The uncertainties surrounding adult neurogenesis in humans (Dennis et al., 2016) also discourage the possible promotion of the birth of new neurons after oral intake of theanine in adult brain. In contrast, a recent report favorable for preserved neurogenesis in the hippocampal DG of healthy older people without cognitive impairment, neuropsychiatric disease or treatment is available in the literature (Boldrini et al., 2018). Accordingly, the conclusion should await the future progress of innovative technology sufficient to monitor *in situ* features of NSCs/NPCs expressed at particular niche regions in human subjects for the significance of adult neurogenesis, in place of the use of postmortem human specimens. Sustained oral ingestion during pregnancy to childhood could be expected to facilitate the construction and harmonization of sound neuronal network for future brain health through a mechanism related to the promotion of embryonic, perinatal and postnatal neurogenesis in NSCs in a particular situation.

8. Future aspects

Evidence is now accumulating for the benefits of oral consumption of theanine for amelioration of functional abnormalities seen in brains of people with impaired cognition, in addition to improving sleep disturbance under different stressful conditions toward mental relaxation in patients with depression, schizophrenia and ADHD as described above. As green tea has been consumed without any serious problems of human health for hundreds of years in Japan, the physical and mental safety of oral intake of theanine in juvenile and senile people with particular brain dysfunctions is highly conceivable so far. The fact that theanine is an ingredient in natural green tea leaves rather than an artificially synthesized compound, gives rise to the importance of the development of innovative nutraceuticals and/or dietary supplements for the prophylaxis and sometimes for the improvement of a variety of unpleasant brain symptoms and syndromes related to the life-style diseases without remarkable side effects seen with most drugs and medicines used for therapy. From a viewpoint of betterment of the quality of life in humans, prophylaxis is much more important than therapy in likely patients suffering from different symptoms of numerous diseases in any situations (Yoneda, 2018).

Conflicts of interest

Dr. Yoneda is playing a role as a scientific advisor in Japan Detox, Co. Ltd. The other authors have no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.104505.

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