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ORIGINAL PAPER

Upregulation of *Slc38a1* Gene Along with Promotion of Neurosphere Growth and Subsequent Neuronal Specification in Undifferentiated Neural Progenitor Cells Exposed to Theanine

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Abstract We have shown marked promotion of both cluster growth and neuronal specification in pluripotent P19 cells with overexpression of solute carrier 38a1 (Slc38a1), which is responsible for membrane transport of glutamine. In this study, we evaluated pharmacological profiles of the green tea amino acid ingredient theanine, which is a good substrate for glutamine transporters, on proliferation and neuronal specification in neural progenitor cells from embryonic rat neocortex. Sustained exposure to theanine, but not glutamine, accelerated the growth of neurospheres composed of proliferating cells and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reducing activity at concentrations of 1–100 μM in undifferentiated progenitor cells. Such prior exposure to theanine promoted spontaneous and induced commitment to a neuronal lineage with concomitant deteriorated astroglial specification. Selective upregulation was seen in the expression of *Slc38a1* in progenitor cells cultured with theanine. Similarly significant increases in cluster growth and MTT reducing activity were found in P19 cells cultured with theanine for 4 days. Luciferase activity was doubled in a manner sensitive to the deletion of promoter regions in P19 cells with a luciferase reporter plasmid of the *Slc38a1* promoter after sustained exposure to theanine for 4 days. Overexpression of X-box binding protein-1 led to a marked increase in luciferase activity in P19 cells transfected with the *Slc38a1* reporter plasmid. These results suggest that theanine accelerates cellular proliferation and subsequent neuronal specification through a mechanism relevant to upregulation of *Slc38a1* gene in undifferentiated neural progenitor cells.

Keywords Theanine · Glutamine transporter · SLC38A1 · Neural progenitors · Neurosphere · Neuronal specification

Abbreviations

AP-1	Activator protein-1
ATF2	Activating transcription factor-2
ATF2	Activating transcription factor-2
ATF3	Activating transcription factor-3
ATRA	All-*trans* retinoic acid
bHLH	Basic helix-loop-helix
BrdU	5-Bromo-2′-deoxyuridine
CaATF6	Constitutive active activating transcription factor-6
CaHIF1α	Constitutive active hypoxia inducible factor-1α
C/EBPα	CCAAT enhancer binding protein-α
CNTF	Ciliary neurotrophic factor
CNTFRα	Ciliary neurotrophic factor receptor alpha
CREB	Cyclic AMP responsive element binding protein

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DMEM/F-12	Dulbecco's modified Eagle medium: Nutrient Mixture F-12
ER	Endoplasmic reticulum
EV	Empty vector
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
Gln	Glutamine
HIF1α	Hypoxia inducible factor-1α
MAP2	Microtubules-associated protein-2
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide
NCAM	Neural cell adhesion molecule
NFAT2	Nuclear factor of activated T cell-1
NF-κB	Nuclear factor-κB
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PA	Paraformaldehyde
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RARα	Retinoic acid receptor alpha
RT-PCR	Reverse transcription polymerase chain reaction
SLC38A1	Solute carrier 38a1
STAT1	Signal transducer and activator of transcription-1
STAT3	Signal transducer and activator of transcription-3
WT	Wild-type
XBP1	X-box binding protein-1

Introduction

The green tea amino acid ingredient theanine (=γ-glutamylethylamide) is a structural analog of glutamine (Gln) rather than glutamate. An intracerebroventricular injection of theanine was effective in protecting hippocampal CA1 pyramidal neurons from delayed neuronal cell death in the brains of gerbils with bilateral forebrain global ischemia in vivo [1]. However, theanine exhibited a very low affinity for different ionotropic glutamate receptor subtypes relevant to ischemic neurotoxicity in rat cortical synaptic membranes in vitro [2]. In contrast, theanine seems to be a good substrate for several Gln transporters in the brain. In rat brain synaptosomes, both [^{3}H]theanine and [^{3}H]Gln were similarly accumulated in a time-, temperature-, sodium- and structure-dependent manner. Theanine markedly inhibited [^{3}H]Gln incorporation without affecting [^{3}H]glutamate influx, while Gln was a potent inhibitor of [^{3}H]theanine uptake under the similar experimental conditions [3]. Theanine intake promoted object recognition memory, in addition to accelerating 5-bromo-2′-deoxyuridine (BrdU) incorporation in the hippocampus, in developing young rats [4]. Similarly, theanine prevented cognitive dysfunction in accelerated-senescence mice [5] in vivo.

Sustained exposure to theanine led to marked promotion of both activities of cellular proliferation and subsequent neuronal specification in pluripotent P19 cells widely used as a neural progenitor cell model [6]. Similarly marked promotion of both activities was seen in the absence of theanine in P19 cells with stable overexpression of solute carrier 38a1 (SLC38A1), which is shown to be responsible for the translocation of both Gln and theanine across cell membranes [6]. However, theanine failed to additionally facilitate both proliferation and neuronal specification activities found in these stable SLC38A1 transfectants [6]. In this study, accordingly, we attempted to evaluate pharmacological profiles of theanine in neural progenitor cells isolated from embryonic rat neocortex enriched of primitive cells immunoreactive for the undifferentiated progenitor cell marker nestin in vitro [7]. To further evaluate possible underlying mechanisms, we also analyzed expression profiles of several genes relevant to cellular maturation and proliferation besides *Slc38a1* in rat neural progenitor cells exposed to theanine.

Materials and Methods

Materials

Materials used were obtained from different sources described below. Theanine, Tokyo Kasei Kogyo (Tokyo, Japan); Pluripotent P19 stem cells derived from murine embryonal carcinoma, Riken Cell Bank (Tsukuba, Japan); Antibodies against microtubules-associated protein-2 (MAP2) and glial fibrillary acidic protein (GFAP), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), ciliary neurotrophic factor (CNTF) and all-*trans* retinoic acid (ATRA), Sigma Chemicals (St. Louis, MO, USA); An anti-mouse IgG antibody conjugated with rhodamine and an anti-rabbit IgG antibody conjugated with fluorescein, ICN Pharmaceuticals (Aurora, OH, USA). Dulbecco's modified Eagle medium: Nutrient Mixture F-12 (DMEM/F-12) 1:1 Mixture and fetal bovine serum (FBS), GIBCO BRL (Gaithersburg, MD, USA), Fibroblast growth factor (FGF), Biomedical Technologies (Stoughton, MA, USA), Dual-Luciferase Reporter Assay System, pGL3-basic vector and pRL-SV40 vector, Promega (Madison, WI, USA). Other chemicals used were all of the highest purity commercially available.

The expression vector for activator protein-1 (AP-1) consisted of c-fos and c-jun was generated by the polymerase chain reaction (PCR)-based cloning method in our laboratory. The expression vectors for nuclear factor of activated T cell-2 (NFAT2) was purchased from Thermo Scientific (Waltham, MA, USA) and cloned into pcDNA3 backbone vector. The expression vector for nuclear factor (erythroid-derived 2)-like 2 (Nrf2) was kindly provided by Dr. J. Alam (Alton Ochsner Medical Foundation, New Orleans, LA, USA) [8]. The expression vector for activating transcription factor-2 (ATF2) was kindly provided by Dr. Z. Ronai (Signal Transduction Program, Sanford-Burnham Medical Research Institute, La Jolla, CA, USA) [9]. The expression vector for ATF4 was kindly provided by Dr. G. Karsenty (Columbia University, New York, NY, USA) [10]. The expression vector for cyclic AMP response element binding protein (CREB) was kindly provided by Dr. G. Thiel (University of Saarland Medical Center, Homburg, Germany) [11]. Expression vectors for hypoxia inducible factor-1α (HIF1α)-wild-type (WT) (addgene#18949), X-box binding protein-1 (XBP1) (addgene#21833) and ATF6 (addgene#27173) were purchased from Addgene (Cambridge, MA, USA). The expression vector for constitutive active form of HIF1α (HIF1α-P564A) was kindly provided by Dr. Elazar Zelzer (Weizmann Institute of Science, Rehovot, Israel) [12]. Expression vectors for signal transducer and activator of transcription-1 (STAT1) and STAT3 were kindly donated by Dr. K. Yokoyama (Tsukuba Life Science Center, Ibaraki, Japan).

Preparation of Rat and Mouse Neural Progenitor Cells

The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University (Permit No. 70093). All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to in vivo techniques. Neocortex was isolated from 18-day-old embryonic Wistar rats or 15.5-day-old embryonic Std-ddY mice, followed by trituration through a Pasteur pipette with enzyme cocktails containing 2.5 U/mL papain, 250 U/mL DNAse and 1 U/mL neutral protease in phosphate-buffered saline (PBS) and subsequent collection of the lower layer enriched of undifferentiated progenitor cells after Percoll centrifugation procedures [13, 14].

As shown in our previous publication [7], particular cells were immunoreactive for either the neuronal marker protein MAP2 or the astroglial marker protein GFAP, but not for the progenitor marker protein nestin, in the upper layer prepared from embryonic rat brains. In the lower layer, by contrast, several cells were immunoreactive for nestin, with expression of either MAP2 or GFAP being not detected. During the culture of the lower cell layer under floating conditions, cells were clustered with each other to form round spheres with increasing sizes proportional to culture periods. These neurospheres were similarly grown during the culture of preparations from discrete brain structures including neocortex, hippocampus, striatum and brain stem. In proportion to increasing culture periods, vital mitochondrial activity determined by MTT reduction was drastically increased in these neurospheres cultured in the presence of FGF along with their sizes [7]. Double immunocytochemical analysis clearly revealed that these neurospheres were immunoreactive for nestin, but not for either MAP2 or GFAP, irrespective of the brain structures when examined on Day 12. Cells were then subjected to the culture in the absence of FGF for an additional 6 days toward spontaneous commitment following dispersion of neurospheres and subsequent seeding on wells previously coated with poly-L-lysine. Marked immunoreactivity was seen for either MAP2 or GFAP, but not for nestin, on double immunohistochemical analysis detection in cells cultured under these conditions. To further evaluate the self-replicating ability, dispersed cells were again cultured in the presence of FGF for an additional 12 days, followed by immunocytochemical detection of nestin on Day 24. Similarly large sized neurospheres were gradually grown during the second culture under floating conditions in the presence of FGF, whereas these secondary neurospheres were again immunoreactive for nestin, but not for either MAP2 or GFAP, on Day 24 DIV. Cells were then dispersed again on Day 24, followed by seeding on wells coated with poly-L-lysine and subsequent culture in the absence of FGF for an additional 6 days toward the second spontaneous commitment. Numerous cells were again immunoreactive for either MAP2 or GFAP in preparations isolated from all discrete brain structures examined without expression of immunoreactive nestin on Day 30. These relevant micrographic pictures were all presented in our previous publications for validation of properties of neural progenitor cells isolated from embryonic rat [7] and mouse [15] neocortex.

Determination of Proliferation Activity

Cells were cultured for 12 days in DMEM/F12 supplemented with 0.6 % (wt/vol) glucose, 15 mmol/L sodium bicarbonate, 250 mmol/L *N*-acetyl-L-cysteine, 20 ng/mL FGF, 20 nmol/L progesterone, 30 nmol/L sodium selenite, 60 nmol/L putrescine, 25 lg/mL insulin and 100 lg/mL apo-transferrin in the absence of FBS at 37 °C under 5 % CO_2 in a humidified CO_2 incubator under floating conditions with a half medium change every 2 days [7]. Cells were not exposed to FBS after seeding at all to avoid possible influences by hitherto unidentified factors present

in FBS during neurosphere formation as described previously [7]. Cultured neural progenitor cells were exposed to either theanine or Gln at different concentrations for 12 days in the presence of EGF. Five different visual fields were chosen at random from each culture well under a phase contrast micrograph in a blinded fashion, followed by calculation of total areas of neurospheres composed of clustered proliferating cells in parallel experiments for summation using Scion Image β 4.02 software as described previously [7]. Proliferation activity was also quantified by MTT reduction assays [6]. A highly positive correlation was confirmed between these two activities and the activity of BrdU incorporation as an index of cellular proliferation in neural progenitor cells isolated from neocortex of embryonic rat [7] and mouse [15] brains. As both the area and volume are invariably a function of a radius of neurospheres, we employed the area instead of the volume as an index of the neurosphere size under our two-dimensional micrographic conditions.

Determination of Specification Activity

After the culture with theanine for 12 days, cells were dissociated by pipetting, followed by seeding at 15,000 cells/well and subsequent culture without theanine to initiate spontaneous specification in the absence of both EGF and theanine for an additional 6 days. Dispersed cells were also cultured without theanine in the presence of 100 ng/mL ATRA to promote neuronal commitment, or 20 ng/mL CNTF to promote astroglial commitment [6]. Cells were then subjected to immunocytochemistry with antibodies against MAP2 (1:500) and GFAP (1:500) overnight at 4 °C and incubated with respective secondary antibodies (1:1000). Quantification was performed by counting the number of cells immunoreactive for either MAP2 or GFAP in a blinded fashion, followed by calculation of the individual percentages over the number of total cells stained with Hoechst33342 [10].

Cultured cells were lysed in CSK buffer [(in mM): NaCl, 100; sucrose, 300; PIPES (pH 6.8), 10; $MgCl_2$, 3; EDTA, 1; 0.5 % Triton X-100]. Each extract was added at a volume ratio of 4:1 to 10 mM Tris–HCl buffer (pH 6.8) containing 10 % glycerol, 2 % sodium dodecyl sulfate, 0.01 % bromophenol blue and 5 % 2-mercaptoethanol, followed by mixing and boiling at 100 °C for 5 min. After determination of each protein content, an aliquot of 10 μg protein was invariably loaded on a 7.5 % polyacrylamide gel for electrophoresis at a constant current of 15 mA/plate for 2 h at room temperature. Gels were then subjected to blotting to a polyvinylidene fluoride membrane previously treated with 100 % methanol. Membranes were reacted with 5 % skimmed milk dissolved in 20 mM Tris–HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05 % Tween 20, and then with an antibody against MAP2 (1:400) or GFAP (1:1000) diluted with buffer containing 1 % skimmed milk. The relative amount of each protein was normalized by the quantitative densitometric analysis using Image J software.

Determination of mRNA Expression

Total RNA was extracted from cultured rat and mouse neural progenitor cells using the standard ISOGEN procedure for the synthesis of cDNA [16]. The individual cDNA species were amplified using primers for *Slc38a1*, *retinoic acid receptor α* (*RARα*), *ciliary neurotrophic factor receptor α* (*CNTFRα*), *β1-Integrin*, *N-Cadherin*, *neural cell adhesion molecule* (*NCAM*), *EGF* or *CNTF* (Table 1), which are previously shown to play a role in mechanisms underlying cellular proliferation, migration and/or differentiation in neural progenitor cells [7]. Quantitative analysis was performed with normalization over the expression

Table 1 Primers used in this study

Gene	Upstream	Downstream
RT-PCR		
Slc38a1	ATGATGCATTTCAAAAGTGGC	TCAGTGGCCTTCACCATTG
RARα	GTACACGCCTGAGCAAGACA	AGAGTGTCCAAGCCCTCAGA
CNTFRα	ATCCTCCAGAAAACGTGGTG	CCAGGGTCACAGATCTTCGT
β1-Integrin	AAACCTGTGTGCCATTGTGA	TGCACTGTGGGTGAATTGTT
N-Cadherin	CTGGGACGTATGTGATGACG	CTGTTGGGGTCTGTCAGGAT
NCA	GGGTGTCACTGCTTCATCCT	CTTGAAGTTCCCTTCGTCCA
EGF	CCAGGACATACGACACATGC	CCGTGTCCGTCCAGAATAGT
CNTF	CACCCCAACTGAAGGTGACT	TGAGCGAATGGCTACATCTG
GAPDH	GGTGAAGGTCGGTGTGAACGGATT	GATGCCAAAGTTGTCATGGATGACC
Real time PCR		
Slc38a1	GGCATCTGTATTTGCTGCTG	CGTTGCTGACGTTGTCATCT
β-Actin	TGTGATGGTGGGAATGGGTCAGAA	TGTGGTGCCAGATCTTCTCCATGT

of the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Quantification of *Slc38a1* levels was also conducted by real time-based reverse transcription (RT)-PCR in neurospheres from murine progenitor cells.

Luciferase Assays

The rat *Slc38a1* promoter fragment (−2959 to −6) was cloned into the promoterless pGL-3 basic vector, to create the recombinant plasmid −2959/−6 GlnT-LUC [11]. Deletion mutants of *Slc38a1* promoter plasmids (−1626/−6 and −768/−6 GlnT-LUC) were made from the recombinant plasmid −2959/−6 GlnT-LUC using restriction enzyme and T4 DNA polymerase. Pluripotent P19 cells were transfected with reporter plasmids of *Slc38a1* promoters using the calcium phosphate method [6]. Cells were cultured in DMEM/F-12 supplemented with 10 % FBS and 0.5 μM ATRA in either the presence or absence of theanine for different days. Similarly, the full length luciferase reporter plasmid was co-transfected with one of expression vectors for different transcription factors in P19 cells, followed by further culture for an additional 3 days in DMEM/F-12 supplemented with 10 % FBS and 0.5 μM ATRA in the absence of theanine. In each situation, cells were lysed for the determination of luciferase activity using specific substrates in a luminometer according to the manufacturer's protocol.

Data Analysis

All results are expressed as the mean ± SE, and the statistical significance was usually determined by the one-way or two-way ANOVA with Bonferroni/Dunnett's post hoc test or two tailed Student's *t* test. In experiments using four different variables, a Chi square test was used for statistical analysis. The level of significance was set at $p < 0.05$.

Results

Exposure to Theanine in Rat Neural Progenitor Cells

In neural progenitor cells from embryonic rat neocortex, theanine significantly increased the size of neurospheres formed of proliferating cells during the culture from 4 to

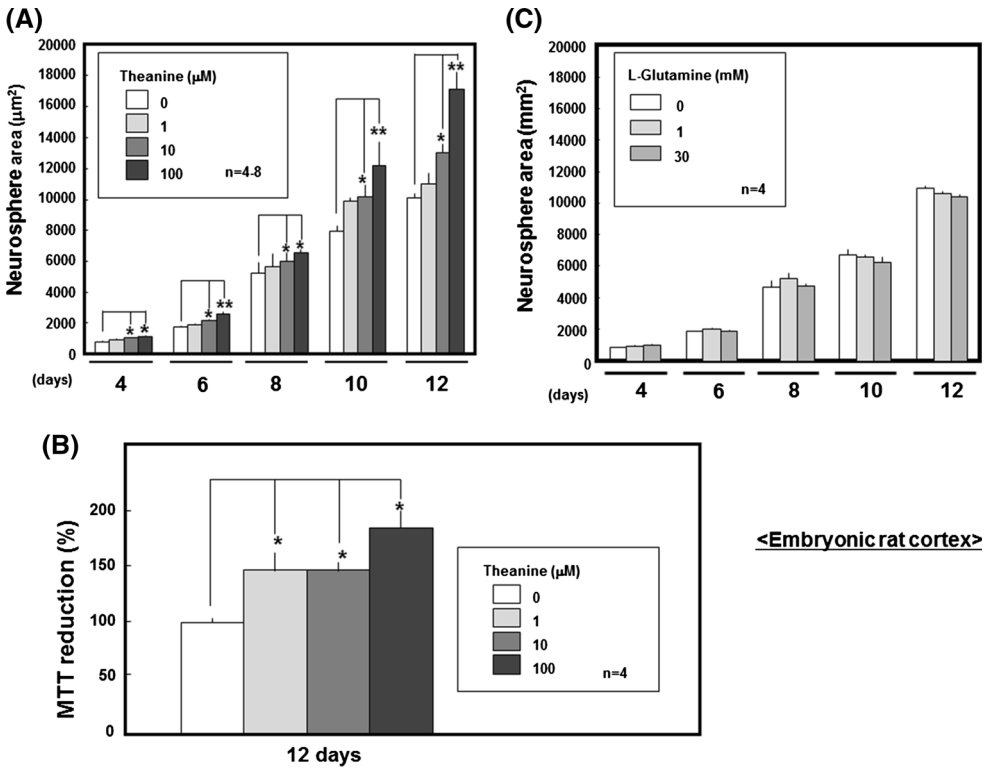

Fig. 1 Effects of theanine on neurosphere growth in rat neural progenitor cells. Neural progenitor cells derived from embryonic rat neocortex were cultured with EGF in the presence of theanine at different concentrations of up to 100 μM for 4–12 days under floating conditions, followed by measurement of **a** neurosphere size on Day 4–Day 12 (n = 4–8). **b** MTT reduction on Day 12 (n = 4). **c** Cells were also exposed to Gln at concentrations of 1–30 mM for 4–12 days under floating conditions, followed by determination of the size of neurospheres formed (n = 4). $*P < 0.05$; $**P < 0.01$, significantly different from each control value in cells not exposed to theanine or Gln. Statistical significance was determined using the one-way (**b**) or the two-way (**a**, **c**) ANOVA with Bonferroni/Dunnett post hoc test

12 days (Fig. 1a) in a concentration-dependent manner at a concentration range of 1–100 μM. Theanine was also effective in significantly increasing the activity of MTT reduction in rat neural progenitor cells exposed to theanine for 12 days in a concentration-dependent manner at concentrations over 1 μM (Fig. 1b). We next employed considerably high concentrations of the endogenous analog Gln, which was present at 4 mM in DMEM/F12 used for the culture of neural progenitor cells. In contrast to theanine, however, Gln was ineffective in significantly affecting the size of neurospheres formed during the culture from 4 to 12 days even at concentrations of 1–30 mM (Fig. 1c).

The cells were then dispersed for culture without theanine in either the presence or absence of ATRA and CNTF for an additional 6 days, followed by double immunocytochemical detection of both MAP2 and GFAP, in addition to Hoechst33342 staining. Around 50 % of cells stained with Hoechst33342 were immunoreactive for GFAP and <30 % for MAP2 after spontaneous specification, respectively. To maximize the concentration-dependent pharmacological action, specification experiments were done using neurospheres previously cultured with theanine at the highest concentration used. Prior culture with 100 μM theanine led to a marked increase in the number of MAP2-positive cells with decreased GFAP-positive cells in a manner independent of the presence of ATRA and CNTF (Fig. 2a). Counting of the number of individual immunoreactive cells clearly revealed significant promotion of subsequent neuronal specification with deteriorated astroglial specification irrespective of the presence of differentiation inducers, in rat neural progenitor cells previously exposed to 100 μM theanine for 12 days (Fig. 2b).

Neurospheres previously cultured with 100 μM theanine for 12 days were similarly subjected to dispersion and further culture for an additional 6 days in either the presence or absence of ATRA and CNTF for Western blotting analysis. In a manner independent of the addition of any differentiation inducers, prior exposure to theanine invariably led to a marked increase in MAP2 levels and a rather mild decrease in GFAP levels on Day 18 (Fig. 3a). Repetition and quantification of these experiments clearly revealed a significant increase in MAP2 levels together with a significant decrease in GFAP levels after spontaneous and induced commitment in cells previously exposed to 100 μM theanine (Fig. 3b).

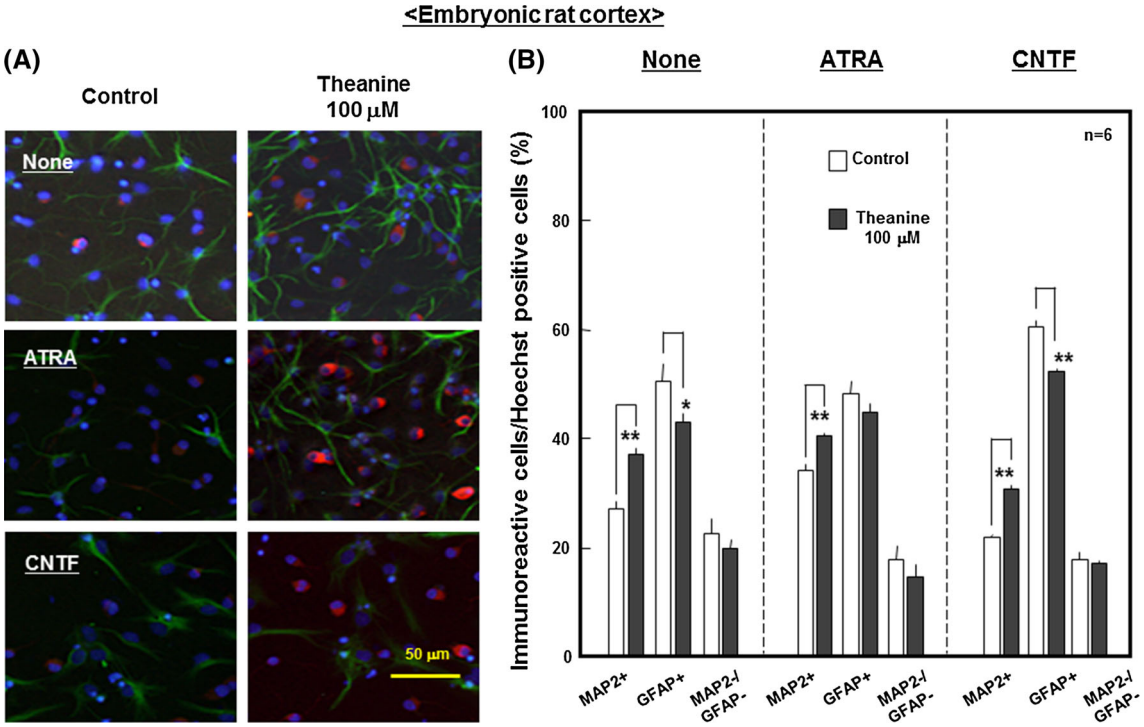

Fig. 2 Effects of theanine on subsequent specification in rat neural progenitor cells. Neural progenitor cells derived from embryonic rat neocortex were cultured with EGF in either the presence or absence of 100 μM theanine for 12 days under floating conditions, followed by removal of EGF and subsequent dispersion for further culture without theanine for an additional 6 days under adherent conditions in either the presence or absence of ATRA and CNTF. Typical fluorescence micrographs are shown in (**a**), while the (**b**) shows percentages of cells immunoreactive for either MAP2 or GFAP over the total number of cells stained with Hoechst33342 in six independent experiments. $*P < 0.05$; $**P < 0.01$, significantly different from each control value in cells not exposed to theanine. Statistical significance was determined using the Chi square test

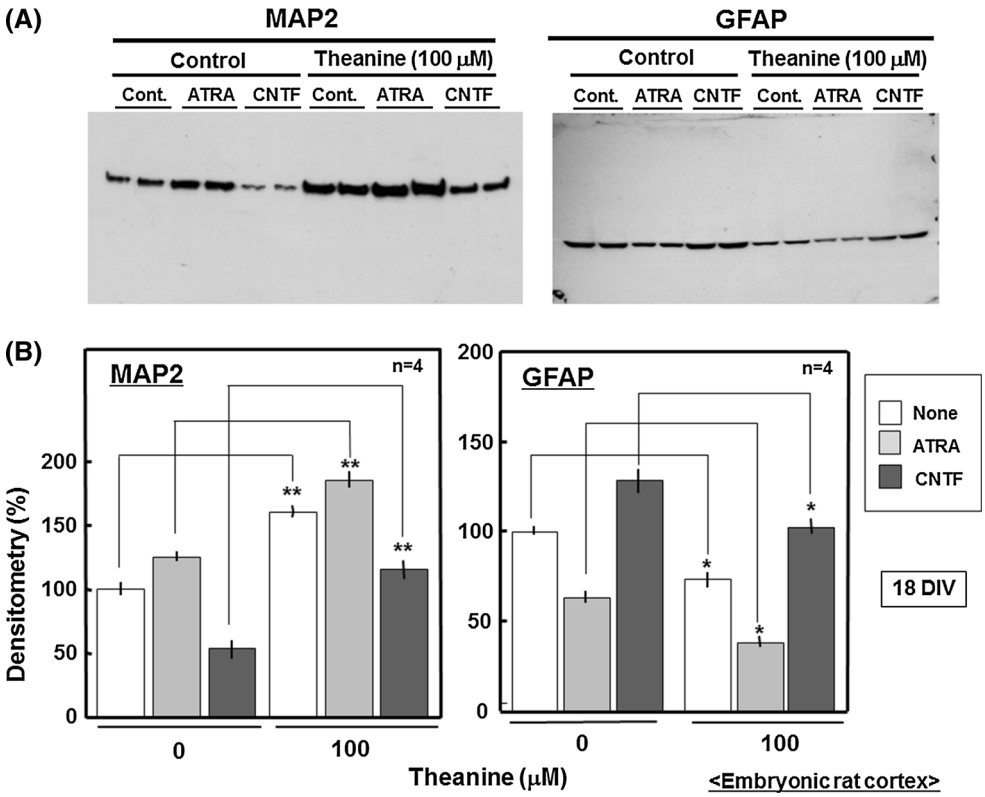

Fig. 3 Effects of theanine on MAP2 and GFAP expression in rat neural progenitor cells. Rat neural progenitor cells were cultured with FGF in either the presence or absence of 100 μM theanine for 12 days under floating conditions, followed by dispersion for further culture for an additional 6 days in either the presence or absence of ATRA and CNTF and subsequent measurement of MAP2 and GFAP levels on western blotting. Typical gel pictures are shown in (**a**), while in (**b**) quantitative data are shown with statistical analysis (n = 4). $^{*}P < 0.05$; $^{**}P < 0.01$, significantly different from each control value obtained in cells not exposed to theanine. Statistical significance was determined using the two-way ANOVA with Bonferroni/Dunnett post hoc test

Expression of Several Genes Relevant to Proliferation and Differentiation

Constitutive expression was seen for *Slc38a1*, *RARα*, *CNTFRα*, *β1-Integrin*, *N-Cadherin*, *NCAM*, *EGF* and *CNTF* in undifferentiated neural progenitor cells cultured for 12 days, while sustained exposure to 100 μM theanine for 12 days led to a prominent increase in *Slc38a1* expression without affecting *mRNA* expression of the other molecules tested in undifferentiated cells (Fig. 4a). Repetition and quantification of these experiments revealed selective upregulation of *Slc38a1* expression in rat neural progenitor cells cultured with 100 μM theanine for 12 days (Fig. 4b). Similar upregulation of *Slc38a1* expression was found in undifferentiated neural progenitor cells isolated from embryonic mouse neocortex after sustained exposure to theanine at 10 or 100 μM for 12 days on real-time based RT-PCR (Fig. 4c). However, our attempt to demonstrate the upregulation of SLC38A1 protein expression in neural progenitor cells was unsuccessful. We could not detect constitutive and inducible expression of immunoreactive SLC38A1 protein on Western blotting and immunohistochemistry analyses using three different anti-SLC38A1 antibodies commercially available (Proteintech, 12039-1-AP; SantaCruz Biotechnology, sc-33439; sc-67080) so far.

Upregulation of *Slc38a1* Gene

In pluripotent P19 cells cultured with ATRA under floating conditions, clustered cells formed were immunoreactive for nestin, but not for MAP2 or GFAP [6]. Sustained exposure to 100 μM theanine significantly increased the size of P19 cell clusters cultured for 4 days, but not in those cultured for 2 days (Fig. 5a), in addition to significantly increasing MTT reduction determined on Day 4 (Fig. 5b). P19 cells were then transfected with a reporter plasmid of the full-length promoter region of *Slc38a1*, followed by exposure to theanine for 1–4 days. A significant increase in luciferase activity was seen in cells with sustained exposure to 100 μM theanine for 4 days, but not in those exposed for 1–2 days (Fig. 5c). Exposure to 100 μM theanine led to a significant increase in luciferase activity in P19 cells transfected with longer promoter plasmids (−2959/−6 and −1626/−6 GlnT-LUC), without significantly affecting the activity in cells transfected with shorter promoter plasmids (−768/−6 GlnT-LUC and empty-LUC) (Fig. 5d).

Responsive Promoter Elements on *Slc38a1* Gene

To search for a transcription factor responsible for the upregulation of *Slc38a1* gene, a luciferase reporter plasmid was co-transfected with one of expression vectors for

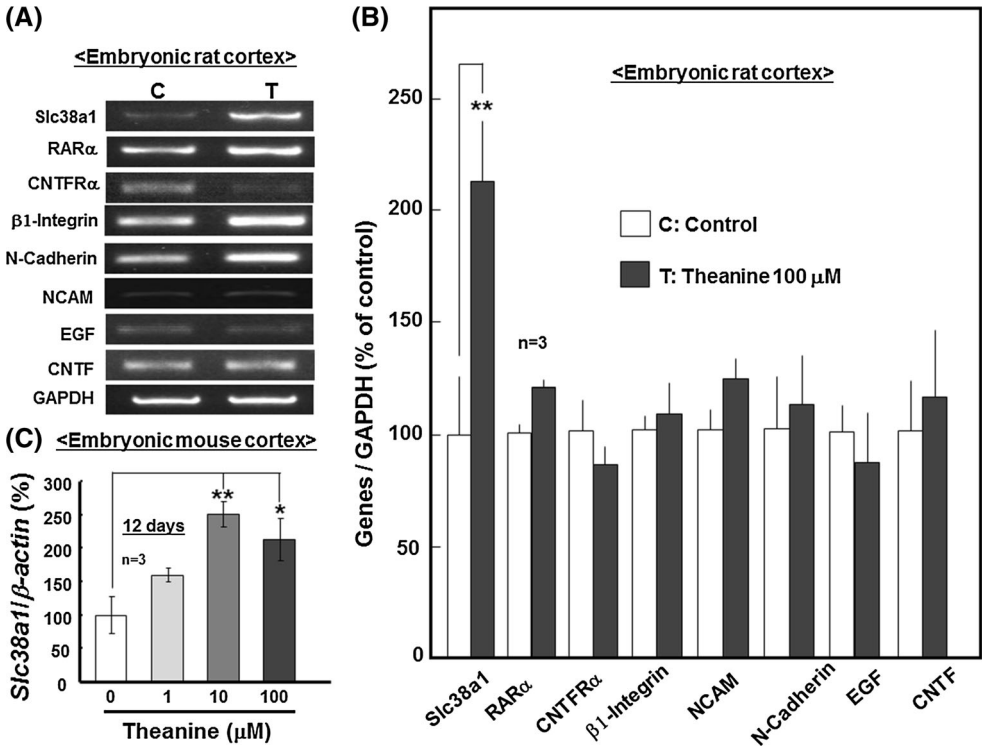

Fig. 4 Effects of theanine on *mRNA* expression of several genes relevant to proliferation and differentiation in rodent neural progenitor cells. Rat progenitor cells were cultured with EGF in either the presence or absence of 100 μM theanine for 12 days, followed by extraction of total RNA and subsequent RT-PCR analysis using primers specific for each gene. Typical pictures are shown in (**a**), while the (**b**) shows quantitative data in three independent experiments. **c** Similar experiments were done in neural progenitor cells isolated from embryonic mouse neocortex after exposure to theanine at concentrations of 1–100 μM for determination of *Slc38a1* expression on real time based RT-PCR (n = 3). $^{**}P < 0.01$, significantly different from each control value obtained in cells not exposed to theanine. Statistical significance was determined using the two-tailed Student's *t* test in (**b**) and the one-way ANOVA with Bonferroni/Dunnett post hoc test in (**c**)

different transcription factors in P19 cells, followed by further culture in the absence of theanine for an additional 3 days. A drastic increase was only seen in the luciferase activity in cells co-transfected with an XBP1 expression vector amongst a variety of vectors tested (Fig. 6). No significant changes were induced in cells with expression vectors of NFAT2, CCAAT enhancer binding protein-α (C/EBPα), STAT1, STAT3, CREB, AP-1, Nrf2, constitutive active ATF6 (CaATF6), ATF2, ATF3, constitutive active HIF1α (CaHIF1α) and nuclear factor-κB (NF-κB).

Discussion

The essential importance of the current findings is that the green tea ingredient theanine, which is an amino acid with a structural analogy to Gln, selectively up-regulated the expression of *Slc38a1* in cultured neural progenitor cells isolated from embryonic rat neocortex. Neocortex was enriched of undifferentiated neural progenitor cells endowed to proliferate and differentiate into neuronal and astroglial lineages during culture, in addition to highly positive immunoreactivity for nestin, in brains of embryonic rats [7] and mice [10]. Fundamentally identical properties were seen in proliferation and specification into neuronal and astroglial lineages between neural progenitor cells from adult mouse hippocampus [8, 9] and embryonic mouse neocortex [10]. In the present study, therefore, we employed neocortex rather than hippocampus of embryonic rodents as a source of neural progenitor cells. It should be emphasized that a clearly positive correlation was invariably seen for the size of round neurospheres formed of clustered cells with both MTT reduction and BrdU incorporation activities as an index of the proliferation activity in cultured neural progenitor cells from neocortex of rodent embryos [7, 10].

Although promoter analysis revealed the importance of the upstream region between −1626 and −768 bp of the *Slc38a1* promoter for the upregulation by theanine in P19 cells, the mechanism underlying upregulation has yet to be clarified. One possible but unproven speculation is that sustained competitive inhibition of Gln incorporation

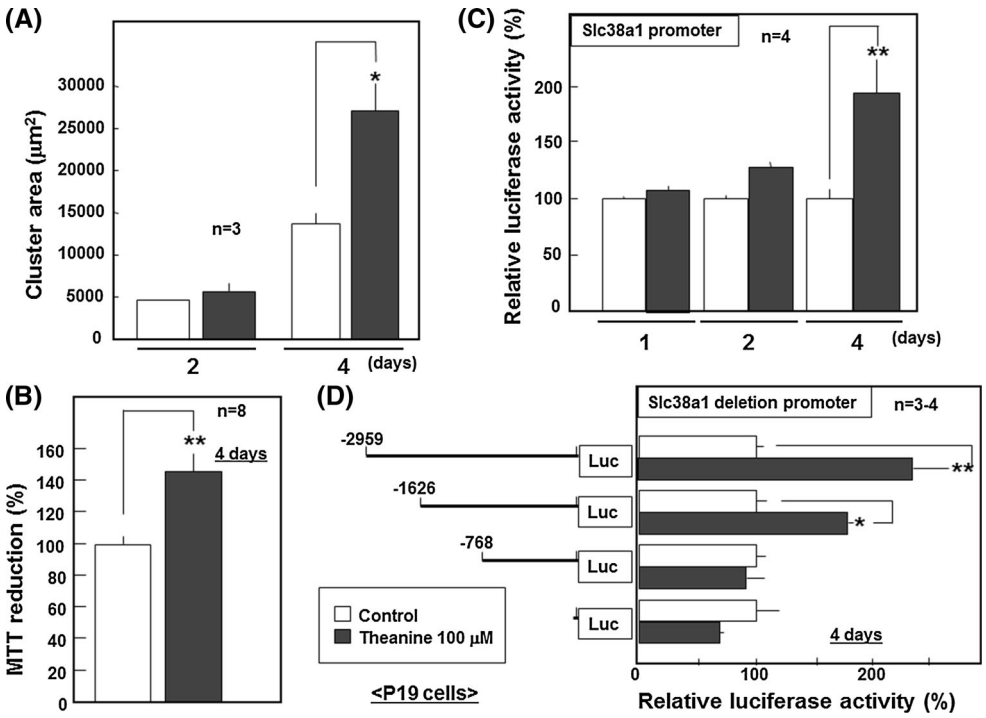

Fig. 5 Effects of theanine on *Slc38a1* gene expression in pluripotent P19 cells. Pluripotent P19 cells were cultured for 2 days, followed by dispersion and culture with ATRA in either the presence or absence of 100 μM theanine for 2 or 4 days under floating conditions for subsequent assessment of **a** the size of cell clusters formed (n = 3). **b** MTT reduction on Day 4 (n = 8). **c** Cells were transfected with a luciferase reporter plasmid linked to the full-length promoter region of *Slc38a1*, followed by culture in either the presence or absence of 100 μM theanine for 1–4 days and subsequent determination of luciferase activity (n = 4). **d** Cells were transfected with each deletion construct of the *Slc38a1* promoter reporter plasmid, followed by culture in either the presence or absence of 100 μM theanine for 4 days and subsequent determination of luciferase activity (n = 3–4). $^{*}P < 0.05$; $^{**}P < 0.01$, significantly different from each control value obtained in cells not exposed to theanine. Statistical significance was determined using the two-tailed Student's *t* test in (**b**) or the one-way ANOVA with Bonferroni/Dunnett post hoc test in (**a**, **c**, **d**)

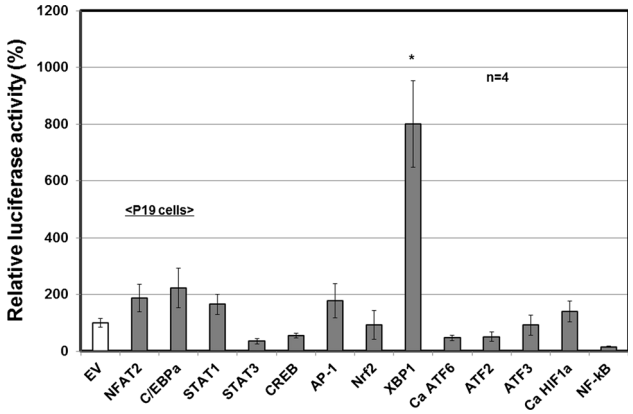

Fig. 6 Effects of different transcription factor expression vectors on *Slc38a1* promoter activity in P19 cells. P19 cells were transfected with a luciferase reporter plasmid of *Slc38a1* along with one of expression vectors, followed by further culture in the absence of theanine for an additional 3 days and subsequent determination of luciferase activity (n = 4). $^{*}P < 0.05$, significantly different from each control value obtained in cells with EV. Statistical significance was determined using the one-way ANOVA with Bonferroni/Dunnett post hoc test

would lead to compensatory upregulation of the *Slc38a1* gene in undifferentiated cells exposed to theanine. The fact that [^{3}H]theanine uptake was highly sensitive to the inhibition by Gln in rat brains [3] undoubtedly gives support to the notion that theanine incorporation is mediated by Gln transporters in the brain. Sustained competitive inhibition of Gln transport by theanine would thus promote gene transactivation of the target transporter SLC38A1, as an upregulation phenomenon often seen in the supersensitivity and/or sensitization for numbers of receptors under persistent exposure to an antagonist. The current promoter reporter analysis clearly revealed the involvement of the transcription factor relevant to endoplasmic reticulum (ER) stress, XBP1, in mechanisms underlying *Slc38a1* upregulation. SLC38A1 is likely to mediate membrane transportation of both theanine and Gln in the brain, however, while there is no direct evidence for this transporter to solely participate in the modulation of proliferation and specification of undifferentiated neural progenitor cells to date. From this viewpoint, it should be noted that stable overexpression of SLC38A1 led to drastic promotion of both proliferation and neuronal specification

in the absence of theanine in pluripotent embryonal carcinoma P19 cells [6]. The late onset of upregulation argues in favor of an idea that sustained exposure to theanine would induce particular unidentified endogenous factor(s) responsible for consequential upregulation of the *Slc38a1* gene in P19 cells.

Alternatively, theanine could be incorporated via particular membrane transporters, including SLC38A1, into the cytoplasm to induce ER stress highly associated with upregulation of XBP1 [17, 18] in neural progenitor cells. In fact, Gln was shown to attenuate ER stress and apoptosis in colons in vivo and in vitro [19]. The possibility that theanine exacerbates ER stress through reduction of intracellular levels of Gln capable of scavenging ER stress in association with competitive inhibition of the incorporation of extracellular Gln is thus not ruled out so far. Final conclusion should await future analyses on profiling of neural progenitor cells with genetic silencing and specific inhibitors of SLC38A1. Since stable overexpression of *Slc38a1* gene more than doubled intracellular Gln levels in P19 cells [6], upregulation of *Slc38a1* gene expression could also increase the endogenous intracellular levels of Gln in undifferentiated neural progenitor cells with sustained exposure to theanine. Glutamine is in fact shown to play a critical role in cell proliferation in a variety of cell types such as lymphocytes, enterocytes and tumor cells [20]. In Caco-2 cells, Gln increased proliferation through stimulation of nucleotide synthesis [21]. Considering the fact that sustained exposure to Gln failed to significantly promote neurosphere growth even at considerably high concentrations, however, upregulation of SLC38A1 expression would be a critical factor for inducing promotion of neurosphere growth and neuronal specification in rat neural progenitor cells exposed to theanine.

Taking into consideration our previous findings that theanine failed to further accelerate the promoted proliferation and neuronal specification in pluripotent P19 cells with stable overexpression of SLC38A1 [6], it is conceivable that SLC38A1 is at least in part responsible for the promotion by theanine of both activities. Although we have already shown promotion by theanine of cluster growth and specification into MAP2-positive cells in pluripotent P19 cells [6], this is the first direct demonstration of the acceleration of proliferation and neuronal specification with concomitant deteriorated astroglial specification in a manner irrespective of the presence of differentiation inducers in neural progenitor cells isolated from embryonic rat neocortex after sustained exposure to theanine. Deteriorated astroglial specification could be brought about as a consequence of facilitated neuronal specification in undifferentiated neural progenitor cells. As stable overexpression of *Slc38a1* led to marked upregulation of the expression of both activator (*Mash1*, *Math3*, *NeuroD1*) and repressor (*Hes5*) types of basic helix-loop-helix domain genes [6], pharmacological profiling on these genes relevant to proliferation and differentiation of neural progenitor cells remains to be elucidated in future studies. Judging from the relatively high effective concentrations, it is unlikely that daily intake of green tea is sufficient to positively regulate proliferation and subsequent specification into neurons of neural stem cells locally expressed in particular brain regions in adult human beings.

It thus appears that the green tea amino acid theanine promotes both neurosphere growth and subsequent neuronal specification through a mechanism relevant to *Slc38a1* upregulation in neural progenitor cells in vitro. Theanine could be beneficial for the discovery and development of supplements and/or drugs useful for the prophylaxis and treatment of patients suffering from different neurodegenerative and neuropsychiatric diseases related to impairment of embryonic neurogenesis in the brain.

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Conflict of interest None.

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