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Catechin hydrate induces GFAP expression and astrocyte branch elongation in rat neural stem cells

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Neural stem cells (NSCs) are multipotent cells capable of self-renewal and differentiate into diverse cell types within the central nervous system, including neurons, astrocytes, and oligodendrocytes. Catechin hydrate (CH) belongs to the flavonoid family and can be found in green tea and grape seeds. In the present study, we have explored the effect of CH on NSC fate at the molecular level. CH increased both mRNA and protein expression of glial fibrillary acidic protein during NSC differentiation. However, the number of astrocytes was not significantly altered. CH enhanced *leukemia inhibitory factor*, and *bone morphogenetic protein* mRNA levels and increased phosphorylation of STAT3 and SMAD1/5/8 in differentiated cells. CH increased *fibroblastic growth factor 2* mRNA levels and induced the formation of thin and elongated branches in differentiating astrocytes. These results suggest that although CH does not increase astrocytogenesis, it contributes to regulating astrocytes to become longer.

Keywords Phenolic compound, Catechin hydrate, Neural stem cells, Differentiation, GFAP, Astrocyte

Abbreviations

AKT	Protein kinase B
BLBP	Brain lipid-binding protein
BMP	Bone morphogenetic protein
CH	Catechin hydrate
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DMSO	Dimethyl sulfoxide
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
FGF2	Fibroblastic growth factor 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
ICC	Immunocytochemistry
IL-6	Interleukin-6
LIF	Leukemia inhibitory factor
NDD	Neurodegenerative disease
NSC	Neural stem cells
PI3K	Phosphatidylinositol 3-kinase
PBS	Phosphate buffered saline
qRT-PCR	Real-time reverse transcription polymerase chain reaction
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SMAD	Suppressor of mothers against decapentaplegic
STAT3	Signal transducer and activator of transcription 3
TUBB3	Class III beta-tubulin

Neural stem cells (NSCs) are multipotent cells capable of self-renewal and differentiate into diverse cell types within the central nervous system (CNS), including neurons and glia such as astrocytes, and oligodendrocytes^{1,2}.

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Neurodegenerative diseases (NDD) such as Alzheimer's disease, Parkinson's disease, and Huntington's disease are characterized by progressive loss of neurons and normal astrocyte function in the brain^{3–5}. NDD has been primarily characterized as neuronal pathology, but recent findings suggest that glial cells, particularly astrocytes might also play a significant role in NDD due to loss of their function like ion homeostasis, gain of function like reactive astrogliosis, and morphological deficit of astrocytes like decreasing fine processes^{3–5}. Since NSCs have the potential in the field of regenerative medicine^{2,6}, the study of chemical compounds in regulating proliferation and differentiation of NSC provides the basis for the development of therapeutic strategies for NDD.

Studies have indicated that dietary flavonoids have beneficial therapeutic effects on NSC through the induction of differentiation, proliferation, and survival enhancement^{7,8}. Catechins, notably abundant in green tea and grape seeds, belong to the flavonoid family and include catechin hydrate (CH), epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate (EGCG)^{9,10}. Catechins have been demonstrated to have effects on stimulating neuronal differentiation and proliferation in NSCs^{8,11}. For example, EGCG facilitated neuronal differentiation in mouse cochlear NSCs via the activation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway¹¹ and protected against apoptosis and promoted NSC proliferation in damaged brain regions after traumatic brain injury in Wistar rats¹². Most studies have been focused on the role of EGCG and their effects on NSC fate determination *in vitro* and *in vivo*^{11,12}. On the other hand, CH showed neuroprotective effect on the rat model of cerebral ischemia injury¹⁰ and pretreatment of CH reduced neurodegeneration and memory impairment in the streptozotocin-induced Alzheimer's rat model¹³. In the present study, we aimed to explore the effects of CH on NSC fate determination. Our findings indicated that CH increased glial fibrillary acidic protein (GFAP) expression and induced morphological changes: producing thin and elongated branches in astrocytes. In addition, CH upregulated the transcripts of *leukemia inhibitory factor (lif)*, *bone morphogenic protein (bmp)2*, *fibroblast growth factor (fgf)2*, and induced phosphorylation of signal transducer and activator of transcription 3 (STAT3) and Suppressor of Mothers against Decapentaplegic (SMAD) 1/5/8.

Results

CH induces GFAP expression without enhancing astrocytogenesis during NSC differentiation

To examine the effects of CH on NSC fate control, we treated NSCs with CH at concentrations of 1, 5, 10 and 20 μ M or DMSO for 2 days in the presence of epidermal growth factor (EGF) and FGF2 to expand the number of NSCs or for 4 days without mitogens to differentiate NSCs. Then cells were prepped for Western blot analysis to determine the protein expression levels of GFAP (an astrocyte marker) and class III beta-tubulin (TUBB3) (a neuronal marker). As shown in Fig. 1A and B, during NSC proliferation, there were no significant differences in GFAP and TUBB3 protein expression between the CH-treated and control groups. However, in differentiated NSCs, the levels of GFAP protein expression were significantly increased in the CH-treated groups at 1, 5 and 10 μ M compared with DMSO-treated control, whereas TUBB3 protein levels showed no significant changes (Fig. 1C–F). We subsequently examined the mRNA levels of *gfap* and *tubb3* after 3 days of CH treatment during NSC differentiation. The *gfap* mRNA levels significantly increased in 10 μ M CH-treated cells (Fig. 1G), whereas *tubb3* mRNA levels did not show any significant changes (Fig. 1H). Since 10 μ M CH significantly induced both GFAP protein and mRNA levels during NSC differentiation, we proceeded to perform immunocytochemistry (ICC) using GFAP and TuJ1 (TUBB3 detecting) antibodies after 4 days of CH treatment. Then we analyzed the number of astrocytes and neurons. However, the ICC data revealed that the percentage of both GFAP- and TuJ1-positive cells was similar between the CH-treated and control groups (Fig. 2A–H). These data suggest that during NSC differentiation, treatment of 10 μ M CH induces both GFAP mRNA and protein expression although it does not increase astrocytogenesis.

CH does not induce radial glial cell differentiation

Next, we investigated whether CH treatment enhances the differentiation of radial glial cells, which are known to express GFAP during development¹⁴. Since radial glial cells are known to express brain lipid-binding protein (BLBP), Vimentin, and Nestin^{14–16}, we performed Western blot analysis to see the expression levels with or without growth factors. As shown in Fig. 3A and B, the protein levels of BLBP, Vimentin and Nestin remain unchanged in CH-treated groups compared with those in the control group. These findings suggest that the increasing GFAP expression upon CH treatment is not due to the induction of radial glial cell differentiation.

CH increases the phosphorylation of STAT3 and SMAD1/5/8 and transcript levels of *bmp2* and *lif*

Next, we explore the mechanism underlying the induction of GFAP expression by CH in differentiated NSCs. The activation of both STAT3 and SMADs has been known to form a STAT3-SMADs co-activating complex on the STAT3 binding site of GFAP promoter, triggering the induction of astrogliogenesis in NSCs^{2,17,18}. As shown in Fig. 4A–D, 4 days of CH treatment increased the phosphorylation of STAT3 and SMAD1/5/8. To explore whether the activation of STAT3 and SMADs phosphorylation is via the increase of astrocytogenic cytokines by CH, we measured the mRNA levels of *lif*, *interleukin-6 (il-6)*, *ciliary neurotrophic factor (cntf)*, and *bmp2* after 3 days of CH treatment in differentiated NSCs. As shown in Fig. 4E–H, the mRNA levels of *lif* and *bmp2* were significantly enhanced by 10 μ M CH. These findings suggest that CH activates the phosphorylation of STAT3 and SMAD1/5/8 and increases the expression of *lif* and *bmp2*.

CH induces thin and elongated primary astrocyte branches

Because CH treatment increased GFAP expression but not the number of astrocytes during NSC differentiation, we decided to observe the morphology of astrocytes under the microscope. In CH-treated NSCs, more numbers of thin and elongated branched GFAP-positive cells were found. Therefore, we then measured the length of

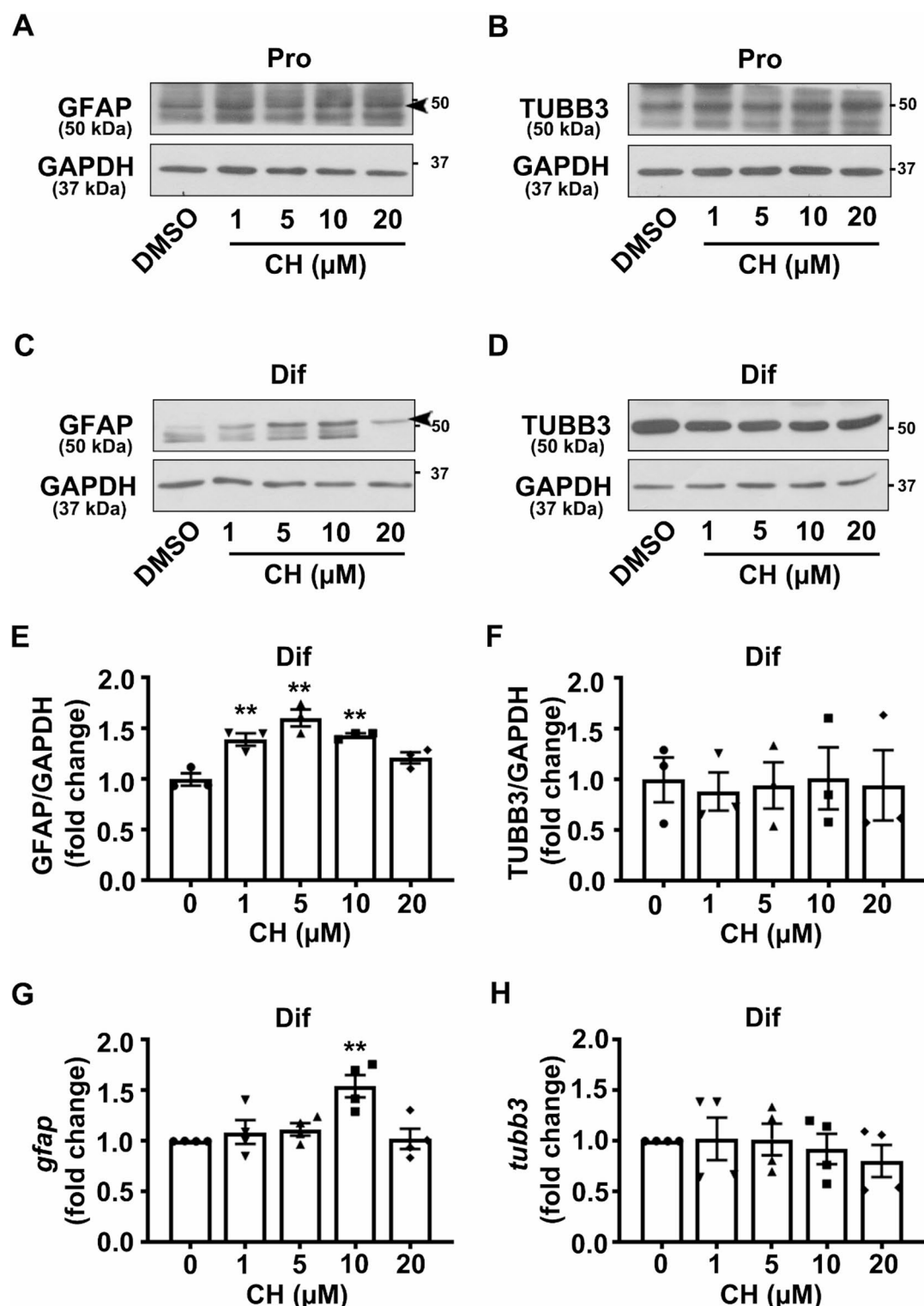


Fig. 1. CH significantly increased both protein and mRNA levels of GFAP in differentiated NSCs. Representative immunoblot images of protein expression in NSCs during proliferation (A, B) and differentiation (C, D). Densitometric analysis of protein expression during differentiation is shown in (E) GFAP, and (F) TUBB3. NSCs were treated with DMSO or CH (1,5,10 and 20 μ M) for 2 days with growth factors (Pro) or 4 days without growth factors (Dif), and cell lysates were subjected to Western blot analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are presented as mean $\pm$ SEM ($n = 3$). The mRNA expression levels of (G) *gfap* and (H) *tubb3* were analyzed by performing real-time reverse transcription polymerase chain reaction (qRT-PCR) after treatment with CH for 3 days in differentiated cells. Data are presented as mean $\pm$ SEM ($n = 4$). Statistical differences were assessed using one-way ANOVA and Dunnett's post-hoc test. (* $p < 0.05$, ** $p < 0.01$ compared with the control group). Uncropped images of Western blot are shown in Supplementary Fig. S1 and S2.

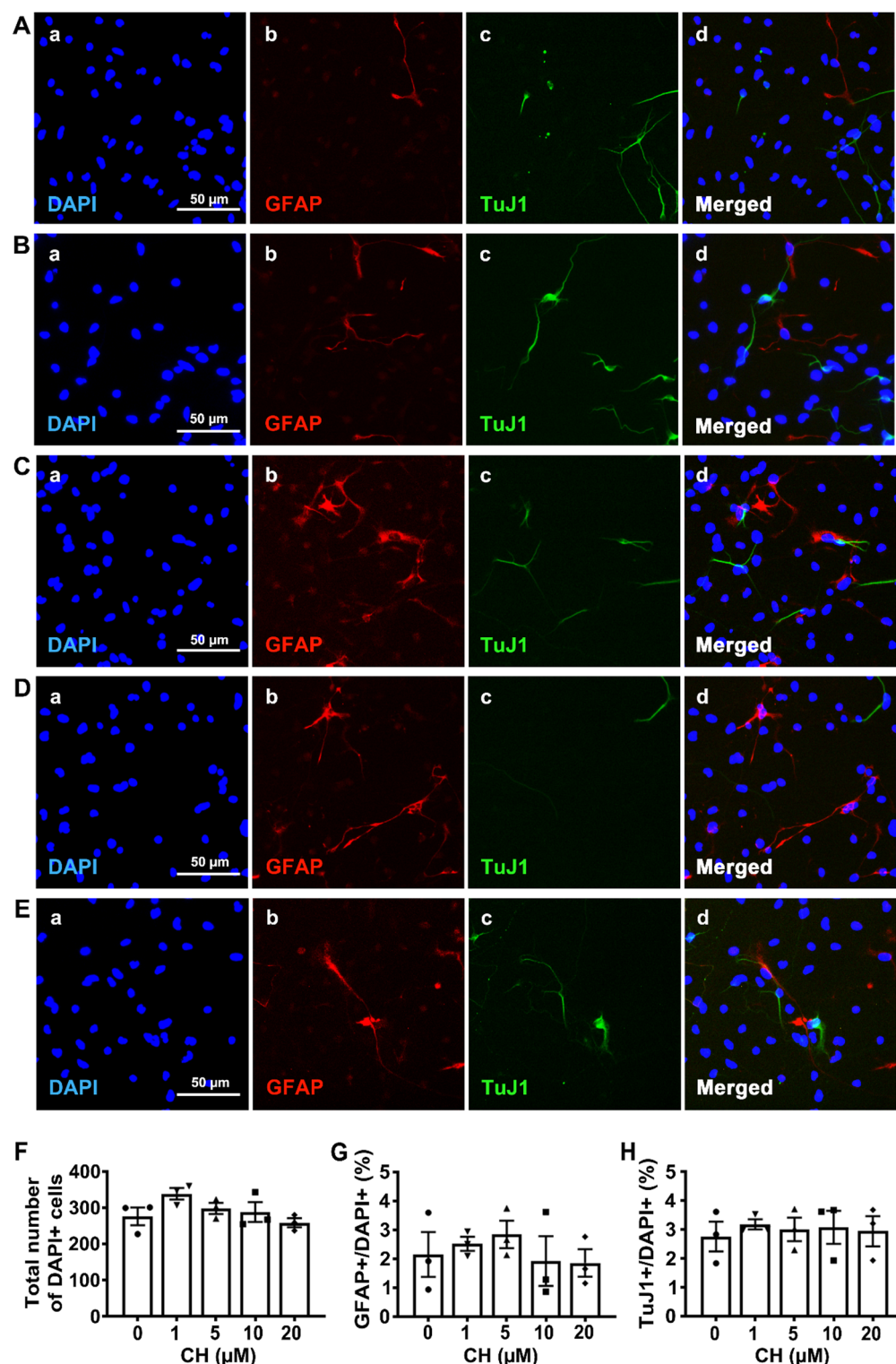


Fig. 2. The number of GFAP-positive astrocytes or TuJ1-positive neurons was not significantly changed in CH-treated groups compared with the control in differentiated cells. Representative immunofluorescence images of (A) DMSO (control), and CH treatment at different concentrations (B) 1 μM, (C) 5 μM, (D) 10 μM, and (E) 20 μM, [DAPI-positive nuclei (a, blue), GFAP-positive astrocytes (b, red), TuJ1-positive neurons (c, green), and merged images (d)]. Scale bar = 50 μm. Quantitative data of (F) DAPI-positive nuclei, (G) GFAP+, and (H) TuJ1-positive cells. NSCs were treated with CH or vehicle for 4 days without growth factors. Data are presented as mean ± SEM ($n = 3$). Statistical differences were assessed using one-way ANOVA and Dunnett's post-hoc test.

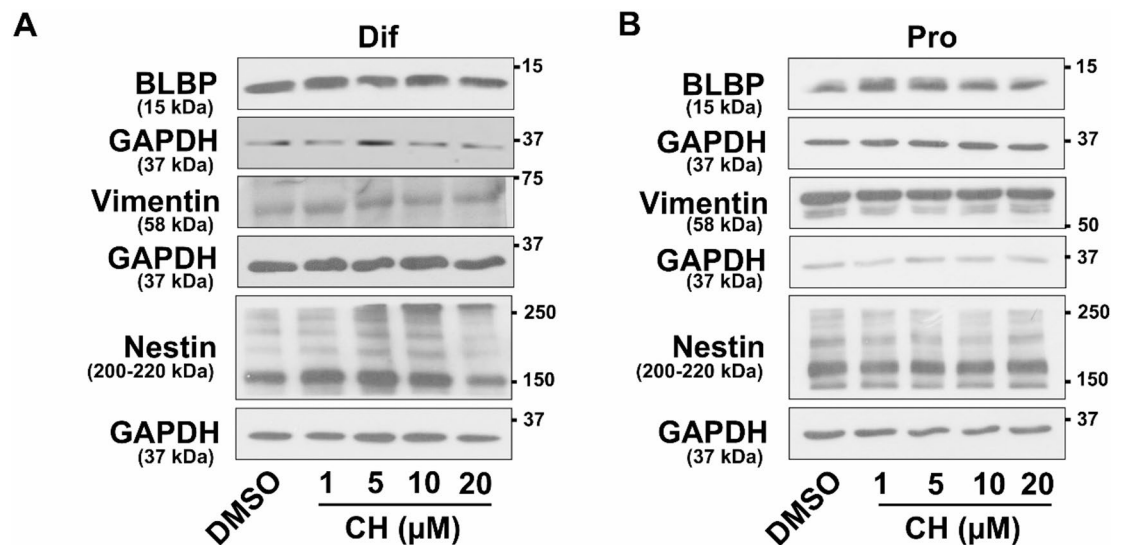


Fig. 3. The expression levels of BLBP, Vimentin, and Nestin were not significantly altered with CH treatment. Representative images of protein bands of BLBP and Vimentin and Nestin in NSCs after treatment with DMSO or CH (A) for 4 days without growth factors (Dif) and (B) for 2 days with mitogens (Pro). GAPDH was used as the loading control. Uncropped images of Western blot are shown in Supplementary Fig. S3, S4, and S5.

primary branches of astrocytes in 10 μ M CH or DMSO treated cells for 4 days. As shown in Fig. 5A and B, 10 μ M CH significantly enhanced the length of primary branches by up to 18% compared with that of DMSO. Because it is known that FGF signaling induces the formation of the branches of the astrocytes^{19,20}, we explored the mRNA expression levels of *fgf2* and *fgf8*. The data showed that *fgf2* transcript levels were significantly upregulated by 10 μ M or 20 μ M CH (Fig. 5C), whereas there was no difference in *fgf8* expression (Fig. 5D). These results suggest that CH increases elongation of astrocyte fibers and induces *fgf2* expression during NSC differentiation.

Discussion

NSCs hold the ability to self-renew and differentiate into diverse cell types including neurons, astrocytes, and oligodendrocytes^{1,2}. In this study, we have found that the dietary flavonoid CH leads to an upregulation of GFAP protein expression but not the number of astrocytes during NSC differentiation. Generally, different types of cells express distinct marker proteins such as GFAP, BLBP, Vimentin, and Nestin during developmental stage of the CNS^{14–16}. In particular, BLBP, Vimentin, and Nestin are found as major intermediate filament proteins in radial glia, NSCs, and immature astrocytes, whereas GFAP mainly holds this role in both immature and mature astrocytes^{14–16}. Moreover, after brain injury, astrocytes can be reactivated, leading to the re-expression of Vimentin and Nestin, along with GFAP overexpression^{15,21,22}. However, in our study, although CH upregulated GFAP expression, no upregulation of other glial markers such as BLBP, Vimentin, or Nestin was observed in differentiated cells. Consequently, it is reasonable to conclude that CH increased GFAP expression but did not contribute to the formation of radial glial cells or reactive astrocytes.

Furthermore, astrocytes hold a pivotal role as the most abundant glial cells, contributing to various aspects of CNS physiology and pathology, rendering them important therapeutic targets for numerous CNS diseases^{22–24}. Under normal physiological conditions, astrocytes uphold homeostasis through the release of antioxidants, neutralization of reactive oxygen species (ROS) and reactive nitrogen species, as well as supplying neurons with energy substrates^{23,25}. Moreover, when damaged, they become activated and degenerate neurons through the secretion of free radicals and pro-inflammatory cytokines^{23,26}. The protective and regulatory roles of catechins in astrocytes have already been documented^{27,28}. For example, catechin protects rat cortical astrocytes by inhibiting palmitic acid-induced ROS production²⁷. Moreover, epicatechin promotes the expression of antioxidant response element-mediated genes and elevates glutathione synthesis in astrocytes, but not neurons²⁸. Our study indicates that CH, apart from its protective function, enhances the expression of GFAP during NSC differentiation.

Interestingly, elongation of astrocyte processes was observed in CH-treated astrocytes. It has been reported that astrocytes respond differently to FGF2 and FGF8; FGF2-treated astrocytes produce long and thin fibrous branches, whereas FGF8-treated astrocytes produce flat and lamellipodial-like branches¹⁹. Finding the increased transcript levels of *fgf2* but not *fgf8* and showing thin and long processes similar to FGF2-induced astrocyte morphology, our results suggest that FGF2 may contribute to CH-induced morphological changes in differentiated NSC.

The classical IL-6 family has eight cytokine members: IL-6, IL-11, IL-27, oncostatin M, LIF, CNTF, cardiotrophin 1, and cardiotrophin-like cytokine factor 1²⁹. Members of IL-6 family: IL-6, LIF, and CNTF and those of BMP family cytokines: BMP2/4 are known as strong inducers of astrocyte differentiation of NSCs¹⁸. In the present study, we observed that CH induced phosphorylation of STAT3 and SMAD1/5/8 and upregulated the transcripts of *lif* and *bmp2* in differentiated NSC. Similarly, it has been previously reported that the complex

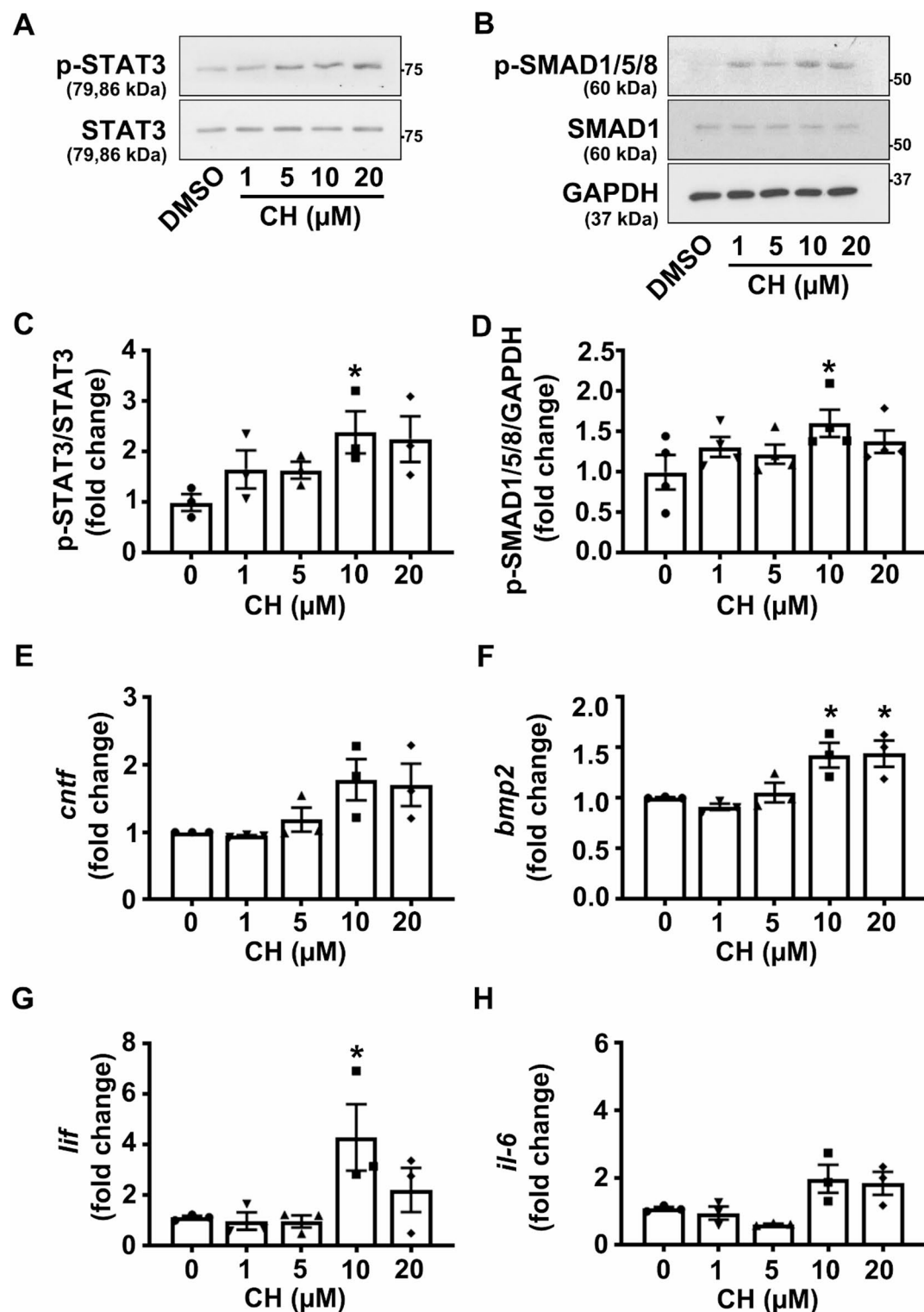


Fig. 4. CH increased phosphorylation of STAT3 and SMAD1/5/8 and enhanced mRNA levels of *lif* and *bmp2*. Representative images of protein bands of (A) p-STAT3 and STAT3, (B) p-SMAD1/5/8, SMAD1 and GAPDH, and (C,D) densitometric data. GAPDH was used as the loading control. Western blot analysis was performed after 4 days of treatment with CH during differentiation. The mRNA expression levels of (E) *cntf*, (F) *bmp2*, (G) *lif*, and (H) *il-6* were determined using qRT-PCR after treatment with CH for 3 days of differentiation. Data are presented as mean $\pm$ SEM ($n=3-4$). Statistical differences were assessed using one-way ANOVA and Dunnett's post-hoc test. (* $p<0.05$, ** $p<0.01$ compared with the control group). Uncropped images of Western blot are shown in Supplementary Fig. S6 and S7.

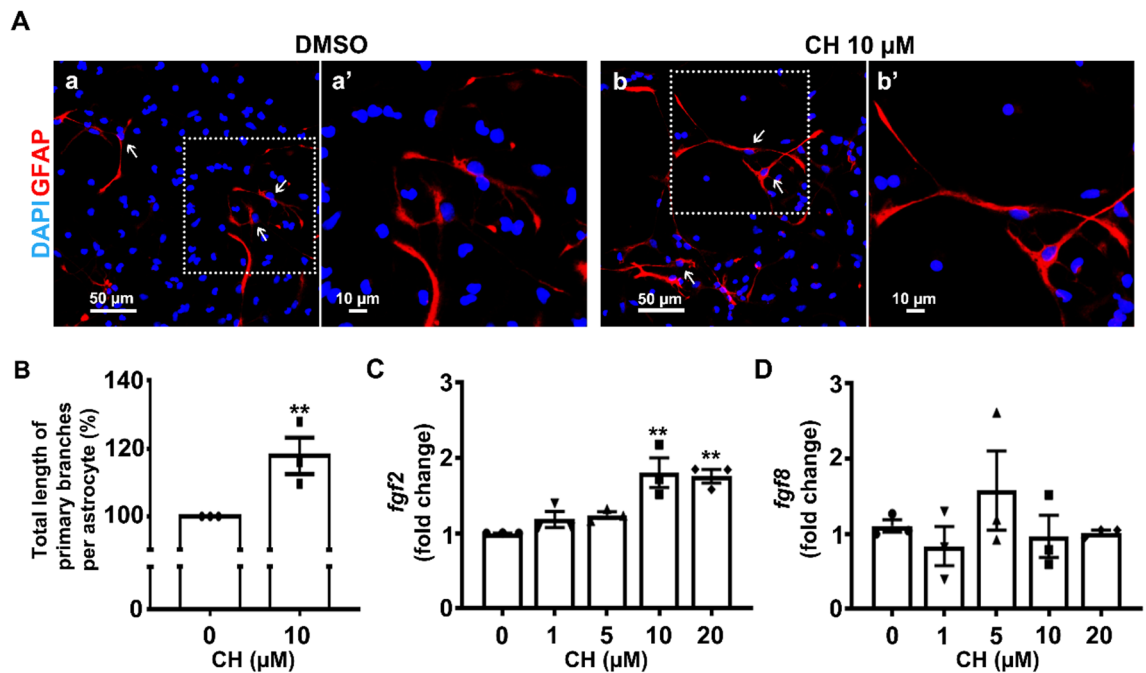


Fig. 5. CH treatment increased elongation of primary branches of astrocytes. **(A)** Representative immunofluorescence images of DMSO (a, a') and 10 μM CH (b, b'). Dotted boxed areas in (a) and (b) were enlarged in (a') and (b'). [DAPI-positive nuclei (blue), GFAP-positive astrocytes (red)]. Scale bar = 50 μm (in a and b) and 10 μm (in a' and b'). **(B)** Quantitative data shown in percentage of total length of primary branches per astrocyte. NSCs were treated with CH 10 μM or DMSO for 4 days without growth factors. **(C)** *fgf2* and **(D)** *fgf8* mRNA expression levels were analyzed by performing qRT-PCR after treatment of CH for 3 days without mitogen. Data are presented as mean ± SEM ($n = 3$). Statistical differences were assessed using one-way ANOVA and Dunnett's post-hoc test. (* $p < 0.05$, ** $p < 0.01$ compared with the control group).

of BMP-activated Smad1 and LIF-activated STAT3 are bridged by a transcriptional coactivator, p300 and synergistically activated GFAP promoter and resulted in astrocytic differentiation in neural progenitors³⁰. Moreover, recent studies reported that BMP2-pSMADs signaling is involved in the regulation of neural stem/precursor cells fate determination and is developmental stage dependent^{31,32}. BMP2-SMADs signaling induce astrocytogenesis by SMADs binding to Sox8 which is expressed during astrocytogenesis and, these complex bind to the target DNA with high affinity to induce astrocytic genes including *GFAP*³¹. In addition, LIF and BMP2 induced mouse embryonic SCs into cardiomyocytes in a synergistic manner through activation of STAT3, and mitogen-activated protein kinase signaling pathways³³. Moreover, several previous studies have shown that IL-6 family cytokines and STAT3 signaling pathways play an important role in the differentiation of various cell types^{18,34,35}. In bone marrow mesenchymal SCs, growth factors and IL-6 family cytokines including IL-6 and LIF-activated STAT3 induced osteogenic differentiation³⁴. In muscle SCs, LIF induced myogenic differentiation through Janus kinase 2/STAT3 signaling pathway³⁵. Taken together, these observations suggest that the fate of SC differentiation can be directed by LIF and BMP. Our result also suggests that CH induced *lif* and *bmp2* and activated STAT3 and SMAD which lead to increasing GFAP expression and the elongation of astrocyte branches in NSCs.

Conclusion

This study demonstrates that CH enhances GFAP expression through the phosphorylation of STAT3 and SMAD 1/5/8 via the induction of *lif* and *bmp2*. CH induces thin and elongated branches in differentiating astrocytes and this may be mediated by *fgf2* induction (Fig. 6). Our findings suggest that CH, beyond the neuroprotective effects of its antioxidant properties, has the potential to increase branching of astrocytes.

Materials and methods

NSC culture and drug treatment

All animal experiments were performed with the guidelines for the Care and Use of Laboratory Animals (The National Academies, 8th edition) and approved by the Animal Care of Chung-Ang University (authorization code: 13-0049, 2014-00032). All methods are reported in accordance with ARRIVE guidelines.

Sprague-Dawley rat (CrI: CD) (Orient Bio Inc., Seongnam-si, Gyeonggi-do, Republic of Korea) were used in this study. Embryonic stage 14 (E14) pregnant rats were euthanized using carbon dioxide (CO₂) inhalation followed by cervical dislocation, in accordance with institutional guidelines. Embryos were then collected, and cortices were dissected to isolate NSCs. Since rat embryos are considered insentient throughout pregnancy due to incomplete neurodevelopment (AVMA Guidelines for the Euthanasia of Animals, 2020), CO₂ exposure

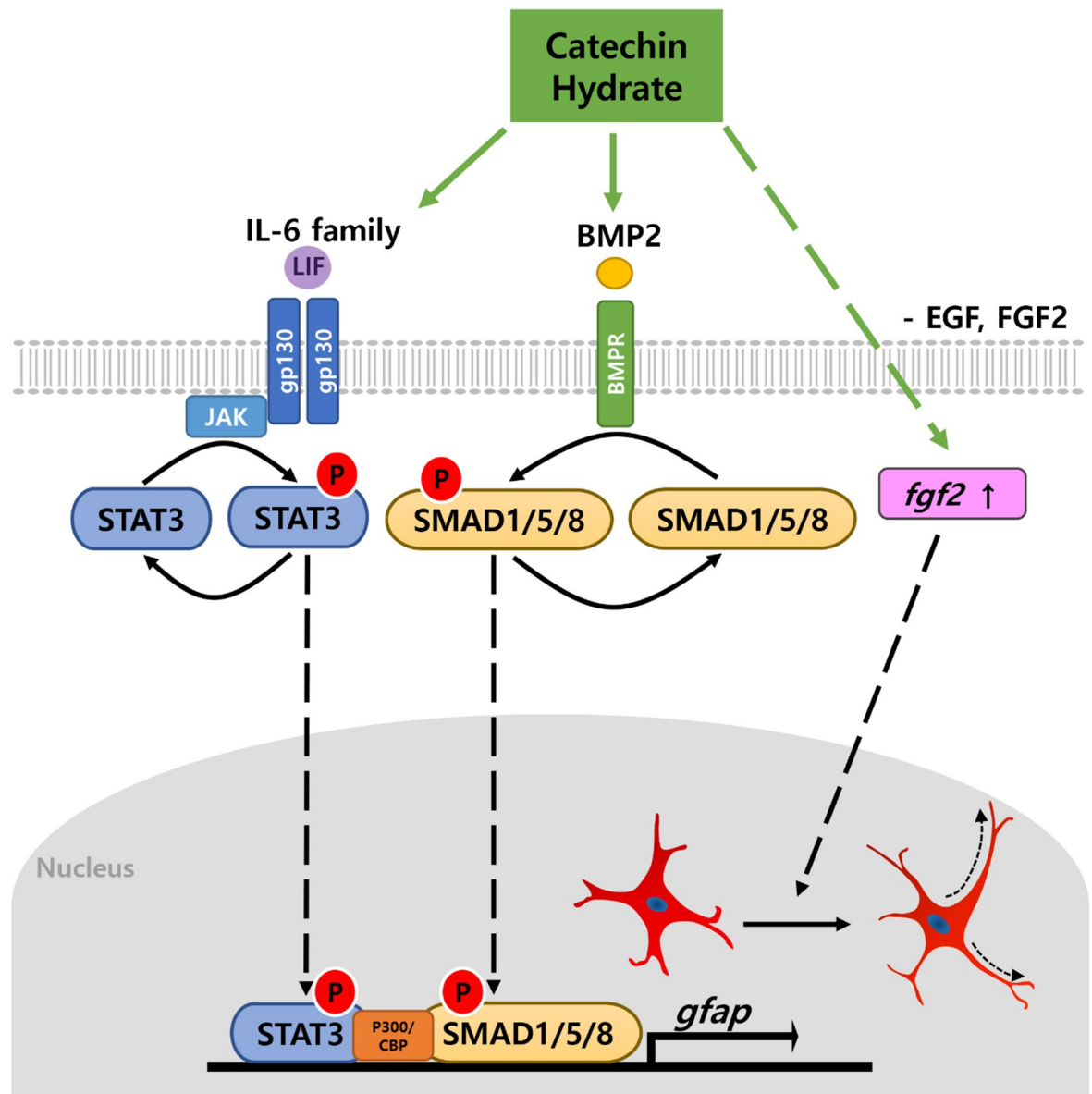


Fig. 6. Catechin hydrate induces GFAP expression and astrocyte branch elongation in rat NSCs. CH increased GFAP expression by enhancing transcript levels of *lif* and *bmp2* and by increasing phosphorylation of STAT3 and SMAD1/5/8. Moreover, CH increased *fgf2* mRNA levels and induced the formation of thin and elongated astrocyte branches.

was not applied to the embryos. As previously reported^{36–38}, NSCs were cultured in a flask and expanded into neurospheres using Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 supplemented with 1% (v/v) antibiotic-antimycotic, 2% (v/v) B27 (12500-062; 15240-062; 17504-044; all from Thermo Fisher Scientific, Waltham, MA, USA), 2 ng/mL of EGF, and 2 ng/mL of FGF2 (GF144; GF003AF; both from Merck Milipore, Burlington, MA, USA) at 37°C in 5% CO₂ for 7 days. The neurospheres were then dissociated using Accutase (SCR005; Chemicon, Temecula, CA, USA), and the cells were seeded onto plates coated with 0.01% poly-D-lysine (P0899; Sigma-Aldrich, MO, USA) and 10 µg/mL laminin (23017-015; Invitrogen, Carlsbad, CA, USA) and allowed to grow for 1 day. After 1 day of proliferation, the cells were cultured with or without growth factors to induce proliferation and differentiation. CH (C1251; ≥ 98% (HPLC)) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Cells were treated with CH at concentrations of 1, 5, 10, 20 µM or vehicle (0.1% dimethyl sulfoxide [DMSO]; 472301; Sigma-Aldrich, Saint Louis, MO, USA).

Western blot analysis

Cells were washed with phosphate-buffered saline (PBS) and lysed using lysis buffer (5 mM EDTA, 50 mM HEPES, 50 mM NaCl, 1% NP-40 Triton X-100 [all from Amresco, Solon, OH, USA], with phosphatase inhibitors [78420; Thermo Fisher Scientific, Waltham, MA, USA], 1 mM phenylmethylsulfonyl fluoride, 0.01 mg/mL each

of leupeptin and aprotinin [P7626; A1153; L9783; all from Sigma-Aldrich, Saint Louis, MO, USA]). Cell lysates were centrifuged at 14,000 rpm for 20 min to remove debris and then boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl of pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue [all from Amresco, Solon, OH, USA], 14.4 mM β -mercaptoethanol [M6250; Bio-Rad, Hercules, CA, USA], and distilled water) to denature the protein. Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Burlington, MA, USA). The membranes were then blocked using 5% non-fat dry milk or bovine serum albumin (82–100-6; Merck Millipore, Burlington, MA, USA) in 20 mM Tris-buffered saline containing 0.03%–0.1% Tween-20 (M147; Amresco, Solon, OH, USA) for 1 h and probed overnight at 4 °C with primary antibodies: GFAP (1:9000), TUBB3 (1:1000) (G9269; T5076; all from Sigma-Aldrich, Saint Louis, MO, USA), BLBP (1:9000; ab32423 Abcam, Cambridge, UK), Vimentin (1:400; V6630; Sigma-Aldrich, Saint Louis, MO, USA), Nestin (1:1000; MAB353; Merck Millipore, Burlington, MA, USA), STAT3 (1:2000), phospho-STAT3 (1:2000), SMAD1, phospho-SMAD1/5/8 (1:1000) (4904 S; 9145 S; 9743; 9511; all from Cell Signaling, Danvers, MA, USA), and GAPDH (1:2000; sc-32233; Santa Cruz, Dallas, TX, USA), followed by incubating with horseradish peroxidase-conjugated secondary antibodies: anti-mouse IgG (1:5000; ADI-SAB-100-J) or anti-rabbit IgG (1:5000; ADI-SAB-300-J; both from Santa Cruz, Dallas, TX, USA) for 1 h at room temperature. Protein bands were visualized using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Dallas, TX, USA) on a CP-BU New X-ray film (Agfa, Mortsel, Belgium). For the quantification, the expression levels of target proteins were normalized to appropriate controls: the level of phospho-STAT3 was normalized to that of total STAT3, while the expression levels of phospho-SMAD1/5/8, GFAP, and TUBB3 were normalized to those of GAPDH.

ICC, cell counting and quantification

Cells were fixed in 4% paraformaldehyde (SC-281692; USB Products, Cleveland, OH, USA) for 30 min, followed by two washes with PBS. Subsequently, the fixed cells were blocked with 5% normal goat serum (S26; Merck Millipore, Burlington, CA, USA) and 0.2% Triton X-100 (M143; Amersco, Solon, OH, USA) for 30 min. Cells were then incubated with primary antibodies against GFAP (rabbit IgG, 1:500, Z0334; Agilent, Santa Clara, CA, USA) and Tuj1 (TUBB3 antibody) (mouse IgG2b, 1:1000, T5076; Sigma-Aldrich, Saint Louis, MO, USA) for 90 min. After washing with PBS, the cells were probed with secondary antibodies conjugated to Cy3 (goat anti-rabbit IgG, 1:1000, 111–165-144; Jackson Immuno Research, West Grove, PA, USA) or Alexa Fluor 488 (goat anti-mouse IgG, 1:1000, A11001; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min. After washing, the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1:10,000 in PBS) (D952; Sigma-Aldrich, Saint Louis, MO, USA) for 5 min. Images were captured using Leica DM IL LED inverted fluorescence microscope (Leica Biosciences, Wetzlar, Germany) equipped with a 10 $\times$ objective lens. Imaging was performed via the camera port in photo (DOC) mode without additional eyepiece magnification. Three independent experiments were performed for ICC. In each batch, three wells/slides were used. Within each slide, we took photos from three random microscopic fields for imaging and quantification. In each field, we counted DAPI-positive nuclei and GFAP- or Tuj1-positive cells, then the percentage was calculated by dividing the number of GFAP+ or Tuj1+ cells to that of total DAPI+ nuclei within the same field. The percentage values from three fields were averaged to obtain a mean percentage value for that slide. Then, the mean value of each batch was calculated from averaging the mean values of the three slides. After obtaining all three mean values from 3 independent experiments, the data was expressed as percentages of GFAP+ or Tuj1+ cells relative to total DAPI+ nuclei.

Measurement and analysis of primary branches of astrocytes

To measure primary astrocyte branches, we analyzed the average numbers (240–280) in each group (10 μ M CH and DMSO). The process originating from the cell body was defined as the primary process, and its length was measured manually with the freehand tool using ImageJ software (Rawak Software Inc, Stuttgart, Germany). We individually measured the lengths of the primary processes in each astrocyte and combined them to determine the total length. The resulting total length is divided by the total number of astrocytes to calculate the average length per astrocyte. The value of the CH-treated group was divided by that of the DMSO-treated group and then multiplied by 100 to obtain the percent value.

qRT-PCR

Total RNA was isolated using the TRIzol reagent (15596026; Invitrogen, Carlsbad, CA, USA). First-strand complementary DNA (cDNA) was generated from 1 μ g of total RNA using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). RT-PCR was performed using the iQ SYBR Green Supermix (#170-8882AP; Bio-Rad, Hercules, CA, USA). The following primer sets were used to amplify cDNA: *gfap*, *acgcgctctgagagagattc* (forward) and *agcaacgtctgtgaggtctg* (reverse); *tubb3*, *agccctctacgacatctgct* (forward) and *attgagctgaccagggaatc* (reverse); *lif*, *aagttggctgagctgtatcg* (forward) and *gagtttgatctggaggctca* (reverse); *il-6*, *tgttctcaggagagattctgg* (forward) and *tttgaagcatccatcattt* (reverse); *bmp2*, *aacaaatgcaggaagctttg* (forward) and *cctgggtgtccaatagtctgg* (reverse); *cnf*, *gcaaggaagattctgtcaga* (forward) and *tcattctcactccaacgatca* (reverse); *fgf2*, *tggctatgaaggaagatgga* (forward) and *tcagtgtccacataccaactg* (reverse); *fgf8*, *accggtctgtacatctgcat* (forward) and *gtgtttctccagcagcatct* (reverse); and *gapdh*, *agttcaacggcagcagctcaag* (forward) and *gtgggtgaagacgcagctaga* (reverse). The reaction conditions were as follows: initial activation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s. The housekeeping gene *gapdh* was used as an internal control.

Statistical analysis

Quantitative data are presented as the mean $\pm$ standard error of the mean (SEM) from three to four independent experiments. Statistical differences were assessed using one-way ANOVA and Dunnett's post-hoc test (* $p < 0.05$, ** $p < 0.01$, compared with the control group) utilizing GraphPad Prism 8 (GraphPad, San Diego, CA, USA).

Data availability

Data supporting the results of this study are available from the corresponding author upon reasonable request.

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Author contributions

HWS: Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing – original draft. H-JK: Conceptualization, Validation, Writing and Editing the final manuscript, Reviewing and Editing, Visualization, Supervision, Project administration, and Funding acquisition.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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