



# Hippocalcin Is Required for Astrocytic Differentiation through Activation of Stat3 in Hippocampal Neural Precursor Cells

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Hippocalcin (Hpca) is a neuronal calcium sensor protein expressed in the mammalian brain. However, its function in neural stem/precursor cells has not yet been studied. Here, we clarify the function of Hpca in astrocytic differentiation in hippocampal neural precursor cells (HNPCs). When we overexpressed Hpca in HNPCs in the presence or absence of bFGF, expression levels of nerve-growth factors such as neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and brain-derived neurotrophic factor (BDNF), together with the proneural basic helix loop helix (bHLH) transcription factors NeuroD and neurogenin 1 (Ngn1), increased significantly. In addition, there was an increase in the number of cells expressing glial fibrillary acidic protein (GFAP), an astrocyte marker, and in branch outgrowth, indicating astrocytic differentiation of the HNPCs. Downregulation of Hpca by transfection with Hpca siRNA reduced expression of NT-3, NT-4/5, BDNF, NeuroD, and Ngn1 as well as levels of GFAP protein. Furthermore, overexpression of Hpca increased the phosphorylation of STAT3 (Ser727), and this effect was abolished by treatment with a STAT3 inhibitor (S3I-201), suggesting that STAT3 (Ser727) activation is involved in Hpca-mediated astrocytic differentiation. As expected, treatment with Stat3 siRNA or STAT3 inhibitor caused a complete inhibition of astrogliogenesis induced by Hpca overexpression. Taken together, this is the first report to show that Hpca, acting through Stat3, has an important role in the expression of neurotrophins and proneural bHLH transcription factors, and that it is an essential regulator of astrocytic differentiation and branch outgrowth in HNPCs.

**Keywords:** hippocalcin (Hpca), hippocampal neural precursor cells, astrocyte, differentiation, signal transducer and activation of transcription 3 (STAT3)

## INTRODUCTION

Neurons, astrocytes, and oligodendrocytes are generated from precursor cells in the neuroepithelium during development of the central nervous system (CNS) (McKay, 1997; Gage, 2000; Anderson, 2001; Merkle and Alvarez-Buylla, 2006). In the mouse cerebral cortex, neurogenesis commences around embryonic day (E) 11, peaks at around E15, and finishes around

birth. While cortical astrocytes are first apparent at E16 and oligodendrocytes are present around birth, the vast majority of both cell types are produced during early neonatal development (McConnell, 1995; Qian et al., 2000). Although the separate timing of neurogenesis and gliogenesis in the CNS has been known for many years, several key questions remain to be addressed regarding the processes by which precursors differentiate into the distinct cell types.

Hippocalcin (Hpca) is a calcium-binding protein mostly found in pyramidal cells of the hippocampus (Saitoh et al., 1993; Palmer et al., 2005; Amici et al., 2009). It belongs to the family of EF-hand-containing neuronal calcium sensor (NCS) proteins that possess a  $\text{Ca}^{2+}$ /myristoyl switch allowing translocation to membranes in response to increased cytosolic  $\text{Ca}^{2+}$  concentration (Kobayashi et al., 1993; Braunewell and Gundelfinger, 1999; O'Callaghan et al., 2002; O'Callaghan et al., 2003; Aravind et al., 2008). In neuronal cells, Hpca is localized in the cytoplasm and plasma membrane of cell bodies and dendrites (Saitoh et al., 1993). We previously reported that it is involved in the PKC-independent  $\text{Ca}^{2+}$ -mediated PLD activation pathway (Hyun et al., 2000) and plays a key role in basic fibroblast growth factor (bFGF)-induced expression of NeuroD, leading to neurite outgrowth in H19-7 cells (Oh et al., 2008). However, the precise mechanism by which Hpca affects neurogenic or astrogenic potential has not been studied.

The neurotrophins comprise a family of four structurally related proteins; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) that exert their effects through high-affinity tyrosine kinase receptors (Trk receptors) (McAllister et al., 1999; Huang and Reichardt, 2001; Chao, 2003). Both the neurotrophins and their receptors are most highly expressed in the developing nervous system during active neuronal growth and differentiation (McAllister et al., 1999). In some cell lines, the relative levels of BDNF and NT-3 may reflect the reciprocal expression of these genes in the developing hippocampus or the differential expression observed for adult hippocampal regions (Ernfors et al., 1990; Maisonpierre et al., 1990). Although multiple effects of neurotrophins in the nervous system have been elucidated, their relevance to Hpca-mediated differentiation remains largely undefined.

Signal transducer and activation of transcription 3 (STAT3) is a key intermediary in signaling by many cytokines and growth factor receptors; it is activated by phosphorylation at Tyr705, thereby inducing dimerization, nuclear translocation and DNA binding (Henze et al., 1996; Mitchell and John, 2005). STAT3 activation is also regulated by phosphorylation at Ser727 via the MAPK or mTOR pathway (Park et al., 2010). STAT3 plays an important role in the differentiation of neurons in response to neurotrophic factors and growth factors (Yu et al., 2009). During brain development, activated STAT3 in conjunction with other cofactors induces expression of glial fibrillary acidic protein (GFAP) in neural precursor cells (Bonni et al., 1997; Nakashima et al., 1999; Takizawa et al., 2001; Wang et al., 2015). In addition, STAT3 signaling

is up-regulated in certain neurodegenerative diseases; for example, spinal cord microglia and reactive astrocytes have increased levels of phosphorylated STAT3 (Shibata et al., 2009). Thus STAT3 activation could be critical for astrocytic differentiation in hippocampal neural precursor cells (HNPCs).

In this study, we found that Hpca expression increased during the differentiation of HNPCs and that overexpression of Hpca stimulated astrocytic differentiation. Overexpression of Hpca also increased the expression of proneural bHLH transcription factors and neurotrophins. In contrast, Hpca deficiency decreased GFAP expression, resulting in defects in astroglialogenesis with decreased expression of neurotrophins, reduced numbers of GFAP-positive astrocytes and lower branch lengths. Furthermore, we found that Hpca specifically promoted astrocytic differentiation by activating STAT3 (Ser727) in HNPCs.

## MATERIALS AND METHODS

### Materials

Dulbecco's modified Eagle medium/Ham's nutrient mixture F-12 (DMEM/F-12 1:1 mixture), penicillin/streptomycin solution, NB (neurobasal medium), human insulin, and B27 were purchased from Gibco (Grand Island, NY, USA), and bFGF was from R&D Systems (Minneapolis, MN, USA). S3I-201 was obtained from Merck Calbiochem/EMD Biosciences (Darmstadt, Germany). Antibodies used were as follows: NT-3, NT-4/5, BDNF, Neurogenin 1,  $\beta$ -actin, and GAPDH were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); p-STAT3 (Ser 727) and STAT3 were from Cell Signaling Technology (Beverly, MA, USA); Hpca and NeuroD were from Abcam (Cambridge, UK); Myc (Bethyl Laboratories, Montgomery, TX, USA); GFAP (Dako, Glostrup, Denmark); Alexa Fluor<sup>®</sup> 488 secondary antibody goat anti-mouse IgG, Alexa Fluor<sup>®</sup> 594 secondary antibody goat anti-rabbit IgG, and GFP monoclonal antibody were from Life Technologies (Paisley, UK). All other chemicals were of analytical grade.

### Primary Culture of Hippocampal Neural Precursor Cells

Pregnant Sprague–Dawley (SD) rats were obtained from Orient Bio Inc. (Seoul, Korea). Briefly, embryonic hippocampi were mechanically dissected from E16.5 rat embryos in chilled Hank's balanced salt solution (Gibco), seeded at  $2 \times 10^5$  cells on 10-cm culture dishes (Nunc A/S, Roskilde, Denmark) precoated with 15  $\mu\text{g}/\text{ml}$  poly-L-ornithine (Sigma-Aldrich, St Louis, Mo, USA) and 1  $\mu\text{g}/\text{ml}$  fibronectin (Invitrogen, Carlsbad, CA, USA), then cultured for 4 days in serum-free N2 medium supplemented with 20 ng/ml bFGF (R&D Systems). The medium was changed every other day, while the bFGF was supplemented every day in order to expand the population of proliferative precursors. For high density cultures, the cells were plated at  $4 \times 10^4$  cells per well on coated 24-well plate,  $6 \times 10^5$  cells per well on coated 6-well or  $1 \times 10^6$  cells on coated 6-cm culture dishes. The plated cells were induced to additional proliferation in

N2+bFGF up to 70–80% cell confluence, before induction of differentiation.

## Immunofluorescence Staining

Cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 20 min followed by washing with 0.1% BSA in PBS three times at room temperature. They blocked with 10% normal goat serum in 0.1% BSA in PBS containing 0.3% Triton X-100 for 1 h at room temperature. Next, the cells were immunostained with following primary antibodies; 1:400 rabbit polyclonal anti-GFAP (Dako), and 1:400 mouse monoclonal anti-GFP (Life Technologies) at 4°C. Cells were then labeled with 1:2000 Alexa Fluor® 488 secondary antibody goat anti-mouse IgG (Life Technologies), and 1:2000 Alexa Fluor® 594 secondary antibody goat anti-rabbit IgG (Life Technologies) for 1 h, before mounting in Vectashield (Vector Laboratories, Burlingame, CA, USA) with 4, 6-diamidino-2-phenylindole (DAPI) mounting medium. Immunoreactive cells were analyzed under an epifluorescence microscope (Nikon Instruments, Melville, NY, USA) at magnifications varying from  $\times 200$  to  $\times 400$ .

## Transient Transfection of HNPCs

Transfection of HNPCs was carried with the Amaxa Nucleofector® Kit V (Amaxa Biosystems, Köln, Germany) according to the manufacturer's optimized protocol. In brief, HNPCs were dissociated using 0.05% trypsin for 5 min followed by the addition of 10% FBS in N2 medium. The resuspended cells were counted using a hemocytometer and  $5 \times 10^6$  cells aliquots were placed in separate tubes (1 tube per nucleofection). The cells were centrifuged at 1000 rpm for 5 min and resuspended in 100  $\mu$ l of pre-warmed Amaxa Nucleofector Solution. Each 100  $\mu$ l of cell suspension was mixed with either 10  $\mu$ g each of pMSCV-IRES-EGFP or pMSCV-Hpca-myc-IRES-EGFP. These DNA-cell suspensions were immediately transferred to Amaxa certified cuvettes and electroporated using the Nucleofector program G-13. The samples were examined by RT-PCR and western blot [see below].

## RNA Interference

For Hpca or Stat3 silencing experiments, Hpca siRNA (ON-TARGET plus SMART pool), and negative control siRNA (ON-TARGET plus non-targeting pool) were purchased from Dharmacon (Lafayette, CO, USA). Stat3 siRNA (5'-CUGUCUUUAGGCUGAUCAU-3') was purchased from Bioneer (Daejeon, Korea). Transient siRNA transfections were performed in 6-well plates; cells were transfected with Lipofectamine RNAiMAX (Invitrogen) and 100 nM siRNA according to the manufacturer's protocol (Invitrogen); and cells were harvested 72 h later.

## Virus Construction and Transduction

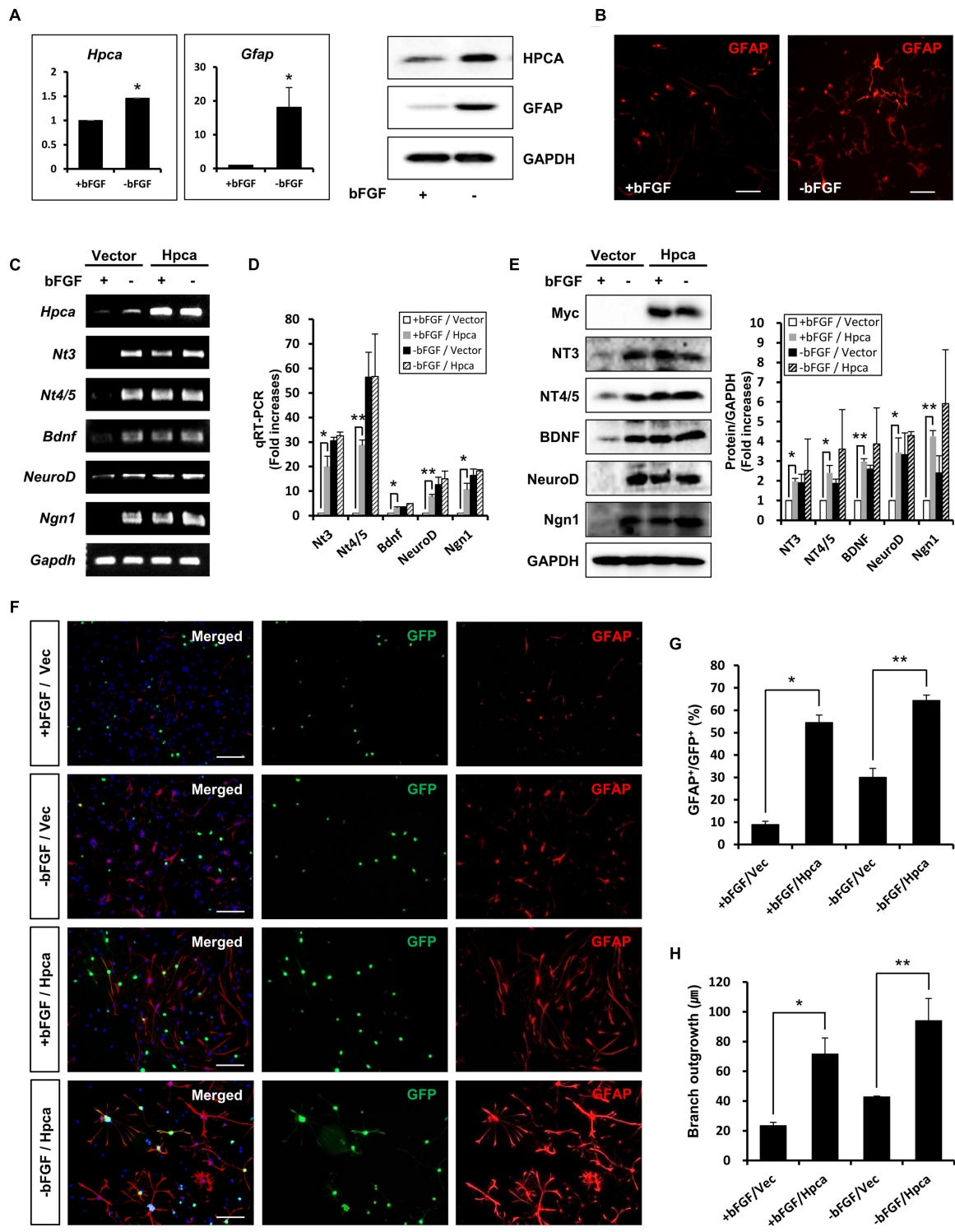
Hippocalcin cDNA was cloned into a retrovirus vector containing IRES-EGFP (obtained from Dr. Hee Yong Chung, Department of Biomedical Science, Graduate School of Biomedical Science and Engineering, Hanyang University, Seoul, Republic of Korea). Virus particles were produced by transfecting the retrovirus packaging cell line 293pgp with the vector using Lipofectamine (Invitrogen), and supernatants containing viral particles were harvested after incubation for 48 h. For virus transduction, prepared HNPCs were incubated with a viral suspension ( $4 \times 10^6$  particles/ml) containing polybrene (1  $\mu$ g/ml; Sigma-Aldrich) for 4 h, followed by transfer to bFGF-supplemented N2 medium.

## Real-Time PCR and RT (Reverse Transcription)-PCR

cDNA was prepared from total mRNA extracted from cells with TRIzol® reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA); 1  $\mu$ g samples of RNA was reverse-transcribed using Superscript II and random primers (Invitrogen). The resulting cDNA was amplified by PCR using the primers listed in **Table 1**. PCR products were analyzed on 1.5% agarose gels and stained with loading star (Dyne Bio, Seoul, Korea). For real-time PCR, 5  $\mu$ l of RT reaction product was amplified in triplicate in a final volume of 20  $\mu$ l iQ™ SYBR® Green supermix (Bio-Rad, CA,

**TABLE 1 | Primers used for RT-PCR.**

Gene symbol	Primer sequences (5'→3')	Annealing Temp (°C)	Cycles	PCR product size (bp)
Hippocalcin (Hpca)	ATGGGCAAGCAGAATAGCAAG TCAGAACTGGGAAGCGCTGCT	56	30	582
Nt3	GGTCAGAATTCCAGCCGATGA GGCACACACAGGAAGTGTC	55	32	515
Nt4/5	CCCTGCGTCAGTACTTCTTCGAGAC CTGGACGTCAGGCACGGCCTGTTC	55	32	249
Bdnf	GTGACAGTATTAGCGAGTGGG GGGTAGTTCGGCATTGC	56	28	213
NeuroD	CTCAGTTCTCAGGACGAGGA TAGTTCTTGCCAAGCGCAG	58	32	368
Ngn1	ATGCCTGCCCTTTGAGAC TGCATACGGTTGCGCTCGC	55	32	320
Gapdh	GGCATTGCTCTCAATGACAA AGGGCCTCTCTTGCTCTC	60	25	165



**FIGURE 1 | Effect of Hippocalcin (Hpca) on GFAP expression during differentiation of HNPCs. (A,B)** HNPCs were induced to differentiate by withdrawal of bFGF. **(A)** After 48 h, mRNA levels of Hpca and Gfap were determined by real-time PCR. \* $p < 0.001$  compared with the +bFGF control. 20 μg aliquots of protein were analyzed by western blotting with anti-hippocalcin, anti-GFAP, and anti-GAPDH. **(B)** The cells were stained with anti-GFAP (red). Bar, 100 μm. **(C–E)** Cells were transfected with pMSCV-IRES-EGFP or pMSCV-Hpca-myc-IRES-EGFP for 48 h and then incubated for 24 h after removal of bFGF. The mRNA levels of neurotrophins and proneural bHLH transcription factors were analyzed by RT-PCR **(C)** and real-time PCR **(D)**. Data are means ± SD of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$  compared with the +bFGF/vector control. **(E)** Proteins were analyzed by western blotting with anti-Myc, anti-NT-3, anti-NT-4/5, anti-BDNF, (Continued)

**FIGURE 1 | Continued**

anti-NeuroD, anti-Neurogenin 1, and anti-GAPDH. Graph shows mean densities as fold increases in three independent experiments (mean  $\pm$  SD). Band intensities were quantified with Quantity Ones software. \* $p < 0.05$ ; \*\* $p < 0.01$  compared with the +bFGF/vector control. **(F)** HNPCs were transduced with EGFP vector or Hpc-myc-EGFP for 48 h and induced to differentiate by withdrawal of bFGF. After differentiation for 72 h, fixed cells were immunostained with anti-GFP (green) and anti-GFAP (red). GFP-stained cells fluoresce green, and GFAP-stained cells fluoresce red, while cells stained with both GFP and GFAP fluoresce yellow. Bar, 100  $\mu$ m. **(G)** Graph shows the percentages of GFAP-positive cells. GFAP-positive cells among GFP-positive cells were counted under a fluorescence microscopy. The proportions of GFAP-positive cells were determined in randomly selected areas from five cultures (means  $\pm$  SD). \* $p < 0.001$  compared with the +bFGF/vector control. \*\* $p < 0.001$  compared with the -bFGF/vector control. **(H)** Branch lengths were measured in random areas from five cultures (means  $\pm$  SD). \* $p < 0.01$  compared with the +bFGF/vector control. \*\* $p < 0.01$  compared with the -bFGF/vector control.

USA). The primer sequences for real-time PCR were the same as for RT-PCR.

### Western Blot Analysis

Cells were lysed in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM PMSF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>. Protein samples (20–30  $\mu$ g) were loaded onto SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, UK) after electrophoresis. After blocking with 5% non-fat dried milk for 1 h, membranes were incubated with primary antibodies against Hpc anti-rabbit (1:500; Abcam), Myc anti-rabbit (1:2000; Bethyl Laboratories), NT-3 anti-rabbit (1:200; Santa Cruz Biotechnology), NT-4/5 anti-rabbit (1:200; Santa Cruz Biotechnology), BDNF anti-rabbit (1:250; Santa Cruz Biotechnology), NeuroD anti-rabbit (1:500; Abcam), Neurogenin 1 anti-goat (1:200; Santa Cruz Biotechnology), phospho-STAT3 anti-rabbit (1:1000; Cell Signaling Technology), STAT3 anti-mouse (1:1000; Cell Signaling Technology),  $\beta$ -actin anti-mouse (1:1000; Santa Cruz Biotechnology), GAPDH anti-rabbit (1:1000; Santa Cruz Biotechnology), GFAP anti-mouse (1:1000; Cell Signaling Technology) followed by HRP-conjugated secondary antibody (1:2000; New England Biolabs, Beverly, MA, USA), and specific bands were detected by ECL (Thermo Scientific, Rockford, IL, USA). Bands were quantified using ImageJ (v1.46r, National Institutes of Health, Bethesda, MA, USA).

### Cell Counting and Measurement of Branch Outgrowth

Cell counting was performed in microscopic field, using an eyepiece grid at a final magnification of 200 $\times$  or 400 $\times$ . Immunostained and DAPI-stained cells were counted in 5–10 random areas of each culture coverslip. All values were confirmed in at least five independent cultures. Photos of the cells were taken with an epifluorescence microscope (Nikon Instruments), and morphological characteristics were

quantitated using Image J software (NIH<sup>1</sup>). The length of branches was defined as the distance from the cell body (proximal) to the tip of the longest branch (distal). For each graph, data on branch length were generated from at least five independent sets of astrocytes (in each condition) and greater than 100 cells were counted for each condition in each experiment.

### Statistical Analysis

Quantitative data are expressed as means  $\pm$  SD of three to five independent experiments. The statistical significance of differences between groups was analyzed using Student's *t*-test and was considered significant at  $p < 0.05$ .

## RESULTS

### Hpc is Required for Glial Fibrillary Acidic Protein (GFAP) Expression during Differentiation of HNPCs

Dissected and mechanically dissociated cells from hippocampi of E16.5 rat embryos were used to isolate NPCs. For growth of these cells, fresh bFGF (20 ng/ml) was present to prevent differentiation and promote proliferation. To investigate the role of Hpc in HNPCs differentiation, bFGF was removed for 48 h. We initially performed real-time PCR analyses and western blotting to determine the mRNA expression and protein level of Hpc. As shown in **Figure 1A**, Hpc and Gfap expression increased in differentiating cultures. When the cells were immunostained with GFAP, we also observed significantly increased GFAP positive cells during differentiation (**Figure 1B**). Increased levels of nerve-growth factors such as NT-3, NT-4/5, and BDNF, together with the proneural bHLH transcription factors neuroD and Ngn1 have been detected in the developing and adult hippocampus and they have major roles in development and maintenance of the hippocampal formation (Ip et al., 1993). Since these factors are closely associated with neural precursor cell differentiation, they can be used as markers of this process (Markus et al., 2002). To examine the effect of Hpc on differentiation, either pMSCV-IRES-EGFP or pMSCV-Hpc-myc-IRES-EGFP was transfected into the HNPCs for 2 days, and then cells were induced differentiation for 1 day. We showed that mRNA levels (**Figures 1C,D**) and protein expression (**Figure 1E**) of NT-3, NT-4/5, BDNF, NeuroD, and Ngn1 were significantly increased by Hpc overexpression in the presence of bFGF compared with vector control in the presence of bFGF. HNPCs have been considered as the primary progenitor cells for the neuronal and glial cell lineages during development (Rietze et al., 2001). Therefore, we examined the role of Hpc in expression of neuronal and glial markers during differentiation. HNPCs were transduced with either pMSCV-IRES-EGFP or pMSCV-Hpc-myc-IRES-EGFP for 2 days and allowed to differentiate for 3 days. We measured the percentage of GFAP-positive cells and Tuj1-positive cells under a fluorescence microscope.

<sup>1</sup><http://rsb.info.nih.gov/ij/>

As shown in **Figures 1E,G**, *Hpca* overexpression resulted in markedly enhanced GFAP-positive astrocytes compared with vector control in the presence or absence of bFGF in HNPCs; at the same time Tuj1-positive neurons were not altered (data not shown). Moreover, *Hpca* overexpression significantly increased the length of branches compared with vector control under the either with bFGF or withdrawal bFGF (**Figures 1F,H**). We also found that overexpression of *Hpca* increased protein levels of GFAP compared with vector control in the presence or absence of bFGF in HNPCs (Supplementary Figure 1). These findings collectively imply that *Hpca*, which regulates the expression of neurotrophins and proneural bHLH transcription factors, promotes astrocytic differentiation of HNPCs.

## Knockdown of *Hpca* Suppresses the Astrocytic Differentiation of HNPCs

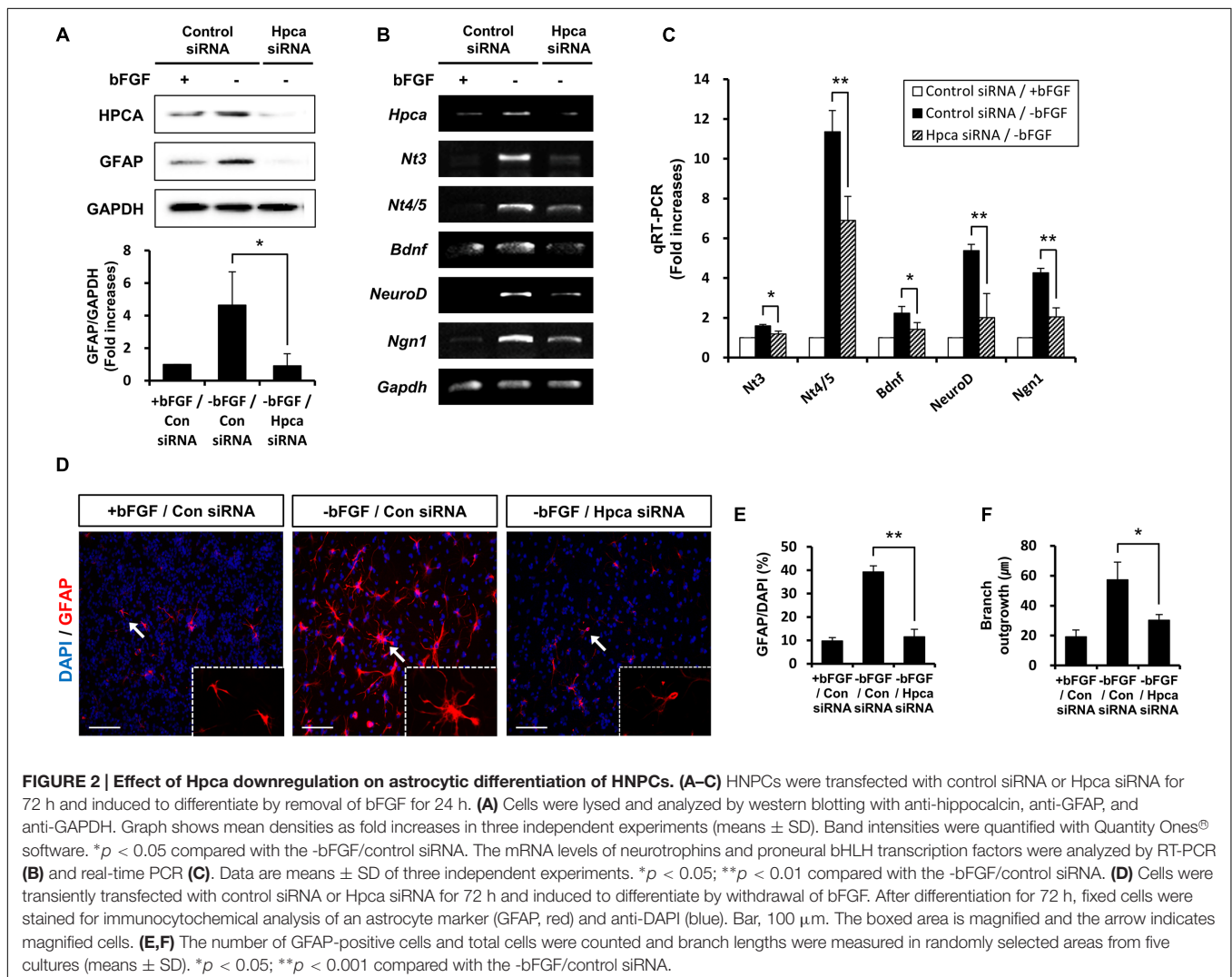
To confirm the role of *Hpca* in the expression of GFAP, we examined the effects of *Hpca* knockdown. 3 days after transfection of HNPCs with *Hpca* siRNA, the cells were allowed to differentiate for 1 day, and *Hpca* expression was examined

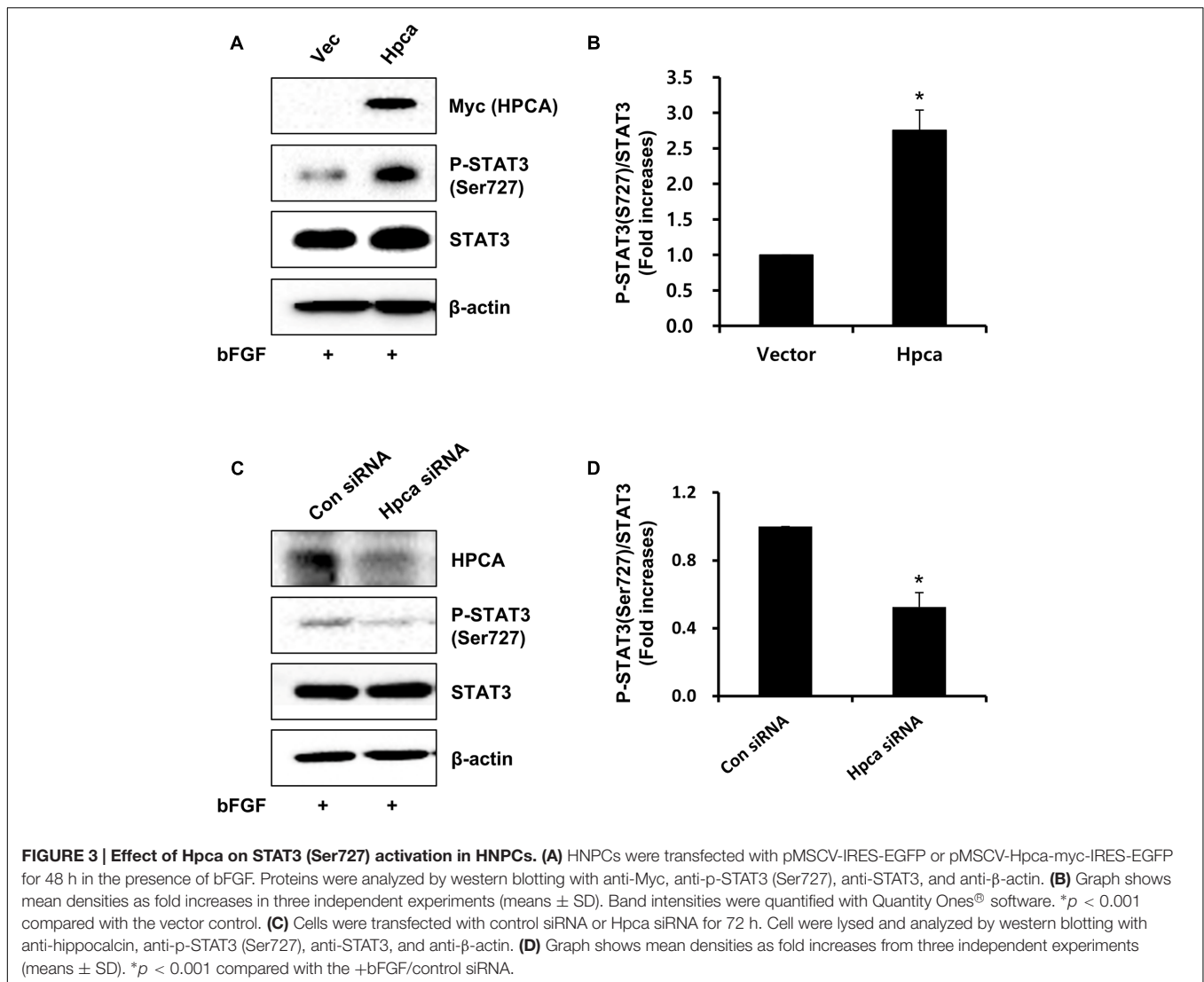
by western blotting. As shown in **Figure 2A**, *Hpca* siRNA markedly decreased expression of GFAP. We also found that knockdown of *Hpca* decreased expression of NT-3, NT-4/5, BDNF, NeuroD, and Ngn1 in the absence of bFGF in HNPCs (**Figures 2B,C**). To confirm the effect of *Hpca* on astrocytic differentiation and branch outgrowth of HNPCs, either control siRNA or *Hpca* siRNA was transfected into the HNPCs for 3 days. Similarly, as shown in **Figures 2D,E**, it reduced the number of GFAP-positive cells as well as branch outgrowth (**Figures 2D,F**).

Taken together, these results indicate that *Hpca* is crucial for the expression of GFAP and for branch formation.

## *Hpca* Increases Activation of Stat3 (Ser727) in Hnpcs

We next asked which signaling pathway was responsible for *Hpca*-mediated astrocytic differentiation of HNPCs. Because the STAT3 activation is associated with GFAP expression in neural precursor cells (Sun et al., 2001), we investigated whether STAT3 was phosphorylated by *Hpca* overexpression. As shown



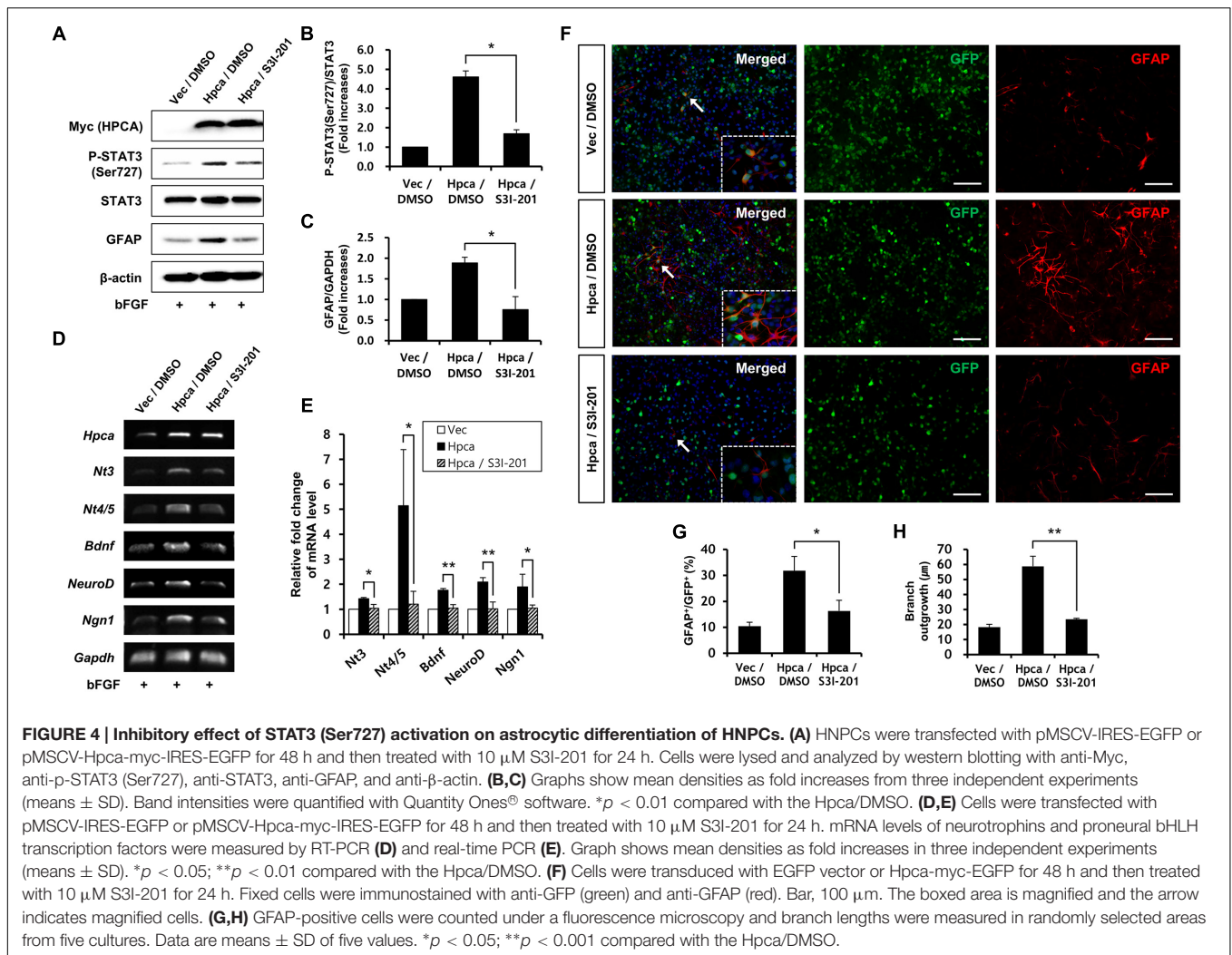


in **Figures 3A,B**, phosphorylation of STAT3 at Ser727 was significantly increased by overexpression of Hpca compared to vector control in HNPCs. On the other hand, knockdown of Hpca decreased the phosphorylation of STAT3 (Ser727) (**Figures 3C,D**). These results suggest that Hpca has an important role in the activation of STAT3 (Ser727) during astrocytic differentiation of HNPCs.

### Inhibition of STAT3 (Ser727) Activation Reduces Astrocytic Differentiation of HNPCs

Next, we determined whether STAT3 (Ser727) activation is required for astrocytic differentiation of HNPCs. Cells were transiently transfected with pMSCV-IRES-EGFP or pMSCV-Hpca-myc-IRES-EGFP for 2 days and treated with the STAT3 inhibitor, S3I-201, for 1 day. Inhibition of STAT3 not only decreased phosphorylation of STAT3 (Ser727) (**Figures 4A,B**) but also reduced the level of GFAP (**Figures 4A,C**) and

transcript levels of NT-3, NT-4/5, BDNF, NeuroD, and Ngn1 (**Figures 4D,E**). We also found that treatment with 10 μM S3I-201 significantly reduced the number of GFAP-positive cells ( $31.8 \pm 1.8\%$  vs.  $16.3 \pm 1.3\%$ , *p* < 0.05; **Figures 4E,G**) compared to the overexpression of Hpca. To test if STAT3 can also promote the branch outgrowth, we calculated the length of branches. The extent of branch outgrowth observed upon overexpression of Hpca was also reduced when STAT3 was inhibited (**Figures 4F,H**). To further determine the involvement of STAT3 in Hpca-mediated astrocytic differentiation, we knocked down STAT3 using siRNA. Stat3 siRNA completely abolished expression of GFAP (**Figures 5A,B**) and transcripts of NT-3, NT-4/5, BDNF, NeuroD, and Ngn1 compared with overexpression of Hpca (**Figures 5C,D**). We also found that knockdown of STAT3 markedly decreased the number of GFAP-positive cells ( $45.0 \pm 4.0\%$  vs.  $15.0 \pm 1.0\%$ , *p* < 0.05; **Figures 5E,F**) compared with overexpression of Hpca. Furthermore, knockdown of STAT3 decreased branch outgrowth (**Figure 5G**). Taken together, our results demonstrate that STAT3



is the signaling molecule that triggers branch outgrowth leading to astrocytic differentiation of HNPCs.

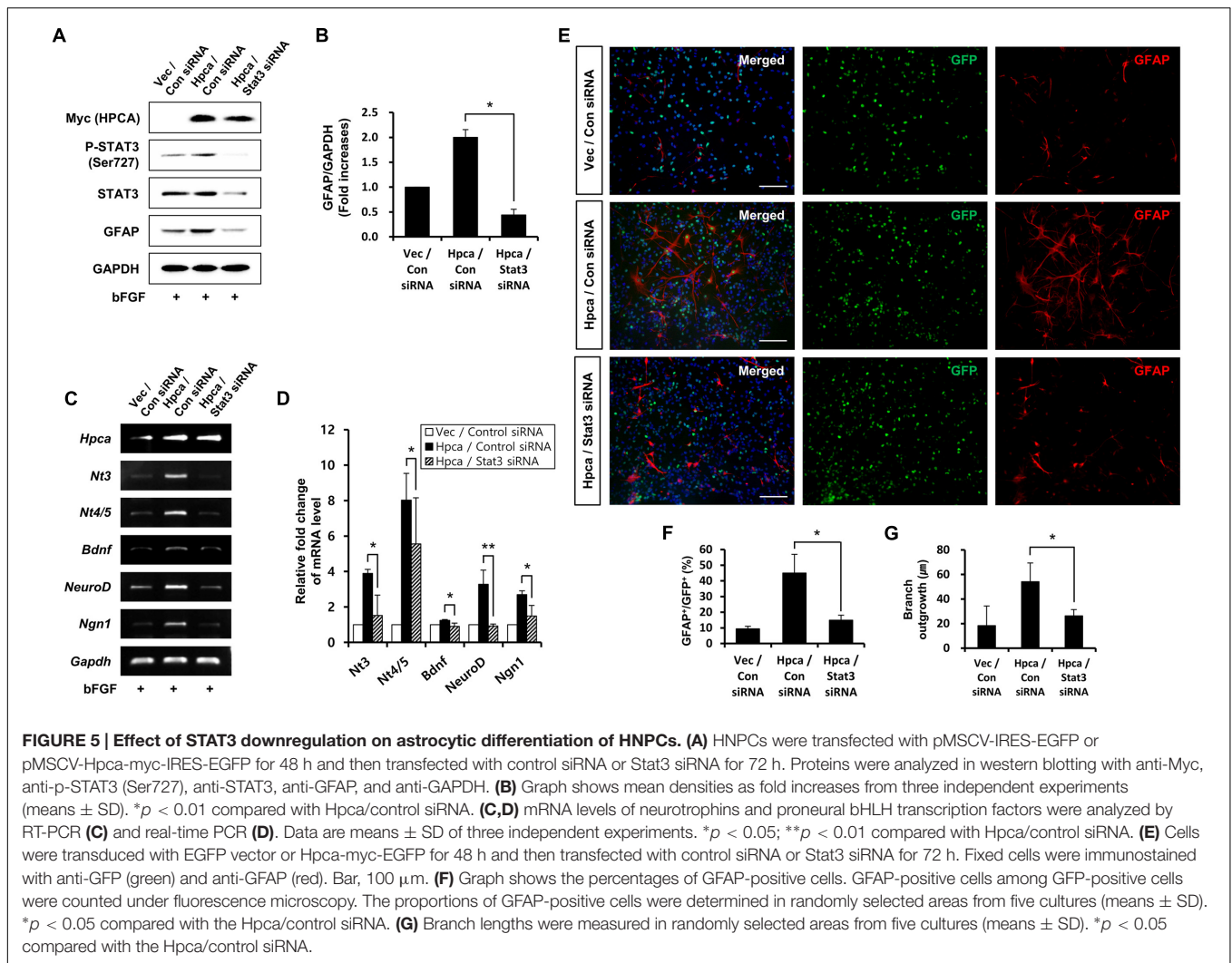
## DISCUSSION

The objective of this study was to elucidate the role of Hpca in neural precursor cell differentiation during hippocampal development. Differentiation of HNPCs was accompanied by upregulation of Hpca expression, and experiments involving gain or loss of Hpca function argued that Hpca was important for the differentiation process. Development of the mammalian nervous system is fulfilled through precursor cells that sequentially generate neurons and glial cells (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Davis and Temple, 1994). *In vivo*, cortical neurogenesis occurs during embryogenesis, while gliogenesis largely occurs postnatally. Remarkably, this precisely timed process can be reproduced in isolated embryonic cortical stem cells (Qian et al., 2000; Ross et al., 2003; Miller and Gauthier, 2007). Therefore, a major question is how the fates of neural precursors

are switched from neurogenesis in the earlier stages to gliogenesis later. One possible explanation is that the precursor cells themselves could change, for example, in response to neurogenic factors (Nieto et al., 2001; Sun et al., 2001; Menard et al., 2002; He et al., 2005), or growth factors (Sauvageot and Stiles, 2002). Here, we found that Hpca controlled the competence of precursors to differentiate into astrocytes by regulating the expression of proneural bHLH transcription factors and neurotrophins that induce GFAP expression in HNPCs. To our knowledge, this is the first example in which Hpca promoted differentiation toward a specific lineage, and particularly, toward astrocytes during development.

Hippocalcin has an important role in AMPAR-mediated endocytosis and NMDAR-mediated hippocampal LTD (Palmer et al., 2005), and its expression is involved in synapse formation and age-related postsynaptic function (Furuta et al., 1999). We have reported that it is one of the major regulatory proteins in the  $Ca^{2+}$ -mediated PLD signaling pathway (Oh et al., 2006) and that it induces neurite outgrowth leading to neuronal differentiation in H19-7 cells (Oh et al., 2008). However, its involvement in





astrocytic differentiation has not been studied. In this study, we found that expression of neurotrophins and proneural bHLH transcription factors are regulated by Hpca-mediated activation of STAT3 (Ser727), which leads to branch outgrowth and astrocytic differentiation in HNPCs. Several studies have reported that expression of neurotrophins was increased in cultured rat hippocampal astrocytes and astrocytes showed expression of neurotrophin receptors. However, the ability of neurotrophins to induce astrocytic differentiation and the effect of proneural bHLH transcription factors on astrocytic differentiation has not been studied. Thus, we will investigate whether Hpca-mediated expression of neurotrophins and proneural bHLH transcription factors has effect on astrocytic differentiation of HNPCs in further study.

The determination of neuronal/glial fate during CNS development involves complex interactions between intrinsic signals acting through many transcription factors (Edlund and Jessell, 1999). Above all, STAT3 plays essential roles in determining the fate of neural stem cells (NSCs) (Han et al., 2006). Inhibition of STAT3 enhances the differentiation of

human NSCs to motor neurons and reduces astrocytosis, and similarly, induces neurogenesis and inhibits gliogenesis (Gu et al., 2005; Cao et al., 2010; Natarajan et al., 2014). Previous studies have shown that GFAP expression in neural precursor cells is dependent on the activation of STAT3 (Bonni et al., 1997; Nakashima et al., 1999; Takizawa et al., 2001; Herrmann et al., 2008). Interestingly, we found that Hpca enhanced STAT3 (Ser727) activation, but did not cause any phosphorylation at Tyr705 (data not shown). Furthermore, inhibition of STAT3 activation by Stat3 siRNA or STAT3 inhibitor decreased transcripts of neurotrophins and proneural bHLH transcription factors and GFAP expression induced by Hpca overexpression, indicating that Hpca-mediated STAT3 (Ser727) activation is essential for astrocytic differentiation. Our results are consistent with earlier reports in which suppression or conditional deletion of STAT3 inhibited glial differentiation (Sun et al., 2001; Gu et al., 2005; Natarajan et al., 2014). However, the kinase involved in Hpca-mediated STAT3 activation remains to be identified. Interestingly, the STAT3 inhibitor and Stat3 siRNA in our

experiments completely inhibit the expression of neurotrophins and proneural bHLH transcription factors. Recent studies have shown that the activities of STAT family proteins can be modulated by interactions with other DNA-binding sites; for instance, STAT3 interacts with c-Jun, c-Fos and the CREB-binding protein co-activators (Zhang et al., 1999; Coqueret and Gascan, 2000). Although the mechanisms underlying this process have not been fully elucidated, our findings offer a theoretical basis for how astrocytic differentiation of HNPCs is regulated.

Taken together, the present study provides the first evidence that Hpca promotes gliogenesis in HNPCs, and induces GFAP expression and branch outgrowth by activating STAT3 (Ser727). Gliogenesis is tightly controlled because of its critical importance in proper physiological function, and multiple signal pathways control the growth and directionality of the relevant cell-fate decision. Recent work has demonstrated abnormalities in the signaling pathways responsible for gliogenesis and neurogenesis could contribute to the pathogenesis of neurodegenerative diseases and tumor development within the nervous system (Lee et al., 2000; Shors, 2004). Recognition of the regulatory mechanisms of gliogenesis provides new direction for intervention of neurogenic disorders. Furthermore, Hpca is implicated in memory formation, neuronal excitability (Kobayashi et al., 2012) and Hpca knockout mice have a defect in spatial and associative memory (Kobayashi et al., 2005). It is possible that Hpca plays an important role in neural plasticity and memory function and further studies should address its possible use as a marker of neurodegenerative disorder and for promoting glial cell generation *in vivo*.

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## AUTHOR CONTRIBUTIONS

M-JK participated in the conception, execution and analysis of experiments and in the writing of the manuscript, then edited in the additions and changes made by the other co-authors. S-YP participated in the conception, analysis of initial experiments and revision of the manuscript. J-SH contributed to the development of the hypothesis and additional text and revision of the manuscript. All authors agree to be accountable for all aspects of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fnmol.2016.00110/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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