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Discovery of a Small Molecule that Enhances Astrocytogenesis by Activation of STAT3, SMAD1/5/8, and ERK1/2 via Induction of Cytokines in Neural Stem Cells

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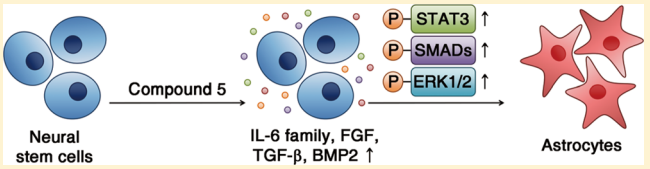
S *Supporting Information*

ABSTRACT: Identification of small molecules that direct neural stem cells (NSCs) into specific cell types would be helpful to understand the molecular mechanisms involved in regulation of NSC fate, and facilitate the development of therapeutic applications. In the current study, we developed and screened small molecules that can modulate the fate of NSCs that are derived from rat fetal cortex. Among these compounds, compounds **5** and **6** successfully differentiated NSCs into astrocytes and neurons, respectively. Compound **5** induced astrocytogenesis by increasing expression of interleukin-6, bone morphogenetic protein 2 and leukemia inhibitory factor and through consequent phosphorylation of signal transducer and activator of transcription 3 and Sma- and Mad-related protein 1/5/8 in NSCs. In addition, compound **5** increased the expression of fibroblast growth factor (FGF) 2 and FGF8 which may regulate the branching and morphology of astrocytes. Taken together, our results suggest that these small molecules can serve as a useful tool to study cell fate determination in NSCs and be used as an inexpensive alternative to cytokines to study mechanisms of astrocytogenesis.



KEYWORDS: *Astrocytogenesis, neural stem cells, neurological agents, structure−activity relationships, neurodegeneration, differentiation*

Neural stem cells (NSCs) are self-renewing cells that can differentiate into multiple cell types such as neurons, astrocytes, and oligodendrocytes. NSCs are found not only in the developing embryos but also in the adult brains.[1,2] Since neurodegenerative diseases are characterized by the loss of specific neurons, identification of small molecules that can stimulate endogenous NSCs to differentiate into neurons would be useful to develop new drugs for neurodegenerative diseases.[3−8] Recent evidence suggests that astrocytes also play important roles in selected pathologies and re-establishing or enhancing normal astrocytic functions may play critical roles in various central nervous system (CNS) disorders.[9] In amyotrophic lateral sclerosis, astrocytes are known to generate toxic materials and in Alzheimer's disease, astrocytes play pivotal roles in the clearance of toxic amyloid beta protein (Aβ).[9−11] Moreover, astrocytes appear to play a crucial role in synapse formation and maturation,[12] suggesting that, to treat neurodegenerative diseases, neurons and astrocytes may need to be simultaneously generated or regulated to re-establish functional neurons. Small molecules that direct NSC differentiation into neurons, astrocytes or oligodendrocytes would also be valuable for elucidating the molecular control mechanisms of NSC fate.

Studies have identified several small molecules and natural products that increase the generation of neurons or astrocytes from NSCs, and investigated their molecular targets.[3,5−8,13−19] For example, KHS101 selectively induced a neuronal differentiation phenotype by linking to cell cycle exit and interacting with transforming acidic coiled-coil-containing protein 3.[20] Phenotypic screening performed by McKnight and his colleagues identified P7C3 and its active aminopropyl carbazole derivatives which protected new born neurons by targeting nicotinamide phosphoribosyltransferase and resulted in increase of the levels of nicotinamide adenine dinucleotide.[21,22] We also previously reported the induction of neurogenesis by P7C3 derivatives[3,23] and increase of astrocytogenesis by a 1,3,4-oxadiazole derivative about 2-fold compared to the vehicle treated control.[24]

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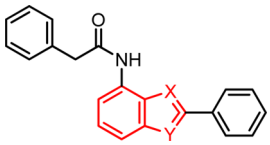
Although the mechanisms of astrocytogenesis have not been fully identified, it is reported that cytokines including interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), and leukemia inhibitory factor (LIF) activate glycoprotein 130 receptors and result in phosphorylation of Janus kinase/signal transducer and activator of transcription 3 (JAK-STAT3). Subsequently, phosphorylated STAT3 translocates into the nucleus and functions as a transcription factor to induce *glial fibrillary acidic protein* (*gfap*).[25,26] Another group of cytokine, bone morphogenetic proteins (BMPs) are known to phosphorylate Sma- and Mad-related protein (SMAD) 1/5/8. The activated SMADs form complex with activated STAT3 and p300/CREB-binding protein and are known to increase *gfap* expression. In addition, 10 of the 18 members of fibroblast growth factor (FGF) family are known to be expressed in the CNS and their receptors are reported to mediate pivotal roles in the generation of neurons and glia.[27−30] Exogenous FGFs are known to promote neuro-regeneration and FGF8-FGF receptor 3 (FGFR3) signaling is reported to control structural changes in astrocytes.[31]

In the current study, we have developed small molecules that enhance astrocytogenesis and neurogenesis. In particular, compound **5**, a benzothiazole derivative, remarkably enhanced astrocytogenesis by increasing transcripts of cytokines such as *il-6*, *bmp-2*, *lif*, *transforming growth factor beta 1* (*tgf-β1*), *fgf 2*, and *fgf 8* followed by activation of STAT3, SMAD1/5/8, and extracellular signal-regulated kinase (ERK) 1/2. Discovery of small molecules that control NSC fate and the underlying mechanisms would be beneficial for understanding how NSCs are regulated and differentiated and the development of new therapeutic or experimental reagents.

RESULTS AND DISCUSSION

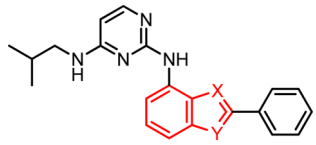
Design and Synthesis of Small Molecules That Can Enhance Astrocytogenesis and Neurogenesis. In an effort to develop small molecules that can modulate stem cell fate, we designed novel heterocyclic analogues as shown in Tables 1 and 2. Previously, we have identified several 1,3,4-oxadiazole derivatives that enhanced astrocytogenesis up to twofold.[24] While a small molecule that can enhance astrocytogenesis is a useful reagent, however, a twofold increase is not ideal for exploring the underlying mechanisms. Therefore, in the current study, we aim to identify small molecules that can increase astrocyte differentiation or NSC fate regulation more efficiently. Since the previously reported compounds contain a freely rotatable oxadiazole core,[20,24] we decided to develop conformationally constrained analogues by generating aromatic heterocycles such as benzoxazole, benzothiazole, and benzimidazole as shown in Figure 1. Additionally, we synthesized a group of derivatives by replacing the benzylamide group in compounds **1**−**5** with an isobutylaminopyrimidine group, which is also found in a known neurogenic inducer.[20] All the listed compounds were synthesized via one-step acylation (compounds **1**−**5**) or a palladium-catalyzed cross-coupling reaction (compounds **6**−**10**) with commercially available heterocyclic amines as described in Scheme 1 and in the Supporting Information.

Table 1. Structures of Small Molecules 1−5



compd	X	Y
1	N	O
2	N	S
3	N	NH
4	O	N
5	S	N

Table 2. Structures of Small Molecules 6−10



compd	X	Y
6	N	O
7	N	S
8	N	NH
9	O	N
10	S	N

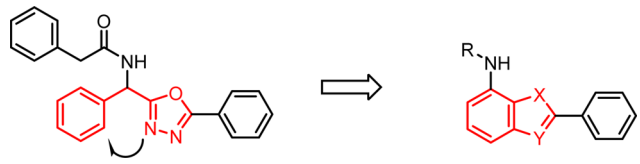


Figure 1. Design of the heterocyclic analogues.

Differentiation of NSCs into Astrocytes or Neurons Is Enhanced by Small Molecules. NSCs proliferate in the presence of mitogens such as epidermal growth factor (EGF) and/or FGF2 and differentiate into neurons or glia when these mitogens are absent in the media. To examine whether the synthesized compounds regulate NSC fate during differentiation, NSCs derived from the cortex of embryonic day (E) 14 rats were expanded for 1 week in the presence of EGF and FGF2 and were treated with 5.0 μM of each compound or with dimethyl sulfoxide (DMSO) for 4 days in the absence of mitogens. Differentiated astrocytes and neurons were identified by immunocytochemistry using anti-GFAP and anti-βIII tubulin antibodies (TuJ1). Total cell numbers were obtained by staining and counting 4′,6-diamidino-2-phenylindole (DAPI)-positive nuclei. Among the small molecules, **5** markedly increased the number of astrocytes by 4.82-fold (Figure 2). Compound **6** enhanced the number of neurons up to 1.43-fold (Figure 3). These data suggest that **5** and **6** significantly enhance astrocytogenesis and neurogenesis, respectively, in NSCs. Compounds **7**−**10** significantly reduced the number of DAPI-positive nuclei and the morphology of the cells appeared unhealthy during NSC differentiation (Figure 2 and 3, data not shown), suggesting that **7**−**10** are toxic to NSCs.

Since **5** increased astrocytogenesis significantly and remarkably, we decided to determine the most effective concentration. NSCs were treated with various concentrations of **5** (5.0, 7.5, 10.0, and 20.0 μM) for 4 days in the absence of EGF and FGF2. Compound **5** enhanced astrocytogenesis at 5.0, 7.5, and 10.0 μM by 3.83-, 6.52- and 6.09-fold, respectively (Figure 4A, B). However, **5** significantly reduced neurogenesis at

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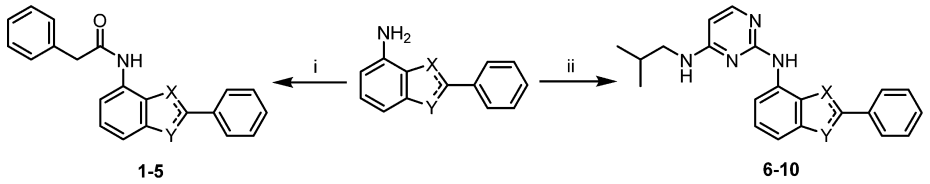
Scheme 1. Synthesis of Compounds 1−10[a]



[a]Reagents and conditions: (i) Phenylacetyl chloride, triethylamine, DCM, 0 °C to RT, 1 h; (ii) (2-chloropyrimidin-4-yl)isobutylamine, Pd_2dba_3, xantphos, Cs_2CO_3, 1,4-dioxane, 100 °C, 18 h.

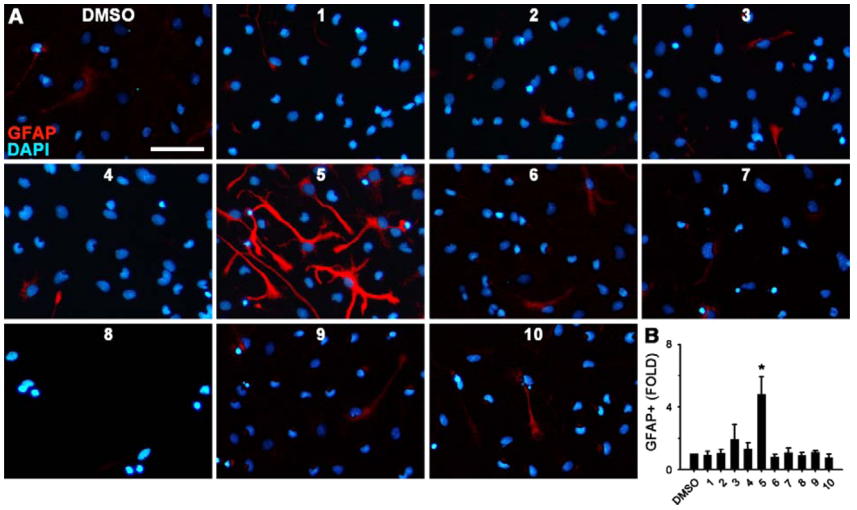


Figure 2. Compound **5** increased astrocytogenesis in NSCs. (A) Rat fetal NSCs were treated with vehicle (0.1% DMSO) or 5.0 μM of each compound (the numbers represent the specific compounds tested) for 4 days in the absence of EGF and FGF2 and were immunostained for GFAP (red). Nuclei were counterstained with DAPI (blue). Scale bar = 50 μm. (B) Quantification of astrocytes (GFAP-positive cells). The percentage of astrocytes obtained from each compound-treated NSCs was divided by that of DMSO-treated NSCs to yield the fold change. The values are presented as mean ± SEM (n = 3 for compounds **1−5**, n = 4 for compounds **6−10**). *P < 0.05 (Student's t test).

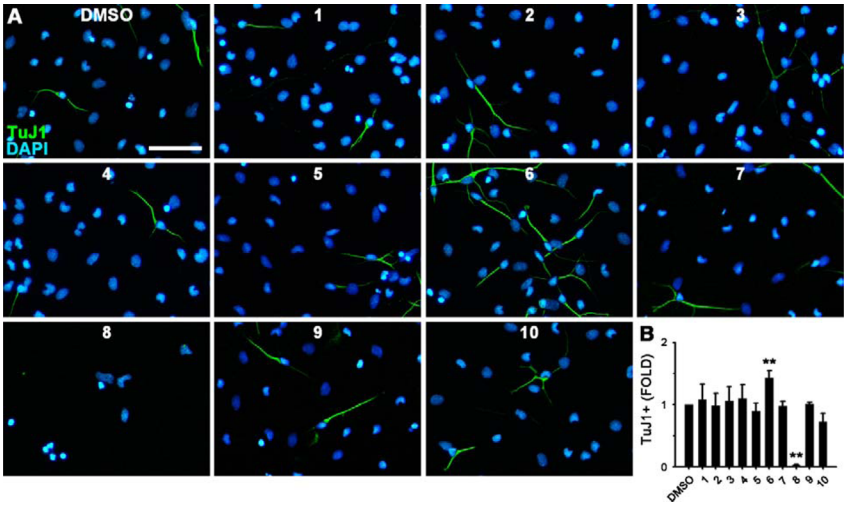


Figure 3. Compound **6** increased neurogenesis in NSCs. (A) Rat fetal NSCs were treated with vehicle (0.1% DMSO) or 5.0 μM of compounds (the numbers represent the specific compounds tested) for 4 days in the absence of EGF and FGF2 and immunostained using TuJ1 (green). Nuclei were counterstained with DAPI (blue). Scale bar = 50 μm. (B) Quantification of neurons (TuJ1-positive cells). The percentage of neurons obtained from compound-treated cells was divided by that of DMSO-treated cells to yield fold change. Values are presented as mean ± SEM (n = 3 for compounds **1−5**, n = 4 for compounds **6−10**). *P < 0.05, **P < 0.01 (Student's t test).

concentrations over 7.5 μM (Figure 4C) and total cell numbers as determined by nuclei staining at concentrations over 10.0 μM (Figure 4D). These data indicate that 7.5 μM is the most effective and nontoxic concentration of **5** to generate astrocytes. Similar to the immunocytochemistry results, after 4 days of treatment during differentiation, **5** at 7.5 μM increased expression levels of GFAP protein and decreased those of βIII Tubulin when detected by Western blot (Figure 4E).

To confirm the effects of **5**, reverse transcription polymerase chain reaction (RT PCR) followed by real-time PCR analysis

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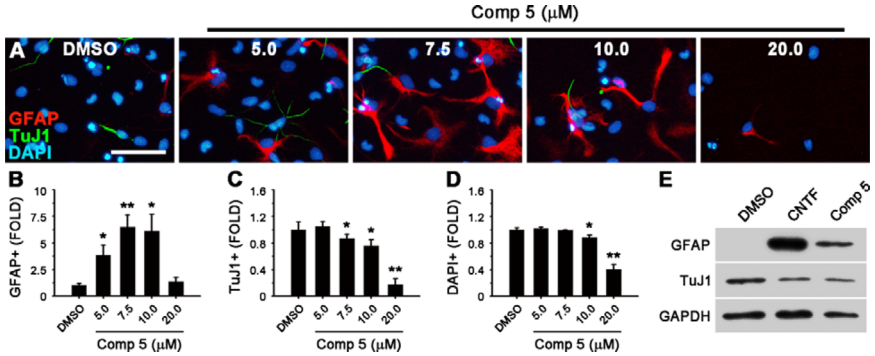

Figure 4. Compound **5** (7.5 μM) efficiently induced astrocytogenesis without toxicity in NSCs. (A–C) Concentration dependent analysis of compound **5**. Rat fetal NSCs were treated with DMSO or 5.0, 7.5, 10.0, and 20.0 μM of compound **5** for 4 days. (A) Representative fluorescence images of GFAP-positive astrocytes (red), TuJ1-positive neurons (green), and DAPI-positive nuclei (blue). Scale bar = 50 μm. Quantification of (B) astrocytes, (C) neurons, and (D) nuclei. In all graphs, values are presented as mean ± SEM (n = 3). (E) GFAP and βIII tubulin proteins were detected by Western blot analysis after 4 days of treatment with DMSO, 10 ng/mL CNTF, or 7.5 μM of compound **5**. *P < 0.05, **P < 0.01 (Student's t test).

was carried out. When NSCs were treated with 7.5 μM of compound **5** or **2** (a structural isomer of **5**) for 4 days during differentiation, **5** significantly increased the mRNA levels of *gfap* by 20.36-fold, while repressing those of *βIII tubulin* by 0.49-fold. (Figure 5A, B). These data suggest that compound **5** enhances astrocytogenesis while reducing neurogenesis.

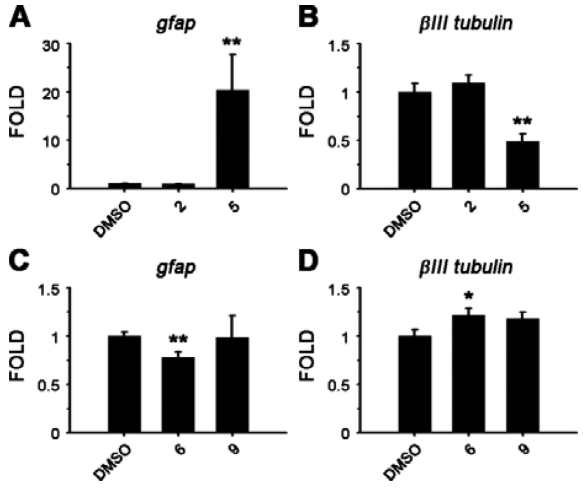

Figure 5. Compounds **5** and **6** increased mRNA expression levels of astroglial *gfap* and neuronal *βIII tubulin*, respectively. After treatment with compounds for 4 days, total RNA from NSCs was extracted and subjected to RT PCR followed by real-time PCR using specific primers for *βIII tubulin* and *gfap*. *Gapdh* was used as an internal control. (A, B) mRNA expression of (A) *gfap* and (B) *βIII tubulin* in NSCs treated with DMSO or 7.5 μM of compound **2** or **5**. (C, D) mRNA expression of (C) *gfap* and (D) *βIII tubulin* in NSCs treated with DMSO or 5.0 μM of compounds **6** or **9**. In all graphs, values are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 (Student's t test).

In the current study, we have discovered compound **5**, a benzothiazole derivative, that enhances production of astrocytes up to 7-fold. Since astrocytes are estimated as the most abundant cell types in the brain providing both structural and functional support for neurons,[32] it is essential to study how astrocytes are generated. Although the function of astrocytes is underappreciated and not identified fully, recently it is known that these cells play a crucial role in the amino acid catabolism and transportation that support the function of neurons. Since many neurotransmitters are amino acids or modified amino acids, the function of astrocytes is indispensable for neurons. For example, astrocytes are involved in the transportation of extracellular glutamate and the conversion of it to glutamine which can be uptaken by presynaptic neurons and used for the generation of excitatory neurotransmitter, glutamate.[33] Astrocytes are not only generated developmentally but also activated during the CNS injury. Weeks after the CNS injury, it is known that the environmental cues associated with inflammation and cell damage activate existing astrocytes to become proliferative and upregulate GFAP, vimentin, and nestin.[34] Although these reactive astrocytes are traditionally considered as bad guys by generation of glial scar and releasing inhibitory factors for axonal growth and regeneration, there is increasing evidence that reactive astrocytes also exert protective role in the CNS including active clearance of extracellular excitotoxic glutamate, protection from ammonium and nitric oxide toxicity, degradation of Aβ, blood brain barrier repair, and so on.[34] Therefore, identification of small molecules such as compound **5** that regulate NSC fate and understanding the differentiation process of not only neurons but also glia is essential for the development of future therapy. In addition, because astrocytes are generated remarkably by the treatment of compound **5**, it may be used as reagent to replace the expensive cytokines such as CNTF, LIF, or IL-6 that are well-known for the induction of astroglia and as an economical and effective drug candidate for Alexander disease. Although the detailed pathology of Alexander disease is still unclear, it is known that the mutation of astrocyte marker GFAP resulted in protein deposits known as Rosenthal fibers (astrocytic inclusions).[35−39] Compound 5 induced astrocytes that are generated from the wild type GFAP containing NSCs may rescue the symptoms of the disease.

Next, we treated NSCs with 5.0 μM of compounds **6** or **9** (a structural isomer of **6**) for 4 days during differentiation and performed RT PCR followed by real-time PCR. Consistent with our immunocytochemistry data, **6** significantly increased the expression levels of *βIII tubulin* by 1.22-fold, while decreasing that of *gfap* by 0.78-fold (Figure 5C, D). These data indicate that **6** increases neurogenesis but decreases astrocytogenesis in NSCs. This is an intriguing result, since compounds **5** and **6** share a structurally similar core, and yet have opposite effects. There are two noticeable differences between these two compounds: first, compound **5** has a benzothiazole core, whereas compound **6** has a benzoxazole

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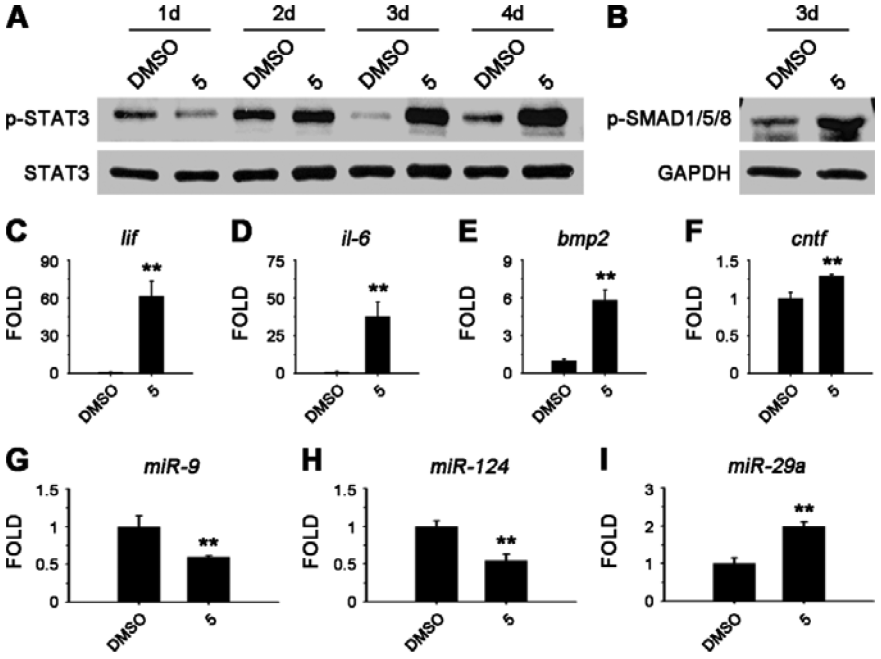

Figure 6. Compound **5** activated STAT3 through inducing LIF, IL-6, BMP-2, and CNTF. (A) Western blot analysis of p-STAT3 and STAT3 in cells treated with DMSO or 7.5 μM of compound **5** for 1−4 days. (B) Western blot analysis of p-SMAD1/5/8 and GAPDH. (C−F) mRNA expression levels of (C) *lif*, (D) *il-6*, (E) *bmp-2*, and (F) *cntf* were determined by real-time PCR upon treatment with DMSO or 7.5 μM compound **5** for 3 days. *Gapdh* was used as an internal control. (G−I) The expression levels of (G) *miR-9*, (H) *miR-124*, and (I) *miR-29a* in NSCs treated with DMSO or 7.5 μM of compounds **5** for 3 days. RNU6 was used as an internal control. In all graphs, values are presented as mean ± SEM (n = 3). $^{**}P < 0.01$ (Student's t test).

core, and second, compound **5** has a benzylamide side chain, whereas compound **6** has an aminopyrimidine side chain. Although compounds **5** and **6** seem to have similar cores such as 2-phenylbenzothiazole (for **5**) and 2-phenylbenzoxazole (for **6**), they contain different side chains (2-phenylacetamide for **5** and *N*-isobutylpyrimidine-2,4-diamine for **6**) at the different positions based on the sulfur and oxygen in the ring (7-position for **5** and 4-position for **6**), suggesting that their structural and positional differences may provide the opposite effect. It appears that compounds sharing the same benzothiazole core, **5** exhibited reducing effects on neurogenesis and **10** showed the similar tendency although not significant, while compounds **1** and **6**, which share the same benzoxazole core, did not seem to exert such correlating effects, suggesting that the benzothiazole core may play an important role in suppressing neurogenesis. It is unclear how the benzoxazole core with a pyrimidine side chain in compound **6** affects its enhancement in neurogenesis, however, previously reported small molecules that can enhance neurogenesis also share a pyrimidine structure,[20,40,41] which may explain the neurogenic activity of compound **6**.

Compound 5 Enhances Astrocytogenesis by Activation of Kinases through Induction of Cytokines. In the present study, **5** increased astrocytogenesis approximately 7-fold, so we used **5** to explore the mechanisms how astroglia are generated. Since most of chemicals that induce astrocytes are known to activate STAT3 or SMADs phosphorylation, we explored whether **5** could phosphorylate STAT3 and SMADs. NSCs were treated with 7.5 μM of compound **5** for different periods of time (1, 2, 3, and 4 days), and the levels of STAT3 phosphorylation and STAT3 expression were detected by Western blot. As shown in Figure 6A, **5** induced phosphorylation of STAT3 after 2 days of treatment, with the maximum effects at 3 and 4 days. In addition, **5** activated phosphorylation of SMAD1/5/8 after 3 days of treatment (Figure 6B). If **5** directly activated STAT3 or SMADs, it would take only a few seconds to minutes or, at most, several hours to activate the signal. However, because it took over 2 days to phosphorylate STAT3 and SMADs by compound **5**, we hypothesized that **5** might indirectly activate STAT3 and SMADs by producing molecules that activate STAT3 and SMADs.

Thus, we decided to test whether **5** induced cytokines that may influence the generation of astrocytes. When NSCs were treated with 7.5 μM of **5** for 3 days, the mRNA levels of *lif*, *il-6*, *bmp2*, and *cntf were* increased by 61.64-, 37.75-, 5.83-, and 1.29-fold, respectively (Figure 6C−F). Upon withdrawal of mitogens, NSCs are known to differentiate into the three major CNS cell types: neurons, astrocytes, and oligodendrocytes.[42] Furthermore, the addition of extracellular factors can efficiently restrict NSCs to become specific types of differentiated cells.[43,44] Compound **5** induced CNTF and CNTF is reported to induce NSCs to an astroglial fate rapidly and robustly via consequent activation of STAT3.[25,26,44] Compound **5** significantly increased BMPs and indeed these are known to instruct NSCs to become astrocytes.[44,45] LIF, upregulated remarkably by **5**, is known to activate STAT3 and induce the expression of BMP2 through STAT3 activation. BMPs are reported to phosphorylate SMAD1 to promote astrocyte differentiation.[46] The proinflammatory cytokine IL-6 is also suggested to have a crucial role in the guidance of NSCs to become both neurons and astrocytes.[47] Collectively, these findings suggest that **5** induces astrocytogenesis through induction of cytokines that are involved in astrocytogenesis and these cytokines appear to activate STAT3 and SMADs.

In addition to activated kinases, recent evidence suggests that cell fate can be modulated by epigenetic control such as histone tail modification and microRNAs (miRNAs). miRNAs are small

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noncoding transcripts that target and degrade or translationally repress mRNAs. They bind to 3′-untranslated regions of target mRNAs in a sequence specific way and prohibit translation or facilitate degradation of target mRNAs.[48] When we treated NSCs with **5**, we observed a significant decrease in *miR-9* and *miR-124* expression (Figure 6G and H). These suggest that the expression of *miR-9* and *miR-124* is reduced during the process of astrocytogenesis. Indeed, *miR-9* is known to be expressed in the brain regions as a regulator of neurogenesis.[49] Since **5** induced the generation of astrocytes and reduced neurogenesis, it appears plausible to observed reduction of *miR-9* expression. *miR-124* is the most abundant miRNA in the brain and although still in debate whether it is a primary determinant of neuronal differentiation or not,[50,51] it is reported that activation of STAT3 signaling is inversely correlated with *miR-9* and *miR-124a*.[52] Similarly we also observed that compound **5** increases STAT3 activation and reduces the expression of *miR-9* and *miR-124a*. In contrast, **5** induced expression of *miR-29a* (Figure 6I). Others also showed that *miR-29a* is restricted in astrocytes.[53] In addition, it is reported that *miR-29a* provides protection of neurons and *miR-29a* inhibitor aggravates cell injury after ischemia-like stresses in vitro.[54] Thus, it appears that the increased expression of *miR-29* by compound **5** may not only be involved in astrocytogenesis but also provide protection for surrounding neurons.

Next, we explored the activated ERK levels after the treatment of compound **5** during NSC differentiation. Since differentiating cells are supplemented with the media without EGF and FGF2, we expected to observe the reduced phosphorylation of ERK. However, interestingly, **5** activated ERK1/2 for 4 days and the maximum activation was observed on third day (Figure 7A), when high levels of STAT3 phosphorylation were seen (Figure 6A). In addition, the mRNA expression of *tgf-β1* was significantly induced by **5** (Figure 7B). These data suggest that **5** increases astrocytogenesis by production of *tgf-β1*. Similar to our results, with a transgenic mouse bearing part of the GFAP promoter linked to the *β*-galactosidase reporter gene, others showed that cerebral cortical neurons secreted TGF-*β*1 whose function is related to the mediation of astroglial maturation.[55] In addition, it is reported that mitogen-activated protein kinase mediates the activation of *gfap* gene promoter in response to TGF-*β*1.[56] In our study, compound **5** may have activated ERK1/2 by production of TGF- *β*1.

Although ERK1/2 mediate TGF-*β*1 induced GFAP activation, it is intriguing to see such remarkable activation of ERK1/2 by **5**, because mitogens were withdrawn from the media to induce NSC differentiation, and ERK1/2 phosphorylation should be reduced under such conditions. Thus, we decided to evaluate the expression levels of other growth factors that may enhance ERK1/2 signaling. RT PCR followed by real-time PCR showed that **5** upregulated the expression of *fgf2* and *fgf8* by 3.78- and 2.14-fold, respectively (Figure 7C, D). In contrast to *fgfs*, egf was significantly down-regulated by compound **5** (Figure 7E). The production of FGF2 and FGF8 in the presence of **5** may be to modulate the structure and number of astrocytes since it is reported that FGF signaling is required for perineural and cortex glia proliferation in the *Drosophila* postembryonic brain.[57] Developmentally, it is widely accepted that neurons are born earlier than astrocytes. It has been postulated that neurons regulate glial proliferation to coordinate proper brain development by releasing FGFs that facilitate glial proliferation.[57] In addition, it is reported that

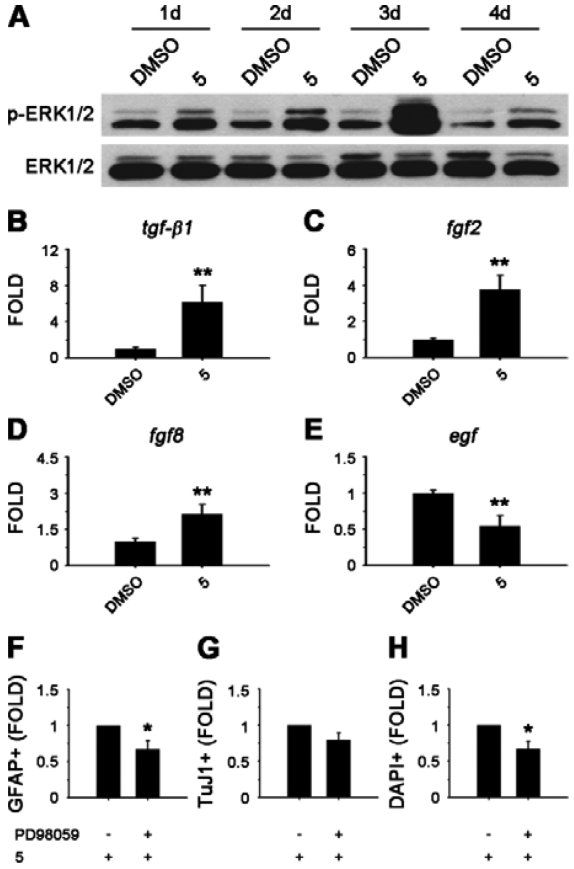

Figure 7. Compound **5** increased phosphorylation of ERK1/2. (A) Western blot analysis of p-ERK1/2 and ERK1/2 in cells treated with DMSO or 7.5 *μ*M of compound **5** for 1−4 days. (B−E) The transcripts of (B) *tgf-β1*, (C) *egf*, (D) *fgf2*, and (E) *fgf8* were detected by RT PCR followed by real-time PCR upon treatment of DMSO or 7.5 *μ*M compound **5** for 3 days. Values are presented as mean ± SD (n = 4). (F−H) The effect of ERK1/2 inhibitor, PD98059 on (F) total cell numbers detected by DAPI staining, (G) neurogenesis, and (H) astrocytogenesis. Cells were treated for 4 days with 7.5 *μ*M of compound **5** in the presence or absence of 20 *μ*M PD98059. Values are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01 (Student's t test).

FGF signaling affects the morphology and branching of astrocytes.[31] FGF8 is known to increase the development of branches in primary astrocytes and the overexpression of a constitutively active form of FGFR3 into the brain increases the structural complexity of astrocytes.[31] Thus, it appears that compound **5** increases FGFs in addition to TGF-*β*1 to generate fully branched mature astrocytes.

If ERK1/2 signaling is important for the induction of astrocytogenesis, treatment with an ERK1/2 inhibitor would reduce the levels of astrocyte production. Thus, we treated NSCs with the ERK1/2 inhibitor PD98059, and observed not only reduced levels of astrocytogenesis but also decreased cell survival while neurogenesis was not affected (Figure 7F−H). These data suggest that ERK1/2 phosphorylation by **5** plays critical roles in both astrocyte differentiation and survival.

In conclusion, we identified two small molecules, **5** and **6**, that increased differentiation of rat fetal NSCs into astrocytes and neurons, respectively, in vitro. Compound **5** enhanced astrocytogenesis by induction of *lif*, *il-6*, *bmp2*, and *cntf*, and indirectly phosphorylated STAT3 and SMAD1/5/8 through generation of these astrocytogenic factors (Figure 8). In

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ACS Chem. Neurosci. 2016, 7, 90−99

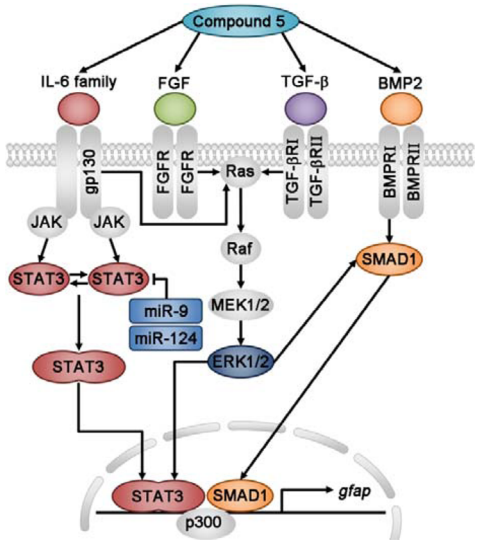

Figure 8. Model: Induction of astrocytogenesis by compound **5**. Upon treatment with **5** during differentiation, NSCs increase the levels of IL-6, FGF2, FGF8, TGF-β1, and BMP2. The released cytokines activate STAT3, SMAD1/5/8, and ERK1/2 and enhance the levels of GFAP to induce the generation of astrocytes.

addition, compound **5** activated ERK1/2 signaling, probably by generation of FGFs and TGF-β1, and regulated the expression of miRNAs that are involved in astrocytogenesis (Figure 8). Compound **5** may substitute astrocytogenic factors to generate astrocytes and will be used as a valuable reagent in future studies to understand how astrocytes are generated from NSCs.

METHODS

Chemistry. Flash column chromatography was performed using silica gel 60 (230−400 mesh; Millipore, Darmstadt, Germany) with the indicated solvents. ^{1}H NMR spectra were recorded on JEOL JNM-LA 300 and Bruker Avance 400 MHz FT-NMR spectrometers. Chemical shifts are reported in ppm units with Me_4Si (Sigma-Aldrich, St. Louis, MO) as a reference standard. Mass spectra were recorded on a VG Trio-2 GC-MS system and a 6460 Triple Quad LC/MS system. Detailed synthetic procedures and characterization data of final compounds can be found in the Supporting Information.

NSCs Culture. The procedures for preparation of rat NSCs were described previously.[58] Animals were treated according to Chung-Ang University and NIH standards of animal care. Briefly, NSCs were isolated from the cortex of E14 Sprague−Dawley rat (Orient Bio Inc., Gyeonggi-do, Korea) embryos. After dissociation, NSCs (200,000 cells/mL) were expanded as neurospheres in Dulbecco's modified Eagle's medium/F12 supplemented with 1% (v/v) PSA, 2% (v/v) B27 (all from Gibco, Grand Island, NY), 20 ng/mL EGF, and 20 ng/mL FGF2 (all from Chemicon, Temecula, CA). The culture was maintained at 37 °C in 5% CO_2 and the medium was replaced every 2 days. After 6 days, neurospheres were incubated with accutase (Chemicon) at 37 °C for 10 min to make a suspension of single NSCs. NSCs were then plated onto tissue culture plates precoated with 0.01% poly-D-lysine (Sigma-Aldrich) and 10 μg/mL laminin (Invitrogen, Carlsbad, CA). After 1 day of proliferation, differentiation was induced without growth factors. For treatment experiments, NSCs were treated with 0.1% DMSO (Sigma-Aldrich), synthetic compounds, 10 ng/mL CNTF or 20 μM PD98059 (all from Millipore, Billerica, MA).

Immunocytochemistry and Cell Counting. Immunocytochemical examination was performed as previously described.[59] Cell cultures were fixed with 4% paraformaldehyde (USB Products, Cleveland, OH) for 30 min and washed with phosphate-buffered saline (PBS). Fixed cells were blocked with 5% normal goat serum (Millipore) and 0.2% Triton X-100 (Amresco, Solon, OH) in PBS for 30 min. The cells were then incubated for more than 1 h with primary antibodies: TuJ1 (mouse monoclonal antibody, 1:1000; Sigma-Aldrich) and anti-GFAP (rabbit polyclonal antibody, 1:1000; Dako, Copenhagen, Denmark). After rinsing with PBS, the cells were incubated for 30 min with secondary antibodies conjugated to Alexa Fluor 488 (goat anti-mouse immunoglobulin G [IgG], 1:1000; Invitrogen) or Cy3 (goat anti-rabbit IgG, 1:1000; Jackson ImmunoResearch, West Grove, PA). DAPI (1:10 000 in PBS; Sigma-Aldrich) was added for 5 min to stain the nuclei. The images were obtained using an inverse fluorescence microscope (DMIL; Leica, Hesse, Germany). To avoid a bias in measurement, the photos were randomly taken and TuJ1-, GFAP-, or DAPI-positive cells were counted. The number of TuJ1- or GFAP-positive cells was divided by that of DAPI-positive cells to obtain the percentage. The percentage value of compound-treated group was divided by that of control to get the fold change.

Real-Time Reverse Transcription Polymerase Chain Reaction. Total RNA from cell cultures was extracted using TRIzol reagent (Invitrogen). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA in a reaction volume of 20 μL using QuantiTect Reverse Transcription Kit (Qiagen, Limburg, Netherlands). Real-time RT PCR was performed using the iQ SYBR Green supermix (Bio-Rad, Hercules, CA). The following primer sets were used to amplify cDNA: *gfap*, agcggctctgagagagattc (forward) and agcaacgtctgtgaggtctg (reverse); *βIII tubulin*, agccctctacgacatctgct (forward) and attgagctgaccagggaatc (reverse); *lif*, aagttggtcgagctgtatcg (forward) and gagtttgatctggaggctca (reverse); *il-6*, tgttctcagggagatcttgg (forward) and tttggaagcatccatcattt (reverse); *bmp-2*, aacaaatgcaggaagc-tttg (forward) and cctggtgtccaatagtctgg (reverse); *cntf*, gcaaggaagatt-cgttcaga (forward) and tcatctcactccaacgatca (reverse); *tgf-β1*, cgga-ctactacgccaaagaa (forward) and ttcccgaatgtctgacgtat (reverse); fgf2, tggctatgaaggaagatgga (forward) and tcagtgccacatatccaactg (reverse); *fgf 8*, accggtctgtacatctgcat (forward) and gttgttctccagcacgatct (reverse); *egf*, cctgacatcagatggtcctc (forward) and atcacattcccaggatgcta (reverse); and *gapdh*, agttcaacggcacagtcaag (forward) and gtggtgaagacgccagtaga (reverse). The PCR 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. The ratio of gene expression between NSCs treated with DMSO and those treated with compound was calculated using the following formula: ratio = $2^{C(t)DMSO/\Delta C(t)compound}$. Here, $C_{(t)}$ DMSO = $C_{(t)}$ target gene − $C_{(t)}$ *gapdh*, from DMSO-treated NSCs and $C_{(t)}$ compound = $C_{(t)}$ target gene − $C_{(t)}$ *gapdh*, from compound-treated NSCs ($C_{(t)}$, threshold cycle)

Western Blot Analysis. The NSCs were washed with PBS and lysed by adding lysis buffer [50 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 1% NP-40 (all from Amresco), Halt Phosphatase Inhibitor Cocktail (Thermo Scientific, Rockford, IL), 1 mM phenylmethylsulfonyl fluoride, 0.01 mg/mL leupeptin, and 0.01 mg/mL aprotinin (all from Sigma-Aldrich)]. The lysates were centrifuged for 30 min at 25 200*g* to remove debris. The proteins were 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), 14.4 mM β-mercaptoethanol (Bio-Rad), and distilled water], separated electrophoretically onto SDS-polyacrylamide gel and transferred to polyvinylidenedifluoride membrane (Millipore). The membrane was blocked with 5% nonfat dry milk or bovine serum albumin (Millipore) in 20 mM Tris-buffered saline containing 0.03−0.1% Tween 20 (Amresco) for 30 min and incubated overnight at 4 °C with primary antibodies: anti-GFAP (1:500), TuJ1 (1:2000) (all from Sigma-Aldrich), anti-STAT3 (1:2000), anti-phospho-STAT3 (Tyr705, 1:2000), anti-phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428) (1:1000), anti-ERK1/2 (1:4000), anti-phospho-ERK1/2 (Thr202/Tyr204, 1:4000) (all from Cell Signaling, Danvers, MA), and GAPDH (1:1000, Santa Cruz, Santa Cruz, CA). Immunoreactivity was detected by sequential incubation with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG antibody (1:5000; Santa Cruz) for 1 h and then Western Blotting Luminol Reagent (Santa Cruz).

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ACS Chem. Neurosci. 2016, 7, 90−99

Detection of miRNA Expression. The miScript PCR system (Qiagen) was used to analyze the expression of miRNAs, including rno-miR-9, rno-miR-124, and rno-miR-29a, according to the manufacturer's instructions. The total RNA including miRNA was extracted using TRIzol reagent (Invitrogen). cDNA was generated from 2 μg of total RNA using the miScript II RT kit. Real-time PCR was performed using the miScript SYBR Green PCR kit with 5 miScript Primer Assays of Rn_miR-9_1, Rn_miR-124*_1, Rn_miR-29a*_2 and Hs_RNU6−2_11. The PCR reaction was conducted at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s. U6 small nuclear RNA (RNU6) was used for normalization.

Statistical Analysis. Values were expressed as means ± standard error of mean (SEM), and statistical significance was determined using Student's *t* test (*$P < 0.05$, **$P < 0.01$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00243.

General synthetic procedures of compounds **1**−**10** (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Aβ, amyloid beta protein; BMP, bone morphogenetic protein; cDNA, complementary DNA; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DAPI, 4′,6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; E, embryonic day; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR3, FGF receptor 3; GFAP, glial fibrillary acidic protein; IgG, immunoglobulin G; IL-6, interleukin-6; LIF, leukemia inhibitory factor; miRNA, microRNA; NSCs, neural stem cells; PBS, phosphate-buffered saline; RNU6, U6 small nuclear RNA; RT PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; SEM, standard error of mean; SMAD, Sma- and Mad-related protein; STAT3, signal transducer and activator of transcription 3; TGF-β1, transforming growth factor beta 1

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