GDNF facilitates differentiation of the adult dentate gyrus-derived neural precursor cells into astrocytes via STAT3

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Abstract

While the pro-neurogenic actions of antidepressants in the adult hippocampal dentate gyrus (DG) are thought to be one of the mechanisms through which antidepressants exert their therapeutic actions, antidepressants do not increase proliferation of neural precursor cells derived from the adult DG. Because previous studies showed that antidepressants increase the expression and secretion of glial cell line-derived neurotrophic factor (GDNF) in C6 glioma cells derived from rat astrocytes and GDNF increases neurogenesis in adult DG *in vivo*, we investigated the effects of GDNF on the proliferation, differentiation and apoptosis of cultured neural precursor cells derived from the adult DG. Data showed that GDNF facilitated the differentiation of neural precursor cells into astrocytes but had no effect on their proliferation or apoptosis. Moreover, GDNF increased the phosphorylation of STAT3, and both a specific inhibitor of STAT3 and lentiviral shRNA for STAT3 decreased their differentiation into astrocytes. Taken together, our findings suggest that GDNF facilitates astrogliogenesis from neural precursor cells in adult DG through activating STAT3 and that this action might indirectly affect neurogenesis.

Key Words: neurogenesis, antidepressant, astrocyte, GDNF, STAT3

Abbreviations: ADP, adult rat DG-derived neural precursor cells; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; DAPI, 4', 6-diamino-2- phenylindole; DG, dentate gyrus; DEX, dexamethasone; FGF2, fibroblast growth factor 2; GDNF, glial cell line-derived neurotrophic factor; IGF, insulin-like growth factor; LIF, Leukemia inhibitory factor; PFC. prefrontal cortex; RA, retinoic acid; RET, rearranged during transfection; shRNA, short hairpin RNA; STAT3, signal transducer and activator of transcription 3; STS, staurosporine; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VEGF, vascular endothelial growth factor

Introduction

Antidepressants increase neurogenesis in the adult DG in rodents [1,2,3], and it is thought that neurogenesis in the adult DG may contribute to the therapeutic actions of antidepressants [4,5,6]. We have previously demonstrated, using a cell culture of the adult rat DG-derived neural precursor cells (ADP)[7], that common mood stabilizers such as lithium, valproate, carbamazepine and lamotrigine promote ADP proliferation, survival and differentiation[8]. Interestingly, however, antidepressants had no effect on ADP proliferation, apoptosis and differentiation *in vitro* [9, our unpublished data], suggesting that antidepressants might promote the proliferation of neural precursor cells in the adult DG *in vivo* through some unknown indirect processes that are not present in the ADP cell culture system.

Neurotrophic/growth factors contribute to adult neurogenesis *in vivo*. Brain-derived neurotrophic factor (BDNF), fibroblast growth factor 2 (FGF2), glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor (IGF) and vascular endothelial growth factor (VEGF) increase the rate of adult neurogenesis in the adult DG [10,11,12,13,14]. Antidepressants increase the expression of these neurotrophic/growth factors [15,16,17,18,19] and BDNF and VEGF mediate the action of antidepressants on neurogenesis in the adult DG *in vivo* [19, 20]. However, it remains unclear whether FGF2, GDNF and IGF mediate the effect of antidepressants on neurogenesis on the adult DG. In the present study, we hypothesized that GDNF and signal transducer and activator of transcription 3 (STAT3) in neural precursor cells in the adult DG mediate the actions of antidepressants on neurogenesis in the adult DG for the following reasons. First, antidepressants increase GDNF expression and secretion in C6 glioma cells derived from rat astrocytes [21]. Second, GDNF increases the proliferation of neural progenitors in the adult DG *in vivo* [11]. Third, the levels of whole blood GDNF in patients with mood disorders are significantly lower than those in healthy control subjects [22]. Fourth, STAT3 might mediate astrogliogenesis from neural precursor cell [23,24,25,26,27] and

GDNF-dependent mechanisms might exist for activation of STAT3 [28,29]. Our data show that GDNF increases ADP differentiation into astrocytes, but has no effect on ADP proliferation or apoptosis and that STAT3 is required for the effect of GDNF on ADP differentiation into astrocytes.

Materials and Methods

Drugs

Basic fibroblast growth factor (bFGF) was purchased from Invitrogen (Carlsbad, CA). GDNF was purchased from Sigma (St. Louis, MO). Dexamethasone (DEX) was purchased from Sigma. STS was kindly donated by Asahi-Kasei Medical Co. Ltd., Shizuoka, Japan. Retinoic acid (RA) was purchased from Sigma. Stattic was purchased from Merck (Darmstadt, Germany).

Isolation and culture of ADP

ADP were isolated from the DG of adult male Sprague-Dawley rats (8 weeks old) as described previously [7]. In brief, ADP were maintained with Neurobasal (Invitrogen)/ B27 supplement minus vitamin A (Invitrogen)/ 1 mM L-glutamine (Invitrogen)/ 20 ng/ml bFGF (proliferation medium) at 37 ℃ on laminin (Invitrogen)-ornithin (Sigma) coated dishes and fed with new medium every two or three days by replacing 50 % of the medium.

Cell counting

To estimate the effects of drugs on the number of cells, we used Alamar Blue assay (Invitrogen) as in the cases of our previous studies [7,8]. 1 x 10^4 cells/well were put in laminin-ornithin coated 96-well plates in 100 µl/well of proliferation medium. After overnight incubation, cells were treated with GDNF at each concentration in the presence or absence of 5 µM DEX. After 3 days, 10 µl/well of Alamar Blue solution were added into medium, and cells were incubated at 37 ℃ for 3 hours. Subsequently, 50 µl of medium were dispensed into plates and the fluorescence of samples was measured and calculated as described in the manufacture's manual.

TUNEL assay

2 x 10^4 cells/well were put in laminin-ornithin coated Lab-Tek□ 8-chamber slides (Nalge Nunc International, Naperville, IL) with proliferation medium. After overnight incubation, cells were treated in proliferation medium with GDNF and 300 nM staurosporine (STS). After 2 days, cells were fixed 4 % paraformaldehyde for 15 min. Permeabilization was performed with PBS containing 0.2 % Triton X-100 for 30 min. Subsequently, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed with DeadEnd Fluorometric TUNEL System (Promega, Madison, WI) as in the case of our previous studies [7,8].

Immunocytochemisry

2 x 10^4 cells/well were put in laminin-ornithin coated Lab-Tek□ 8-chamber slides with proliferation medium without bFGF (differentiation medium). After overnight incubation, cells were treated in differentiation medium with 1 μM RA/ 0.5% fetal bovine serum (Invirogen), GDNF and/or stattic. After 7 days, immunocytochemistry was performed with our previour studies [7,8]. Primary antibodies were used at the following concentrations: mouse anti-nestin (1:2000; BD Biosciences, Franklin Lakes, NJ), rabbit anti-glial fibrillary acidic protein (GFAP) (1:2000; Dako, Glostrup, Denmark), mouse anti-Tuj1 (1:5000; Covance, Princeton, NJ). Secondary antibodies were used at following concentrations: FITC-conjugated goat anti-mouse IgG antibody (1:100; Jackson Immuno Reserch, West Grove, PA), Cy3-conjugated goat anti-rabbit IgG antibody (1:100; Jackson Immuno Reserch).

Western Blotting

1 x 10^5 cells/ well were seeded in proliferation medium on laminin-ornithin coated 6-well plates. After overnight incubation, cells were treated with each drug at each concentration. After 3 days, lysis of cells and preparation of total proteins were performed with Mammalian Cell Lysis

Kit (Sigma). Western Blotting of pSTAT3 and total STAT3 was performed with mouse monoclonal anti-STAT3 antibody (1:1000; Cell Signaling, Danvers, MA) and rabbit polyclonal anti-phospho-STAT3 (Tyr705) antibody (1:1000; Cell Signaling) as described in our past study (Boku et al, 2009). The pictures were converted to digital files and the intensity of each band was analyzed with Image J (National Institutes of Health, Bethesda, MD).

Knockdown of STAT3 with lentiviral shRNA

The shRNA plasmid for STAT3 and the plasmid for negative control were purchased from Thermo Scientific (Waltham, MA). Lentivirus was prepared with Trans-Lentiviral shRNA Packaging Kit with Calcium Phosphate Transfection Reagent (Thermo Scientific) as the manufacuturer's manual. Lentiviral shRNAs for STAT3 and negative control were infected to ADP at 1 x 10^5 TU/ml. After 1 day of infection, lentivirus was removed and flesh medium was added to ADP. After 3 days of infection, cell lysate was prepared and the effect of lentiviral shRNA was examined with Western Blotting as described above. After 7 days of infection, immunostaining was performed with anti-GFAP antibody as described above.

Statistical analysis

In Alamar Blue assay and TUNEL assay, statistical analysis was performed by one-way ANOVA and Dunnet post hoc test. In immunocytochemistry and western blotting, Statistical analysis was performed by unpaired *t*-test. Significance was defined as $p<0.05$. Data are expressed as the means ± SEM.

Results

GDNF has no effect on ADP proliferation and apoptosis

The effects of GDNF on ADP proliferation was examined with Alamar Blue assay both in the presence and absence of 5 μM DEX for the following reasons. First, the proliferation potency of ADP is saturated under the culture condition described in Materials and Methods. So, it is necessary for the investigations of the positive effects of drugs and/or factors on ADP proliferation to decrease it. Second, it has been shown that glucocorticoids are the physiological negative regulator of neurogenesis in the adult DG [30,31] and DEX is a specific agonist of glucocorticoid receptor. GDNF had no significant effect on the number of cells at any dose in the presence (One-way ANOVA and Dunnett's post hoc test, $F(3, 28)=0.885$, $p=0.4608$, Fig.1A) or the absence (data not shown) of 5 μM DEX. Next, we examined the effects of GDNF on ADP apoptosis. Apoptosis was induced by 300 nM STS, an inhibitor of protein kinase C for the following reasons and estimated with TUNEL staining. First, STS is often used as an induced of apoptosis via the internal pathway [32]. Second, the internal pathway, but not the external pathway, is involved in the apoptosis of neural progenitor cells [33,34]. Third, we have already shown that Tumor Necrosis Factor-α, a ligand of death receptors and an inducer of the external pathway, did not induce apoptosis on ADP (data not shown). GDNF had no significant effect on the ratio of TUNEL-positive cells/ DAPI-positive cells at any dose (One-way ANOVA and Dunnett's post hoc test, $F(3, 28)=2.420$, $p=0.0871$, Fig.1B).

GDNF facilitates ADP differentiation into astrocytes

ADP differentiation was induced by 1 μM RA, which differentiates ADP into both neurons and astrocytes, for the following reasons. First, ADP are not able to be differentiated into neurons and/or astrocytes by the removal of bFGF alone. Second, RA is often used as a inducer of neural differentiation *in vitro* [35,36]. Third, it has been shown that endogenous RA is involved in neural differentiation in adult hippocampus *in vivo* [37,38]. The effect of GDNF on RA-induced ADP differentiation was estimated with immunocytochemistry of Tuj1 (a marker of neuron) and GFAP (a marker of astrocyte). While GDNF decreased the ratio of Tuj1-positive cells/ DAPI-positive cells (unpaired t-test, $t=5.140$, $df=5$, $p<0.01$, Fig.2), it increased the ratio of GFAP-positive cells/ DAPI-positive cells (unpaired t-test, $t=7.260$, $df=5$, $p<0.01$, Fig.2).

Involvement of STAT3 in the effect of GDNF on ADP differentiation into astrocytes

We examined the effects of Stattic, a specific inhibitor of STAT3, on GDNF- promoted ADP differentiation into astrocytes with immunostaining of anti-GFAP antibody. Stattic significantly decreased the ratio of GFAP-positive cells/ DAPI-positive cells (unpaired t-test, $t=4.705$, $df=14$, $p<0.01$, Fig.3A). Because it is well established that STAT3 is activated by phosphorylation, we next examined the effects of GDNF on the phosphorylation of STAT3 with Western Blotting. GDNF significantly increased the phosphorylation of STAT3 (unpaired t-test, $t=3.761$. $df=8$, $p<0.01$, Fig.3B). We then examined the effects of STAT3 inhibition by shRNA on GDNF-promoted ADP differentiation into astrocytes. STAT3 shRNA significantly decreased the expression of STAT3 (data not shown) and the ratio of GFAP-positive cells/ DAPI-positive cells (unpaired t-test, $t=4.482$. $df=6$, $p<0.01$, Fig.3C).

Discussion

Our data show that GDNF facilitates differentiation of ADP into astrocytes, but has no direct effect on proliferation or apoptosis of ADP. More over, this process seems to require STAT3. Our observation suggests that GDNF might increase proliferation and survival of neural precursor cells in the adult DG through its effect on astrocytes in neurogenic niches, underscoring the critical role played by astrocytes in the neurogenic niches in the adult DG [39,40]. Previous studies have shown that astrocytes derived from the adult hippocampus promote proliferation of adult hippocampal neural precursors in co-culture [41]. and that GDNF increases BrdU-positive cells and protects cell death in the adult rat DG *in vivo* [11,42]. While these data make an apparent contrast to our observation that GDNF has no effect on ADP *in vitro*, our data provide a plausible mechanism underlying this seemingly conflicting results; GDNF might act on astrocytes, in a STAT3-depednent manner, to have an indirect impact on ADP *in vivo*. However, our present discussion is still speculative and our data were derived from an *in vitro* culture system of neural precursor cells. Therefore, a future challenge is to directly test this hypothetical mechanism *in vivo*.

Previous studies have shown that GDNF activates STAT3 [23, 28, 29] and that STAT3 promotes differentiation of neural precursors into astrocytes [24, 25, 26, 27]. On the other hand, it was not known whether GDNF promotes differentiation of neural precursors into astrocytes through STAT3. Therefore, our present study is the first one to demonstrate that STAT3 is functionally required for astrogliogenesis *in vitro*. However, previous studies and our present study cited above are *in vitro* studies and it remains unclear whether STAT3 is actually involved in astrogliogenesis *in vivo*. Therefore, more work is needed to examine the effects of GDNF on the phosphorylation of STAT3 and astrogliogenesis in the adult DG *in vivo.*

The original motivation of our present study was to identify an antidepressant-induced mediator, which directly increases ADP proliferation. We hypothesized that GDNF is such a mediator, but our present results do not support the hypothesis; GDNF has no significant direct effect on ADP proliferation. Therefore, other neurotrophic/growth factors, such as BDNF, FGF2, VEGF and IGF can be such a mediator. We have observed that FGF2, but not BDNF, increases ADP proliferation and that ADP has no VEGF receptors (our unpublished data). Moreover, a recent study has shown that amitriptyline, a tricyclic antidepressant, increases the expression of BDNF, FGF2, GDNF and VEGF in primary cultured astrocytes [43]. Taken together, FGF2 might be a possible mediator through which antidepressants increases proliferation of neural precursor cells in the adult DG *in vivo*. However, our present discussion is exclusively based on the data derived from an *in vitro* culture system of neural precursor cells. Therefore, a future challenge is to directly test the possibility of FGF2 as such a mediator *in vivo*.

In our present study, we focused on the adult DG because involvement of neurogenesis in this structure in depressive-like behaviors and actions of antidepressants are supported [1, 2, 3, 4, 5, 6], but we do not rule out the possibility that other structures and processes are also involved.

The expression of GFAP is decreased in both the hippocampus and prefrontal cortex (PFC) in a rat model of depression [44]. The inhibition of astrocytic function in the hippocampus or PFC induces depressive-like behaviors and abolishes the effects of antidepressants on them [45,46]. Moreover, FGF2 mediates the actions of antidepressants through gliogenesis in the PFC [47].

These suggest that astrocytes in both the PFC and DG may play roles in the action mechanisms of antidepressants and the pathophysiology of depression. If so, increasing astroglionegenesis in the PFC and DG through activating STAT3 pathway might serve as a therapeutic option for depression. However, it remains unclear whether STAT3 pathway is involved in astrogliogenesis in the PFC and DG in *vivo* and more work is needed to critically evacuate whether STAT3 is required for astrogliogenesis in the PFC. A further understanding of these molecular steps may contribute to the development of new therapy for depression and a better understanding of the pathophysiology of depression.

Our data show GDNF has no direct effect on proliferation or survival of ADP, but increases ADP differentiation into astrocytes through activation of STAT3. These results suggest that the GDNF may indirectly affect proliferation and survival of neural precursor cells in the adult DG through STAT3-depedent astrogliogenesis. However, all experiments in our present study were performed *in vitro* and it remains unclear whether GDNF increases astrogliogenesis from neural precursor cells through STAT3 in adult DG *in vivo* as in the case of ADP. This is the limitation of our present study. Therefore, *in vivo* studies are needed to confirm the results presented in our present study. A future challenge is to evaluate this hypothetical mechanism *in vivo*.

Acknowledgements

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Figure legends

Figure 1. GDNF has no effect on ADNP proliferation and apoptosis

A. GDNF had no significant effect on ADNP proliferation at any dose in the presence of 5 μM DEX. Alamar Blue assay was perfomed 3 days after drug treatments for four independent cultures.

B. GDNF had no significant effect on ADNP apoptosis induced by 300 nM STS. TUNEL assay was performed 2 days after drug treatments for four independent cultures. positive

Figure 2. GDNF facilitates ADNP differentiation into astrocytes

50 ng/ ml GDNF increased the ratio of astrocyte-like GFAP- positive cells induced by 1 μM RA and decreased that of Tuj1- positive cells. Immunocytochemistry was performed 7 days after drug treatment for four independent cultures. Scale bar = 120 μm.

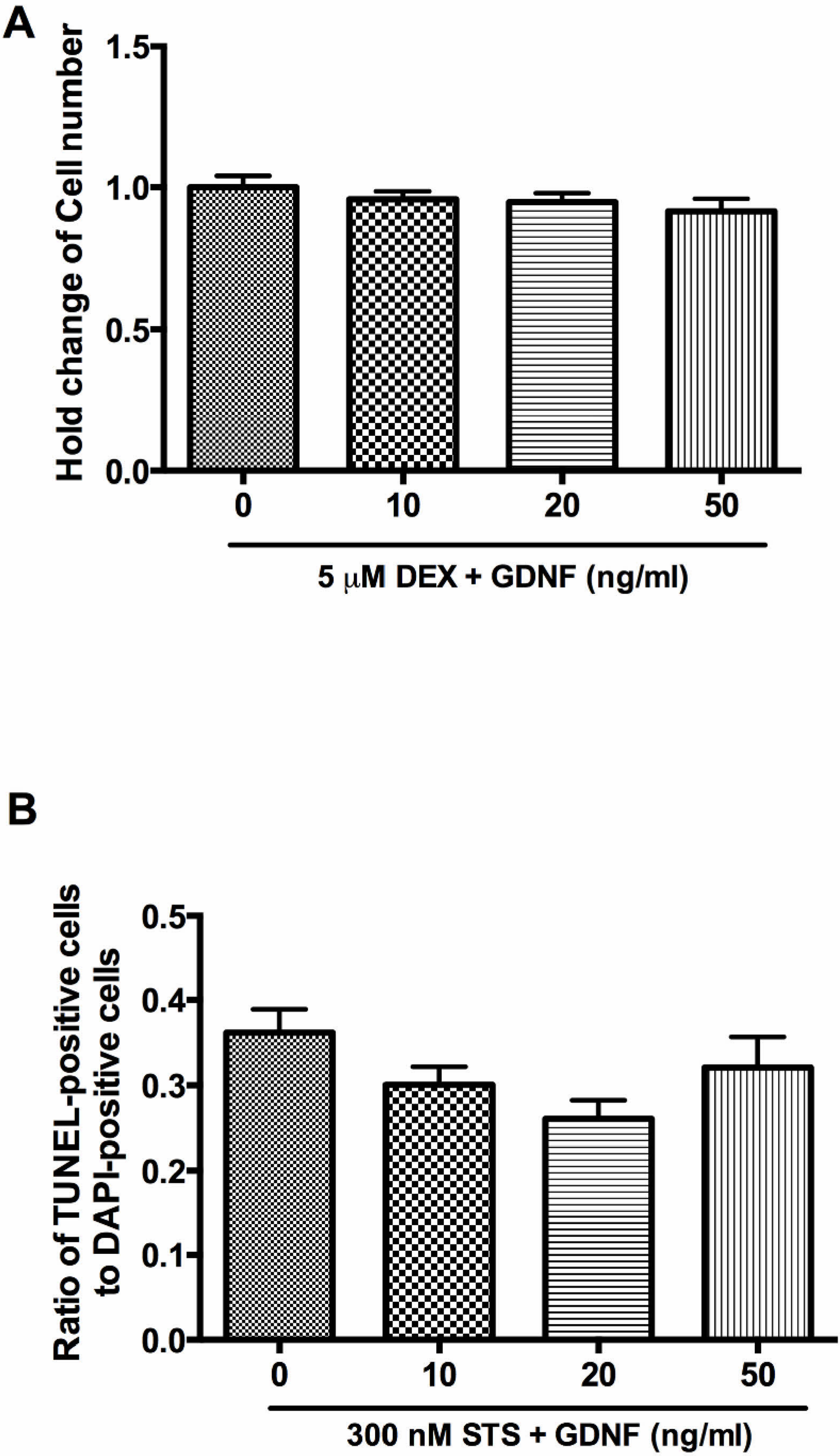
Figure 3. GDNF-induced activation of STAT3 is involved in the effects of GDNF on facilitating ADNP differentiation into astrocyte

A. Stattic, a specific inhibitor of STAT3, inhibits GDNF-induced ADNP differentiation into astrocytes. Immunocytochemistry was performed 7 days after treatment of 50 ng/ml GDNF and 500 nM Stattic for four independent cultures. Scale bar = 120 μm.

B. GDNF increases the phosphorylation of STAT3. Preparation of cell lysates was performed 3 days after treatment of 50 ng/ml GDNF. Values are shown as the ratio of phosphorylated STAT3 versus total STAT3.

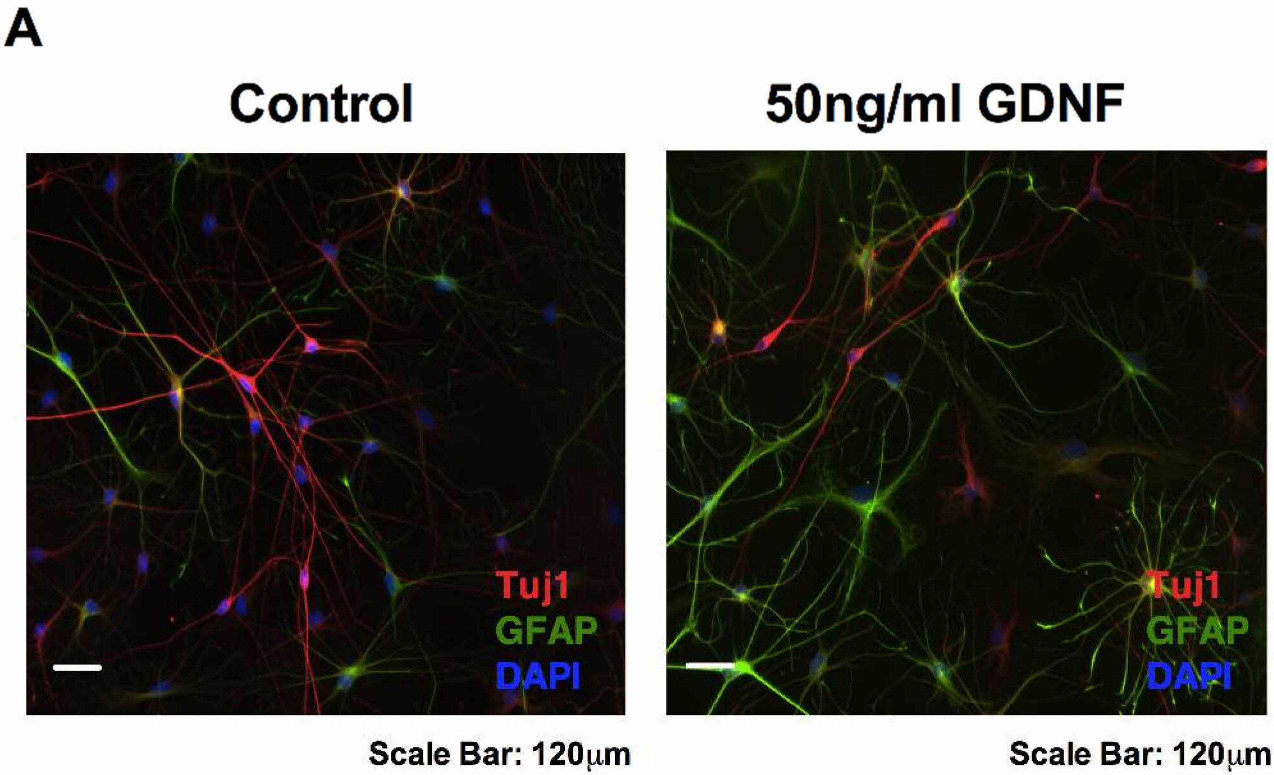
C. Lentiviral shRNA for STAT3 inhibits GDNF-induced ADP differentiation into astrocytes. Immunocytochemistry was performed 7 days after infection of lentivirus for four independent cultures. Scale bar = 120 μm.

Figure 1



A
Hold change of Cell number
1.5
1.0
0.5
0.0
0
10
20
50
5 μM DEX + GDNF (ng/ml)
B
Ratio of TUNEL-positive cells to DAPI-positive cells
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
50
300 nM STS + GDNF (ng/ml)

Figure 2


A
Control
50ng/ml GDNF
Tuj1
GFAP
DAPI
Tuj1
GFAP
DAPI
Scale Bar: 120μm
Scale Bar: 120μm

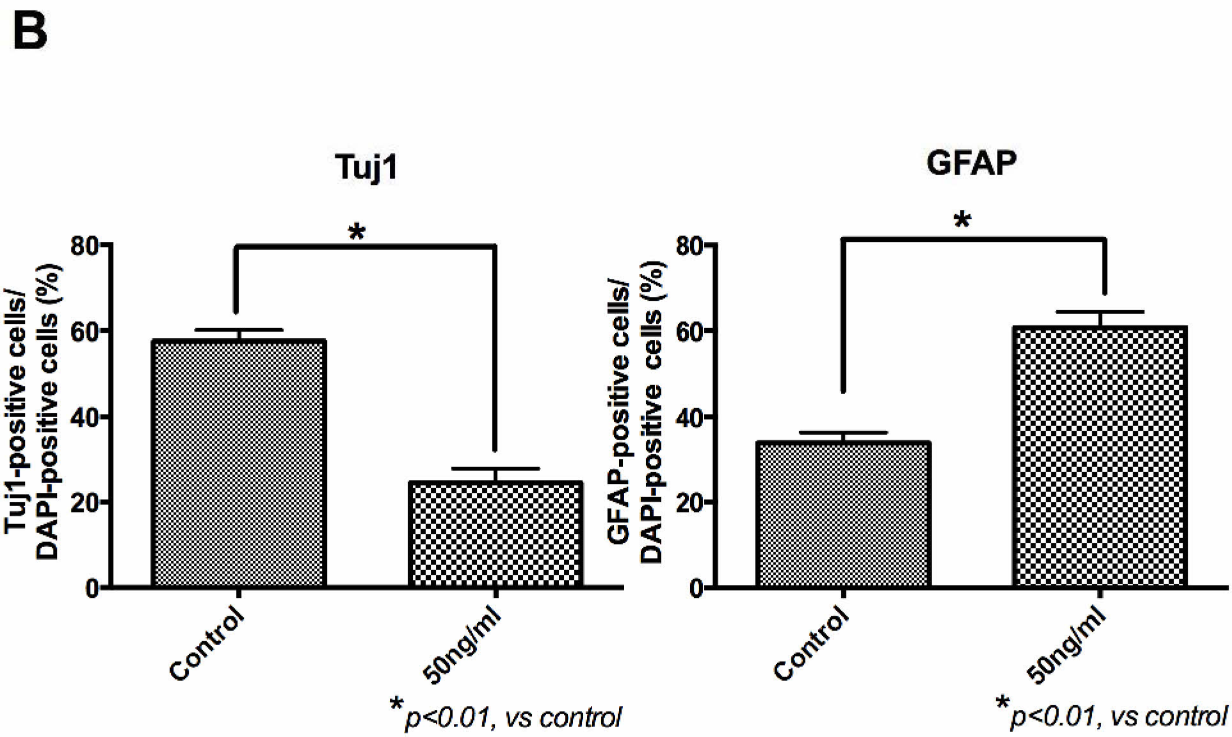
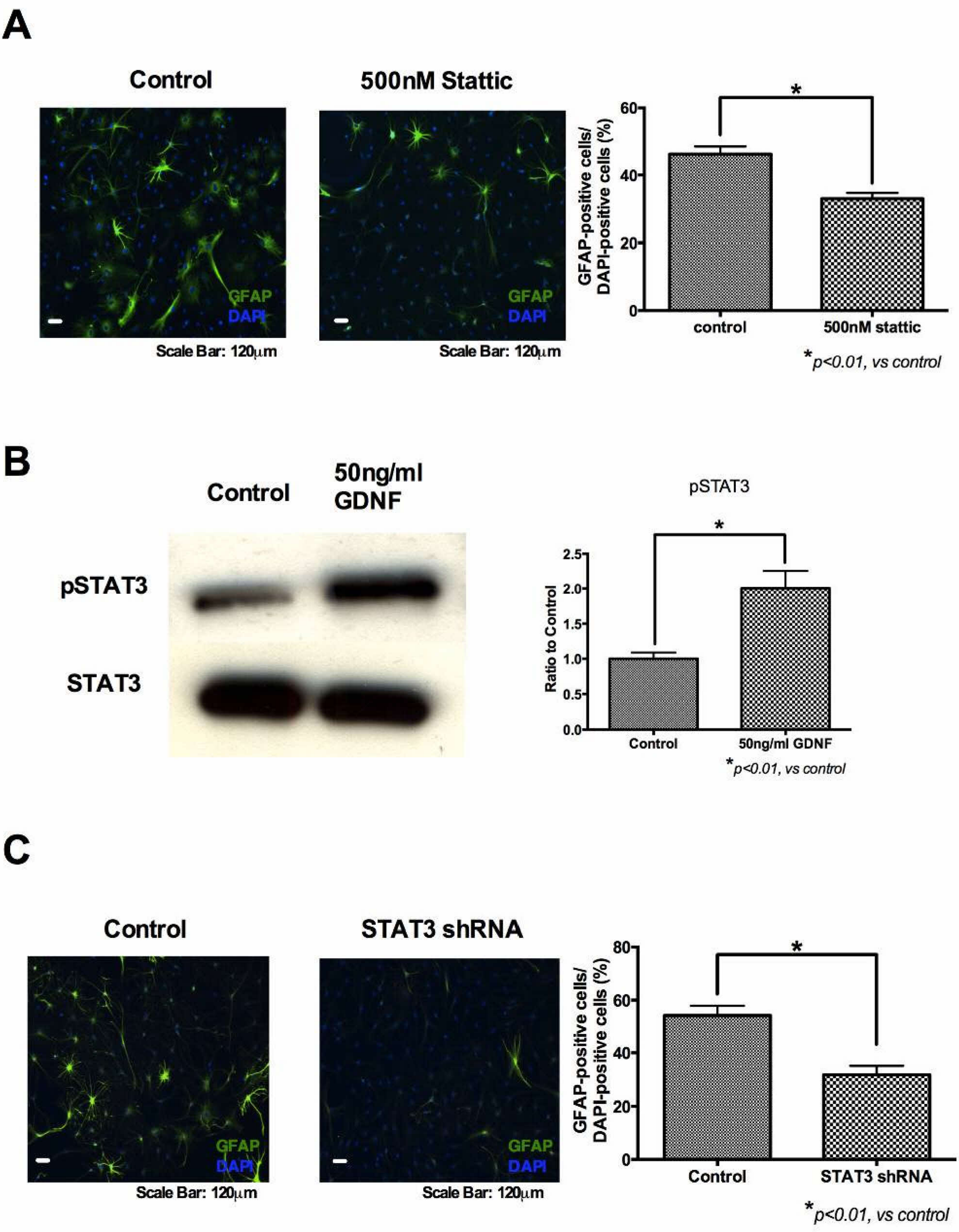

B
Tuj1
GFAP
*
*
Tuj1-positive cells/
DAPI-positive cells (%)
GFAP-positive cells/
DAPI-positive cells (%)
0
20
40
60
80
Control
50ng/ml
*p<0.01, vs control
*p<0.01, vs control

Figure 3



A
Control
500nM Stattic
GFAP
DAPI
Scale Bar: 120μm
GFAP-positive cells/
DAPI-positive cells (%)
60
40
20
0
*
control
500nM stattic
*p<0.01, vs control
B
Control
50ng/ml
GDNF
pSTAT3
STAT3
pSTAT3
Ratio to Control
2.5
2.0
1.5
1.0
0.5
0.0
Control
50ng/ml GDNF
*p<0.01, vs control
C
Control
STAT3 shRNA
GFAP
DAPI
Scale Bar: 120μm
GFAP-positive cells/
DAPI-positive cells (%)
80
60
40
20
0
Control
STAT3 shRNA
*p<0.01, vs control