Neurobiology of Disease

Activation of Phosphatidylinositol-Linked D_1-Like Receptor Modulates FGF-2 Expression in Astrocytes via IP3-Dependent Ca^{2+} Signaling

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Fibroblast growth factor-2 (FGF-2) is predominantly synthesized and secreted by astrocytes in adult brain. Our previous study showed that activation of classical dopamine receptor D_1 or D_2 elicits FGF-2 biosynthesis and secretion in astrocytes. Here, we report that astrocytic FGF-2 expression is also regulated by phosphatidylinositol (PI)-linked D_1-like receptor. SKF83959, a selective PI-linked D_1-like receptor agonist, upregulates the levels of FGF-2 protein in striatal astrocyte cultures in classical dopamine D_1 and D_2 receptor-independent manner. The conditional medium derived from SKF83959-activated astrocytes promoted the number of TH^+ neurons *in vitro*. Treatment of astrocytes with SKF83959 increased intracellular calcium in two phases. Inhibition of intracellular calcium oscillation by inositol 1,4,5-triphosphate (IP3) inhibitors blocked the SKF83959-induced increase in FGF-2 expression. Moreover, intraperitoneal administration of SKF83959 reversed l-methyl-4-phenyl-l,2,3,6-tetrahydropypridine (MPTP)-induced reduction in FGF-2 expression in both the striatum and ventral midbrain and resulted in marked protection of dopaminergic neurons from MPTP-induced neurotoxicity. These results indicate that IP3/Ca^{2+}/calmodulin-dependent protein kinase is an uncharted intracellular signaling pathway that is crucial for the regulation of FGF-2 synthesis in astrocytes. PI-linked D_1-like receptor plays an important role in the regulation of astrocytic FGF-2 expression and neuroprotection which may provide a potential target for the drug discovery in Parkinson's disease.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by selective and progressive loss of nigral dopaminergic (DA) neurons and the formation of Lewy bodies in pathology (Vila et al., 2001). Although symptoms of this devastating disease may be temporally controlled by drug therapy, an effective cure for PD, however, has not been established. Thus, there is currently great interest in the development of more effective approaches for the alteration of PD pathological progression.

Recent studies demonstrated that astrocytes play an important role in neuroprotection via neurotrophic factors. In this regard, some of DA receptor agonists have drawn great attention. For examples, Pramipexole, a dopamine D_2/D_3 receptor agonist used in the treatment of PD, has been reported to exert neuroprotective action on DA neurons via astrocyte-produced factors including brain-derived neurotrophic factor (BDNF) (Imamura et al., 2008). Apomorphine (APO), a D_1/D_2 receptor agonist, also provides symptomatic relief by directly stimulating postsynaptic DA receptors. Meanwhile, our previous studies showed that APO stimulates biosynthesis of BDNF and glial cell line-derived neurotrophic factor (GDNF) in rat mesencephalic as well as striatal neuronal cultures (Guo et al., 2002). Furthermore, APO was found to enhance biosynthesis and release of fibroblast growth factor-2 (FGF-2) which promotes the survival and protection from neurotoxin-induced death of DA neurons (Chadi et al., 1993; Grothe et al., 2000). Enhanced FGF-2 expression is further demonstrated to be mediated through activation of DA receptors in striatal astrocytes (Li et al., 2006). Together, these findings suggest a crucial role of nigrostriatal astrocytes in DA neuronal survival and the nigrostriatal astrocytes emerge as a promising target for drug development of PD. Astrocytic DA receptors (Bal et al., 1994; Luo et al., 2009) may be important mediators regulating astrocytic activities and bridging neuron–glia interactions.

In addition to classical DA receptors, regulation of phosphatidylinositol (PI)-linked D_1-like receptor has recently been identified as a novel approach for drug discovery of PD. SKF83959 [3-methyl-6-chloro-7,8-hydroxy-1-(3-methylphenyl)-2,3,4,5-

Received Jan. 24, 2009; revised May 5, 2009; accepted May 5, 2009.

This work was supported by grants from the Chinese Ministry of Science and Technology (nos. 2006AA02Z184, 2006AA02A114, 2007AA02Z163), Shanghai Metropolitan Fund for Research and Development (no. 07DJ14005), Natural Science Foundation of China (nos. 30525041, 30623003, 30721004), and State Key Program for Basic Research of China (nos. 2006CB500704, 2009CB522201). We thank S.-L. Qian (Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China) for excellent assistance in HPLC analysis.

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DOI:10.1523/JNEUROSCI.0389-09.2009

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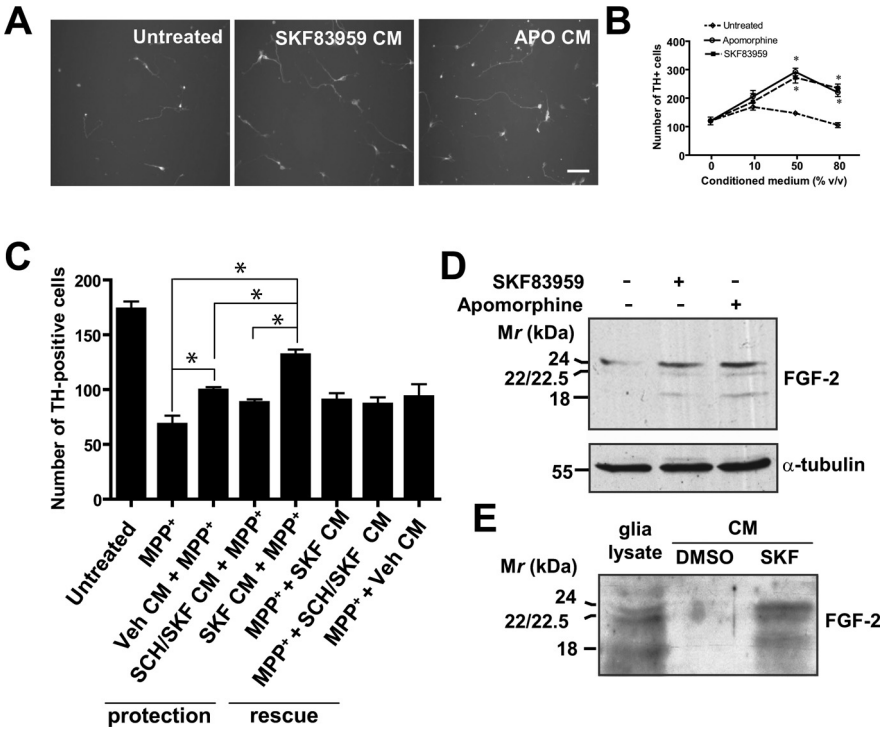

Figure 1. Increased FGF-2 levels in the CM from primary striatal astrocytes elevated the number of TH^+ neurons. ***A***, VM cultures were incubated with 50% (v/v) untreated, SKF83959- and APO-stimulated CM from donor striatal astrocytes for 24 h and were then fixed for TH immunofluorescent staining. Scale bar, 100 μm. ***B***, Dose–response curves show the effect of SKF83959-induced CM from donor striatal astrocytes on the number of TH^+ neurons. The CM was prepared from donor striatal astrocytic cultures by incubating cultures with or without SKF83959 and APO (positive control). The CM was added to recipient cultures of E14 VM plated at 1×10^5 cells/cm^2. Cultures were fixed 24 h after cultures were switched to serum-free medium, and the number of TH^+ neurons was counted. Data represent means ± SEM from at least three independent experiments performed in triplicates; *$p < 0.01$, compared with untreated. ***C***, Recipient VM cultures are incubated for 48 h with 50% (v/v) SKF83959-, SCH23390 plus SKF83959-stimulated and untreated CM from donor striatal astrocytes before or after administration with 10 μM MPP^+ and then fixed for TH immunostaining. The number of TH^+ neurons was counted, and data represent means ± SEM; *$p < 0.05$, $n = 3$. SCH, SCH23390; SKF, SKF83959; Veh, vehicle. ***D***, Levels of FGF-2 protein are determined using Western blotting. Cell lysates were from striatal astrocytes treated with SKF83959 or APO. ***E***, Both high- and low-molecular-weight forms of FGF-2 were released into the media after SKF83959 treatment. CM derived from donor striatal astrocytic cultures treated with or without SKF83959 was concentrated and subjected to Western blot analysis using FGF-2 pAb.

tet-rahydro-1*H*-3-benzazepine], a recently identified PI-linked D_1-like receptor agonist, selectively stimulates PI hydrolysis via Gq-mediated activation of phospholipase C β (PLCβ) (Frail et al., 1993; Jin et al., 2003). The powerful antiparkinsonian action of SKF83959 is believed to associate with the drug-elicited PLCβ/PI-hydrolysis (Zhen et al., 2005; Zhang et al., 2007). Moreover, chronic SKF83959 treatment was found to attenuate the development of levodopa-induced dyskinesia (Andringa et al., 1999; Zhen et al., 2005; Zhang et al., 2007). The latter is thought to attribute to the drug's potent neuroprotection (Yu et al., 2008). However, the detailed mechanism for SKF83959-mediated neuroprotection remains elusive.

In the present study, we tested how striatal astrocytes respond to specific PI-linked D_1-like receptor agonist SKF83959 and how this D_1-like receptor-mediated signal pathway in astrocytes contributes to the modulation of FGF-2 expression. Our results demonstrated that treatment of astrocytes with SKF83959 elevated FGF-2 expression which was associated with the activation of inositol 1,4,5-triphosphate (IP3)/Ca^{2+}/calmodulin-dependent protein kinase (CaMK) signaling. Our data provide a novel mechanism for PI-linked D_1-like receptor in the regulation of astrocyte function which may, in turn, underlie the potent antiparkinsonian neuroprotection.

Materials and Methods

Animals. Adult or neonatal C57BL/6 mice (weighing 20–22 g) or Sprague Dawley rats (weighing 200–225 g) were from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. They were maintained on a 12 h light/dark cycle at 23°C with food and water available *ad libitum*. All procedures performed were approved by the Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Mesencephalic neuronal culture. Primary ventral mesencephalic (VM) cell cultures were prepared as described previously (Guo et al., 2002). Briefly, fetuses obtained from pregnant rat on the 14th gestational day (E14, where E0 is the day of the vaginal plug) were used for preparation of VM cell cultures. Briefly, after injection with overdose of pentobarbital sodium, the ventral mensencephalons were collected and digested with trypsin. Cell suspension was plated onto poly-D-lysine coated 96-well plates at a density of 100,000 cells/cm^2 in DMEM/Ham's F12 medium (Invitrogen, Life Technologies) containing 10% fetal bovine serum (FBS; Invitrogen). Cells were incubated at 37°C in a 95% air-5% CO_2 humidified atmosphere and maintained for 3 h, and the cells were then switched to serum-free Neurobasal medium with 2% B27 supplement (Invitrogen).

Primary astrocytic culture. Astrocytes were prepared from the striata of 1- to 2-d-old neonatal or adult Sprague Dawley rats, as described previously by Menet et al. (2001). The neonatal striata were trypsinized for 10 min, whereas the adult striata were digested by 0.1% papain, 0.02% cysteine, 0.02% EDTA, and 0.001% DNase in sterile D-Hank's buffer for 30 min. Tissues were dissociated and plated at a density of 5×10^7 cells per 75 cm^2 flask (Corning) in DMEM/Ham's F12 containing 10% FBS. Culture media were changed 24 h later to complete medium and subsequently once a week. Cultures were shaken to remove the top layer of cells sitting over the astroglial monolayer to yield mainly type-1 astrocytes with a flat morphology between day 5 and 7.

Before experimental treatments, astrocytic cultures were passaged once. Cells were allowed to reach 90% confluence. Cultures were treated with compounds at various concentrations for the indicated incubation times. Untreated cells were included as controls in all experiments.

Preparation of conditioned medium. The conditioned medium (CM) was prepared as described previously (Li et al., 2006). Postnatal striatal astrocytes were allowed to grow to 90% confluence and maintained with FBS-free DMEM/Ham's F12 medium containing SKF83959 or APO for 24 h after pretreatment with or without 50 μM SCH23390. The CM was centrifuged and concentrated by a factor of 10 using an Amicon Ultra Unit (molecular cutoff, 5 kDa; Millipore Bioscience Research Reagents).

In vitro *experiment treatments.* To determine the protective effects of CM on tyrosine hydroxylase-positive (TH^+) neurons in cultures, primary VM cultures in 96-well plates were maintained in CM for 24 h followed by treatment with 10 μM 1-methyl-4-phenylpyridnium (MPP^+; Sigma) for 48 h. To determine the rescue effects of CM on TH^+ neurons, separate sets of VM cultures were treated with 10 μM MPP^+ for 24 h followed by 48 h incubation with CM.

SKF83959 (kindly provided by the National Institute of Mental Heath synthesis program, 333 Ravenswood Avenue, Menlo Park, CA), APO (4 μM), clonidine (100 μM), and DA receptor agonist quinpirole (50 μM)

were added to cultures starting the day after plating out the culture (day 0). DA receptor antagonists SCH23390 (10–50 μM), SKF83566 (10 μM), haloperidol (1 μM) and spiperone (10 μM) and LY294002 (20 μM), PD98059 (50 μM), KT5720 (50 μM), GF109203X (2 μM) and yohimbine (Sigma) were applied to cultures at least 2 h before SKF83959, APO, or clonidine was added. Except for SKF83959, all compounds were from Sigma.

In vivo *experiment treatments.* To test *in vivo* effects of SKF83959 on FGF-2 expression, adult rats were administered three intraperitoneal injections of 0.5 or 2 mg/kg SKF83959 ($n = 8$ for each group), 1.5 mg/kg APO ($n = 7$), or vehicle [0.2% dimethylsulfoxide (DMSO) in saline solution] ($n = 6$) at 8 h intervals. Twenty-four hours later, the animals were killed by rapid decapitation, and the striatum and ventral brain were dissected and processed for Western blot analysis.

To investigate the *in vivo* neuroprotective effects of SKF83959 on the nigral DA neurons, the mice were injected daily with SKF83959 (0.5 or 2 mg/kg) or the vehicle for 7 consecutive days. On day 3 of SKF83959 treatment, mice were given intraperitoneal injections of l-methyl-4-phenyl-l,2,3,6-tetrahydropypridine (MPTP; 20 mg/kg) administered for four times at 2 h intervals as described previously (Kurosaki et al., 2004), and the total dose per mouse was 80 mg/kg. Animals in control group received 0.9% NaCl injection at the same time when SKF83959 or MPTP was administrated. On day 7 after MPTP treatment, animals were perfused with 0.9% NaCl and 4% paraformaldehyde in 0.1 M phosphate buffer (PB) orderly, and brains were rapidly removed and coronal sections were made with a freezing microtome at a thickness of 25 μm. Sections through VM were processed for immunohistochemistry.

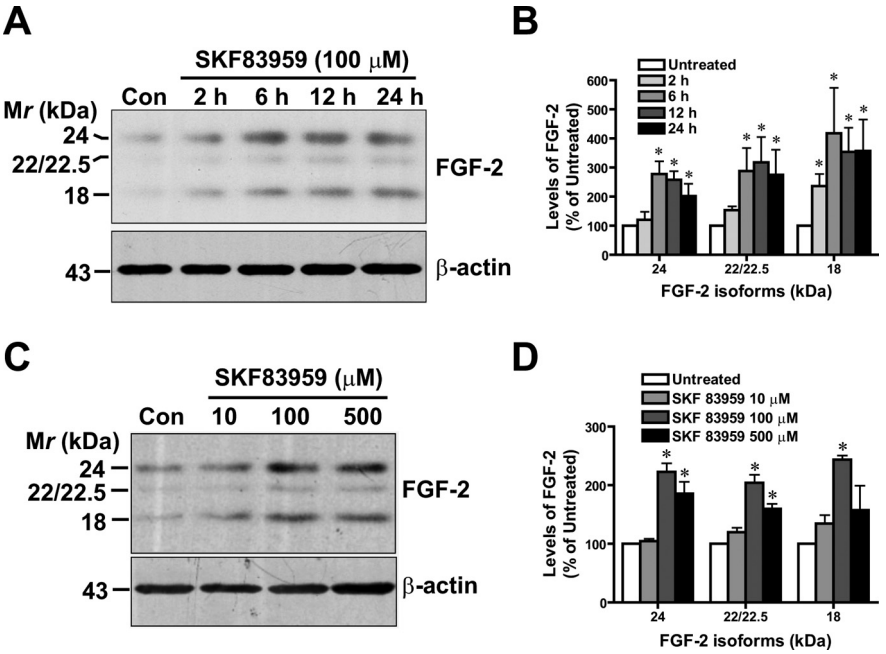

Figure 2. Treatment of astrocytic cultures with SKF83959 increased protein levels of FGF-2 in dose- and time-dependent manners. ***A***, Time dependency of FGF-2 expression. FGF-2 levels in cultured striatal astrocytes were assessed by Western blot after SKF83959 (100 μM) treatment at various time points (0, 2, 6, 12, and 24 h). β-Actin serves as a loading control. ***B***, Quantitative data as shown in ***A***. $^*p < 0.05$, compared with control. ***C***, Dose dependency of SKF83959-induced FGF-2 expression. Cultured striatal astrocytes were treated with various concentrations of SKF83959 (10, 100, and 500 μM). Six hours later, FGF-2 levels were assayed by Western blot analysis. The bottom shows β-actin levels. ***D***, Quantitative data as shown in ***C***. $^*p < 0.05$, compared with control. Con, Control.

Immunostaining and cell count. VM neuronal cultures in 96-well plates were fixed with 4% paraformaldehyde in 0.1 M PB, pH 7.4. The fixed cultures or brain sections were immunostained with rabbit anti-TH polyclonal antibodies (pAb; 1:1000 dilution; Millipore Bioscience Research Reagents). After washing, the VM cultures were incubated with a FITC-conjugated anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories), whereas cryosections were incubated with a biotin-conjugated anti-rabbit IgG and then with avidin–biotin–peroxidase complex (ABC Elite; Vector Laboratories). Signals were detected with 3,3-diaminobenzidine (Sigma).

Numbers of TH^+ neurons in the recipient VM cultures were counted in entirely well. Controls included samples without primary antibodies. The number of TH^+ cells and Nissl-stained neurons were quantified in brain cryosections with typical morphology of the substantia nigra (SN) and ventral tegmental area, as described previously (Sauer et al., 1995).

Western blot analysis and semiquantification. Western blotting was performed as described previously (Li et al., 2006). The following primary antibodies were used: (a) rabbit anti-FGF-2 pAb (1:300; Santa Cruz Biotechnology); (b) rabbit anti-BDNF pAb (1:200; Santa Cruz Biotechnology); (c) rabbit anti-GDNF pAb (1:500; Santa Cruz Biotechnology); (d) rabbit anti-neurotrophin-3 (NT-3; 1:200; Santa Cruz Biotechnology); (e) mouse anti-α-tubulin monoclonal antibody (mAb; 1:4000; Sigma); (f) mouse anti-β-actin mAb (1:5000; Sigma); (g) rabbit anti-phospho-CaMKIIα pAb (1:500; Cell Signaling Technology). The membrane was washed and incubated for 1 h at room temperature with the corresponding secondary antibodies: (a) HRP-conjugated goat anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Laboratories); (b) HRP-conjugated goat anti-mouse IgG (1:10,000; Jackson ImmunoResearch Laboratories). Peroxidase activity was detected with SuperSignal WestPico chemiluminescent substrate (Pierce Biotechnology) and visualized with Kodak BioMax film. Bands were digitized, and optical densities were analyzed by using ImageMaster 2D Platinum (version 5.0; GE Healthcare). FGF-2 protein isoform and other protein levels, quantified by computer analysis as the ratio between each immunoreactive band and the levels of α-tubulin or β-actin, were expressed as a percentage of untreated cultures.

To assay FGF-2 protein secreted into culture medium, proteins in samples were precipitated with acetone as described previously (Li et al., 2006) before Western blotting analysis.

Protein kinase A phosphorylation assay. The phosphorylation assay to detect cAMP-dependent protein kinase was performed according to the manufacturer's instructions. Briefly, astrocytes stimulated with or without SKF83959, SKF38393, or APO for 30 min were washed once in 1× PBS and lysed, and supernatants were assayed for protein kinase A (PKA) activity using the PepTag nonradioactive PKA assay kit (Promega). Samples were loaded onto 0.8% agarose gels and electrophoresed at 100 V for 15 min.

Calcium imaging. Striatum astrocytes, grown on coverslips and serum starved for 2 h at 37°C, were incubated with 4 μM fluo-3 AM (dissolved in DMSO; Dojindo Laboratories) in serum-free medium for 30 min at 37°C and washed three times with HEPES-buffered saline. In some cases, astrocytes were pretreated with IP3 inhibitors 2-aminoethoxydiphenyl borate (2-APB) and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octylester (TMB-8) and calcium chelators 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid acetoxymethyl (BAPTA-AM) or EGTA for 2 h before incubation with fluo-3 AM. Cells in coverslips were mounted on a perfusion chamber containing HEPES-buffered saline and were scanned every 5 s with a 488 nm excitation argon laser and a 515 nm long-pass emission filter under a confocal laser-scanning microscope (Bio-Rad Radiance 2000; Bio-Rad). In later stage of this study, a confocal laser-scanning microscope (TCS SP5; Leica) was also used. At least 40 cells under 20× objective were examined in each independent experiment. After the baseline of $[Ca^{2+}]_i$ was observed for ~30–50 s, SKF83959, glutamate, quinpirole, and SKF38393 were added gently to the perfusion chamber. Up to 50–100 images were acquired from single experiment. Image processing was per-

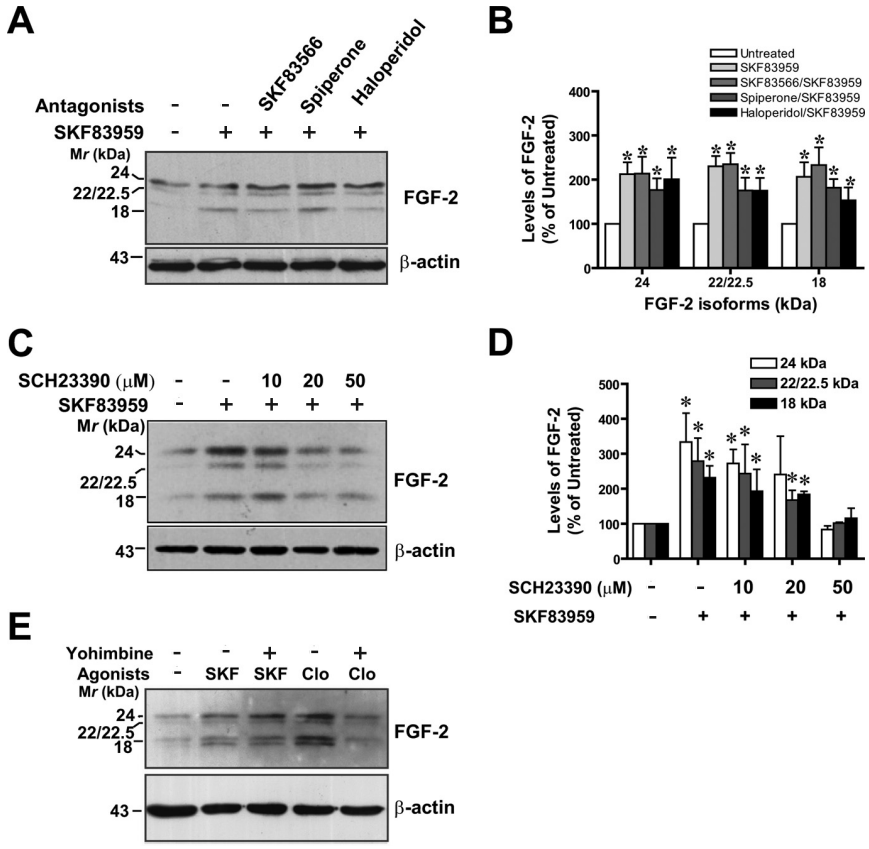

Figure 3. Neither classical D_1 or D_2 dopamine receptor nor α_2-adrenoceptor is involved in SKF83959-modulated FGF-2 biosynthesis. ***A***, Dopamine receptor antagonists failed to block SKF83959-induced increase of FGF-2 protein. Striatal astrocyte cultures were stimulated with SKF83959 for 6 h after treatment with SKF83566 (D_1 receptor antagonists), spiperone, and haloperidol (D_2 receptor antagonists) for 2 h. Six hours later, cells were harvested, and FGF-2 levels were assayed by Western blot analysis. β-Actin serves as a loading control. ***B***, Quantitative data as shown in ***A***. $n = 5$; $^*p < 0.05$, compared with untreated group. ***C***, SCH23390 blocks SKF83959-stimulated FGF-2 expression in a dose-dependent manner. Striatal astrocyte cultures were stimulated with SKF83959 for 6 h after pretreatment with 10, 20, and 50 μM SCH23390 for 2 h. Six hours later, cells were harvested, and FGF-2 levels were assayed by Western blot analysis. ***D***, Quantitative data as shown in ***C***. $n = 3$; $^*p < 0.05$, compared with untreated group. ***E***, α_2-Adrenoceptor antagonist did not inhibit SKF83959-increased FGF-2 expression. After preincubation with or without yohimbine (α_2-adrenoceptor antagonist), cultured striatal astrocytes were stimulated with SKF83959 (SKF) or clonidine (Clo, α_2-adrenoceptor agonist). Cell lysates were subjected to Western blot using FGF-2 Ab. β-Actin levels are shown as a loading control.

formed using software LaserSharp 2000 (Bio-Rad), and the changes of $[Ca^{2+}]_i$ at the single-cell level were analyzed. Results were expressed as the relative fluorescence intensity (RFI).

Statistical analysis. Statistical analysis was performed using GraphPad software (GraphPad Prism v4.0; GraphPad Software). Data presented as mean ± SEM were submitted to one-/two-way ANOVA followed by either Dunnet test or Student–Newman–Keul's test (as a *post hoc* test). $p < 0.05$ was considered as significant in statistics.

Results

SKF83959 induces FGF-2 expression and release in astrocytes and elevates the number of TH$^+$ neurons *in vitro*

We have previously demonstrated that activation of $D_{1/2}$ receptor by APO enhances the survival of cultured DA neurons via increased synthesis and release of FGF-2 from astrocytes (Li et al., 2006). We hypothesized that activation of specific PI-linked D_1-like receptor in astrocytes may exert a similar effect. To test this hypothesis, various percentages (10, 50, and 80%) of CM from astrocytic cultures treated with either SKF83959 (10–50 μM), APO (as a positive control), or vehicle (DMSO) were used to culture DA neuron-enriched recipient VM cells. Cultures were maintained for 1 d and then fixed for TH immunostaining. As shown in Figure 1, *A* and *B*, CM derived from either SKF83959- or APO-stimulated astrocytic cultures increased the number of TH$^+$ neurons in recipient VM cultures in a dose-dependent manner. The number of TH$^+$ neurons in these cultures increased 1.9- or 2.3-fold when exposed to 50 or 80% SKF83959-stimulated CM, respectively, compared with that of vehicle-treated CM. This promoting effect was not the direct effect of SKF83959 or APO, because the incubated CM have been concentrated through a 5 kDa molecular cutoff Ultra Filter by centrifugation, and SKF83959 or APO was removed by discarding <5 kDa fraction. These results indicate that soluble neurotrophic factors in the CM from the astrocytic cultures exposed to SKF83959 may be responsible for the elevated number of TH$^+$ neurons.

To determine whether the SKF83959-stimulated CM can rescue TH$^+$ neurons *in vitro* against MPP$^+$-induced neurotoxicity, recipient VM cultures were treated with 10 μM MPP$^+$ before or after incubation with the CM derived from donor astrocyte cultures either treated with SKF93959 alone or cotreated with SCH23390 and SKF83959. Compared with MPP$^+$ treatment alone, the SKF83959-situmlated CM significantly protected but not rescued those TH$^+$ neurons in cultures from MPP$^+$ neurotoxicity (Fig. 1*C*). These results indicate that soluble neurotrophic factors in the CM from the astrocytic cultures exposed to SKF83959 may be responsible for the elevated number of TH$^+$ neurons.

To elucidate the potential role of FGF-2, the CM was concentrated with the addition of acetone, and precipitates were subjected to Western blot analysis. As shown in Figure 1, *D* and *E*, the levels of FGF-2 isoforms were markedly increased in both the lysates and CM of striatal astrocytes treated with SKF83959 for 18 h. FGF-2 antibodies recognized four FGF-2 isoforms (24, 22.5, 22, and 18 kDa). In the contrast, FGF-2 isoforms were not detected in the CM of untreated astrocytic cultures (Fig. 1*E*). These data suggest that FGF-2 is present in the CM of SKF83959-treated striatal astrocytic cultures.

We further characterized the time course and dose–response for SKF83959-induced FGF-2 expression in striatum-derived astrocytes. A significant enhanced expression of FGF-2 isoforms was already evidenced at 6 h after exposure to SKF83959 and maintained to 12 and 24 h onward (Fig. 2*A*,*B*). The maximal increase in FGF-2 expression was observed at 100 μM SKF83959 (Fig. 2*C*,*D*). Moreover, in contrast to upregulated FGF-2 expression induced by SKF83959 treatment (Fig. 1*D*,*E*), the mRNA and protein levels of known neurotrophic factors including GDNF, BDNF, and NT-3 were not significantly increased in SKF83959-treated astrocytes (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). For the following experiments, unless indicated, 100 μM SKF83959 and 6 h treatment were used.

Classical D_1 and D_2 DA receptors and α_2-adrenergic receptor are not involved in SKF83959-induced modulation of FGF-2 expression in astrocytes

In addition to D_1-like receptor, SKF83959 also exhibits moderate or weak affinities to D_2 receptors, α_2-adrenoceptors (Andringa et al., 1999b). To clarify which receptor mediates the SKF83959-stimulated FGF-2 expression in astrocytes, astrocytic cultures from neonatal striatum were pretreated with various respective receptor antagonists for 2 h and then incubated with SKF83959 for additional 6 h. As shown in Figure 3, FGF-2 expression in cell lysates was dose-dependent attenuated by selective D_1 receptor antagonist SCH23390. However, neither the D_2 receptor antagonists nor the α_2-adrenoceptors antagonist altered SKF83959-stimulated FGF-2 expression, although activation of α_2-adrenoceptor by clonidine enhanced FGF-2 expression which was blocked by yohimbine. Together, these results suggest that SKF83959-stimulated FGF-2 upregulation was associated with PI-linked D_1-like receptor activation but independent of either D_2 receptor or α_2-adrenoceptor.

SKF83959 modulates FGF-2 biosynthesis via PLC/IP3/Ca^{2+} signal pathway

Activation of PI-linked D_1-like receptor is known to stimulate PI-hydrolysis via PLCβ and results in the production of IP3 and diacylglycerol (DAG). IP3 subsequently induces intracellular calcium release and the activation of CaMKII, whereas DAG activates protein kinase C (PKC). We next asked which intracellular signaling pathways mediate SKF83959-induced FGF-2 expression. Preincubation of astrocytic cultures of the striatum with either IP3 inhibitor 2-APB or TMB-8 completely abolished SKF83959-enhanced FGF-2 expression (Fig. 4*A*,*B*), whereas the IP3 inhibitors significantly suppressed glutamine- and SKF83959-induced Ca^{2+} oscillation (Fig. 4*C*,*D*). In accordance with enhanced FGF-2 expression, we also detected 4.7-fold increase in phosphorylation of CaMKIIα protein in astrocyte cultures compared with untreated (Fig. 4*F*). Together, SKF83959 stimulates astrocytic Ca^{2+} oscillation and CaMKII activation that is associated with the drug-upregulated FGF-2.

Intracellular calcium release mediates astrocytic FGF-2 expression

In contrast to single calcium spike evoked by glutamate, SKF83959 elicited two phase

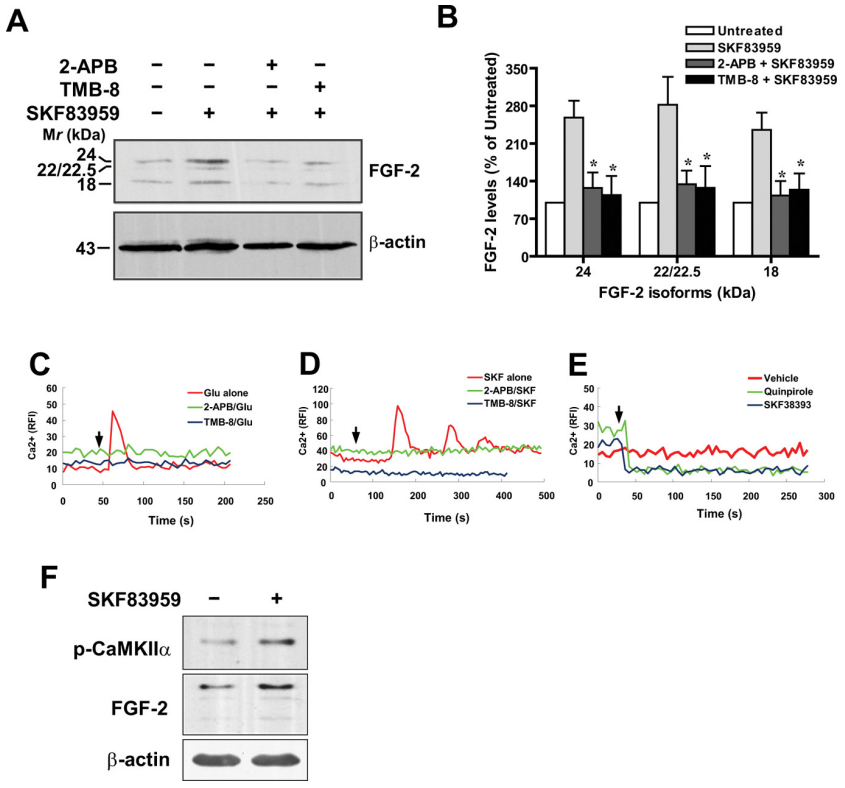

Figure 4. IP3/CaMK pathways are involved in SKF83959-modulated FGF-2 expression. ***A***, IP3 inhibitors completely blocked SKF83959-induced FGF-2 expression. Striatal astrocytes are stimulated with SKF83959 after a 2 h treatment with 2-APB and TMB-8. Six hours later, FGF-2 levels are assayed by Western blot analysis using FGF-2 pAb. β-Actin serves as a loading control. ***B***, Quantitative data as shown in ***A***. $n = 4$; $^*p < 0.05$, compared with the SKF83959-treated astrocytes. ***C–E***, Calcium monitoring in striatal astrocytes stimulated by glutamate (***C***), SKF83959 (***D***), or selective dopamine receptor agonists (***E***). Serum-starved striatal astrocytes labeled with 4 μM fluo-3 AM is monitored by using a laser-scanning confocal microscope. Levels of $[Ca^{2+}]_i$ are presented as the RFI. ***C***, $[Ca^{2+}]_i$ changes when treated with glutamate in presence of IP3 blockers 2-APB (green) or TMB-8 (blue) or in their absence (red). ***D***, Levels of $[Ca^{2+}]_i$ changes when treated with SKF83959 in the presence of 2-APB (green) or TMB-8 (blue) or in their absence (red). Approximately 40 or 50 astrocytes were observed under a 20× objective, and approximately ≥80% of the observed cells exhibited intracellular calcium increase. Traces were recorded on representative astrocytes. ***E***, Levels of $[Ca^{2+}]_i$ changes when treated with vehicle DMSO (red), D_1 dopamine receptor agonist SKF38393 (blue), or D_2 dopamine receptor agonist quinpirole (green). Each trace is a single-cell representative from at least four independent experiments. Glu, Glutamate; SKF, SKF83959. ***F***, SKF83959 activates phosphorylation of CaMKIIα. After 6 h stimulation with SKF83959, striatal astrocytes were harvested, and supernatants were assayed for level of phospho-CaMKIIα.

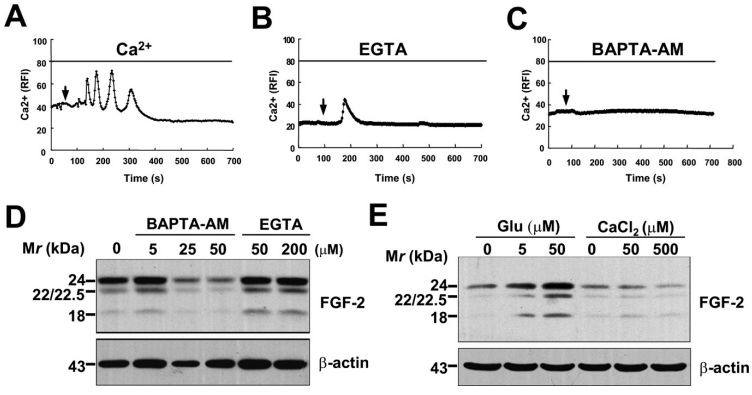

Figure 5. Intracellular calcium release mediates astrocytic FGF-2 expression. Cytosol calcium in striatal astrocytes is monitored after treatment with SKF83959. ***A***, Serum-starved striatal astrocytes labeled with 4 μM fluo-3 AM are stimulated directly by SKF83959. Calcium imaging is performed under confocal laser-scanning microscope (TCS SP5; Leica). Note that although the calcium fluctuation pattern is not exactly the same as that shown in Figure 4*C*, the timing for the first calcium spike is approximately the same as that in Figure 4*C*, i.e., 100 s. ***B***, Calcium in medium is removed by 2 h pretreatment with EGTA before SKF83959 stimulation. ***C***, Intracellular calcium is chelated by 2 h pretreatment with BAPTA-AM before SKF83959 treatment. ***D***, Starved striatal astrocytes are treated with BAPTA-AM or EGTA at indicated concentrations for 6 h, and cell lysates are assayed by Western blot by using FGF-2 Ab. ***E***, Administration of glutamate but not $CaCl_2$ upregulates FGF-2 expression in a dose-dependent manner. Striatal astrocytes are treated with gradient concentration of glutamate (Glu, 5 and 50 μM) and $CaCl_2$ (50 and 500 μM) for 6 h, and cell lysates are assayed by Western blot analysis using FGF-2 Ab.

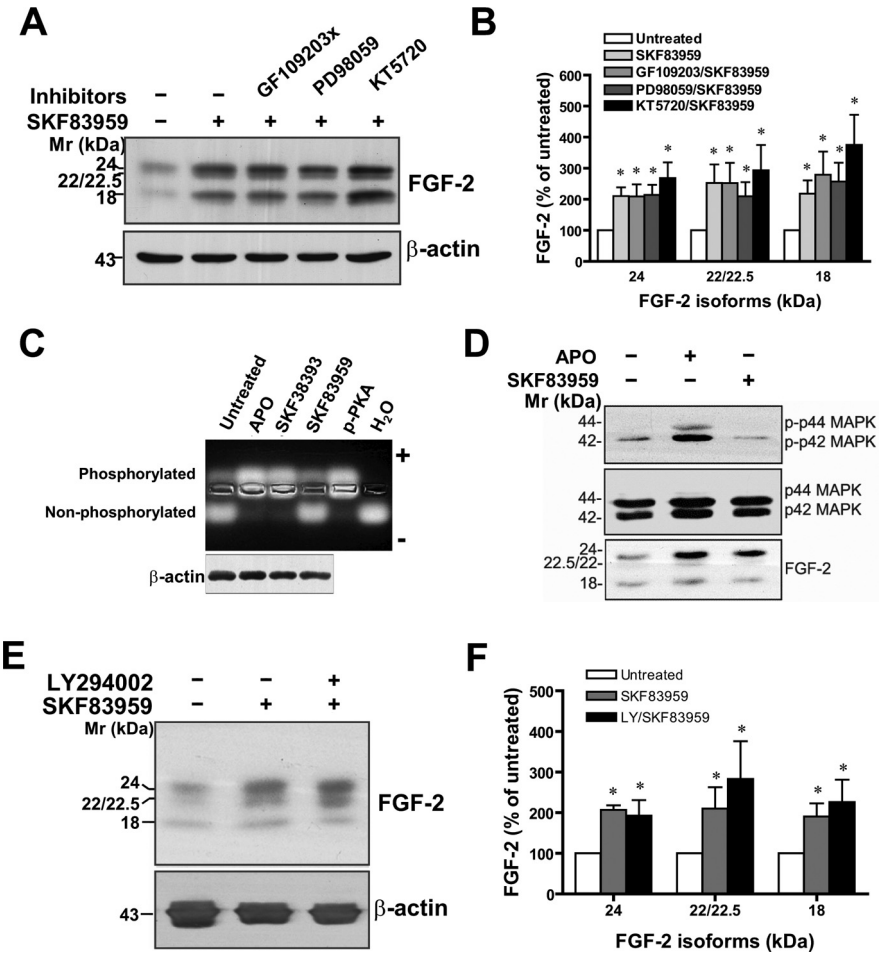

Figure 6. SKF83959-modulated FGF-2 expression is independent of cAMP/PKA, PKC/MAPK, and phosphatidyl inositol 3-kinase (PI-3K)/Akt pathways. ***A***, PKC, MAPK, and PKA inhibitors did not block the effect of SKF83959. Striatal astrocytes were stimulated with SKF83959 after a 2 h treatment with GF109203x (PKC inhibitor), PD98059 (MAPK inhibitor), and KT5720 (PKA inhibitor). Six hours later, FGF-2 levels were assayed by Western blot analysis using FGF-2 Ab. Bottom shows β-actin levels as a loading control. ***B***, Quantitative data as shown in ***A***. $n = 5–6$; $^*p < 0.05$, compared with the untreated. ***C***, SKF83959 fails to activate phosphorylation of PKA. After 30 min stimulation with SKF83959, APO, and SKF38393, striatal astrocytes were harvested, and supernatants were assayed for level of PKA phosphorylation. Phosphorylated PKA and H_2O served as positive and negative controls, respectively. ***D***, MAPK phosphorylation was not activated by SKF83959. Serum-starved striatum astrocytes were stimulated with SKF83959 and APO, and levels of phospho-MAPK and MAPK were assayed by Western blot analysis. ***E***, PI-3K inhibitor did not attenuate SKF83959-induced FGF-2 expression. Striatal astroglial cultures were stimulated with SKF83959 for 6 h after treatment with LY294002 (PI-3K inhibitor), and cell lysates were assayed by Western blot analysis using FGF-2 Ab. β-Actin serves as a loading control. ***F***, Quantitative data as shown in ***E***. $n = 3$; $^*p < 0.05$ compared with the untreated.

responses in Ca^{2+}. An initial calcium spike was evoked up to average threefold increase followed by several delayed calcium fluctuations (Fig. 4*D*, red trace). We thus further characterized the Ca^{2+} components contributing to the SKF83959-induced two-phase responses. Removal of the extracellular calcium by EGTA resulted in disappearance of delayed oscillations, whereas the initial spike of calcium remained (Fig. 5*A*,*B*), indicating that the later components of calcium oscillations were resulted from extracellular calcium influx, whereas application of selective chelation of intracellular calcium BAPTA-AM completely blocked the SKF83959-trigered calcium fluctuations (Fig. 5*C*). These results demonstrated that the initial spike wave resulting from the intracellular store release was necessary for the followed calcium influx.

To determine which calcium component is responsible for SKF83959-stimulated astrocytic FGF-biosynthesis, extracellular calcium was altered by addition of $CaCl_2$ or EGTA into culture medium. These treatments did not change FGF-2 expression (Fig. 5*D*,*E*). In contrast, intracellular calcium depletion by BAPTA-AM significantly downregulated FGF-2 levels (Fig. 5*D*). As expected, treatment with glutamate, which was shown to increase intracellular calcium levels (Fig. 4*C*), also enhanced the FGF-2 expression (Fig. 5*E*). These data indicate that SKF83959-elevated FGF-2 expression in astrocytes is associated with the drug-induced intracellular calcium release.

PKA, PKC, and phosphatidyl inositol 3-kinase do not mediate SKF83959-induced FGF-2 expression

Activation of DA receptors have shown to activate PKA, PKC, mitogen-activated protein kinase (MAPK), and PI-3 kinase pathways, we sought to further verify whether those DA receptor-mediated signal pathways are involved in SKF83959-induced enhancement of FGF-2 expression. Application of respective protein kinase inhibitor KT5720, GF109203x, PD98059, or LY294002 to astrocytic cultures did not alter SKF83959-stimulated FGF-2 expression, which is in agreement with the data that SKF83959 produced no stimulation on all those signal pathways (Fig. 6). In contrast, APO-induced FGF-2 expression was confirmed to be dependent on PKA via Gs-coupled DA D_1 receptor. This further supports that that Gs- or Gi-coupled classical DA receptor signal pathways are not involved in SKF83959-stimulated FGF-2 expression.

SKF83959 promotes astrocytic FGF-2 expression in the striatum and ventral midbrain *in vivo*

Our *in vitro* data demonstrated that SKF83959 markedly promoted striatal astroglial FGF-2 biosynthesis and release, which are beneficial to cultured VM-derived DA neurons. Next, an *in vivo* assay was designed to determine whether SKF83959 also increases FGF-2 biosynthesis. As shown in Figure 7, administration of SKF83959 significantly upregulated FGF-2 protein levels in both the striatum and ventral midbrain ($n = 6–8$ for each group, $p < 0.05$ vs vehicle). These effects were comparable with that of APO treatment (Fig. 7*A–D*). To elucidate whether the contribution of striatal astrocytes in adult mice brain is responsible for the enhanced FGF-2 expression *in vivo*, the striatal astrocytes were cultured *in vitro* and exposed to SKF83959. This treatment significantly increased astrocytic FGF-2 expression (Fig. 7*E*), indicating that astrocytes in the striatum contributed, at least in part, to the upregulation of FGF-2 protein by SKF83959 treatment.

SKF83959 protects nigral DA neurons from MPTP-induced neurotoxicity

Next, we determined whether SKF83959 is effective in neuroprotection of DA neurons *in vivo* using an MPTP-induced PD mouse

model. Mice receiving total dose of 80 mg/kg MPTP exhibited ~50% reduction in the number of TH^+ neurons in the substantia nigra pars compacta (SNc) (Fig. 8*A*,*B*). Levels of low molecular weight FGF-2 (18 kDa) in the striatum, but not in the ventral midbrain, were markedly decreased after MPTP treatment (Fig. 8*C*,*D*). Pretreatment with 0.5 mg/kg SKF83959 remarkably increased the number of TH^+ neurons in the SNc. Higher dose of SKF83959 (2.0 mg/kg) completely prevented the MPTP-mediated DA neuronal loss (Fig. 8*A*,*B*). Accordingly, SKF83959 treatment markedly reversed MPTP-induced downregulation of 18 kDa isoform of FGF-2 in the striatum. Notably, the dramatic elevations in FGF-2 (high molecular forms) levels were also observed in the striatum and ventral midbrain (Fig. 8*D*). Nissl staining revealed that total number of neurons in the SNc decreased markedly after MPTP administration. In contrast, treatment with SKF83959 dramatically increased the number of neurons in this region at a dose-dependent manner (supplemental Fig. S2, available at www.jneurosci.org as supplemental material), suggesting that SKF83959 protects nigral TH^+ neurons not by merely upregulating TH expression *in vivo*.

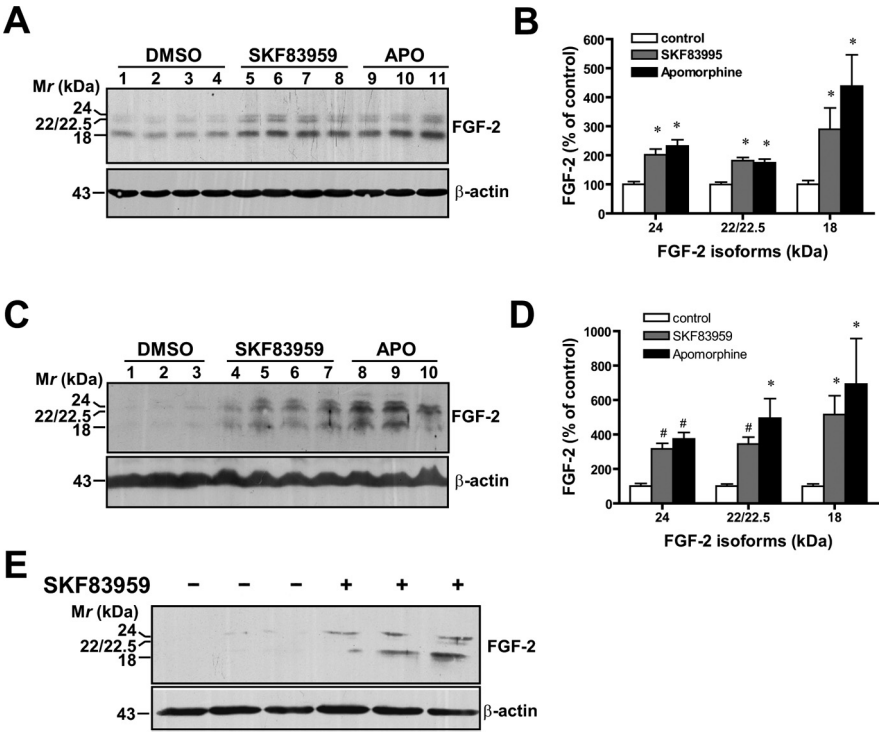

Figure 7. SKF83959 enhances FGF-2 expression in the striatum and ventral midbrain *in vivo*. After intraperitoneal administration of SKF83959 and APO, FGF-2 was assayed in the lysates of the striatum and ventral midbrain using Western blotting. ***A***, Upregulation of FGF-2 in striatum induced by SKF83959 and APO. Lanes 1, 2, 3, and 4 were control animals administrated with vehicle DMSO. Lanes 5, 6, 7, and 8 were those that received SKF83959 injections. Lanes 9, 10, and 11 were those treated with APO, as positive controls. ***B***, Quantitative data as shown in ***A***. $^{\#}p < 0.01$; $^{*}p < 0.05$, compared with control. ***C***, Upregulation of FGF-2 in ventral midbrain induced by SKF83959 and APO. Lanes 1, 2, and 3 were control animals administrated with DMSO. Lanes 4, 5, 6, and 7 were the animals that received SKF83959 injections. Lanes 8, 9, and 10 were the animals treated with APO. ***D***, Quantitative data shown in ***C***. $^{\#}p < 0.01$; $^{*}p < 0.05$, compared with control. ***E***, SKF83959 stimulation promotes adult striatal astrocytic cultures to express FGF-2. Serum-starved adult astrocytes from striatum are treated with SKF83959 for 6 h and harvested for Western blot analysis. Cultures are from three independent mice. β-Actin is used as loading control in ***A***, ***C***, and ***E***.

SKF83959 does not block MPTP conversion to MPP^+ *in vivo*

To investigate whether SKF83959 affects MPTP conversion into MPP^+, MPP^+ was assessed in MPTP-treated mice using high performance liquid chromatography-photodiode assay. The MPP^+ peaks in the striatum matched well with the standard MPP^+ peak at 285 nm (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Sixty minutes after MPTP administration, the conversion of MPTP to MPP^+ in the striatum was markedly blocked by R-(-)-deprenyl, a monoamine oxidase B inhibitor. In contrast, SKF83959 did not alter the levels of MPP^+ ions in the striatum of MPTP-treated mice (supplemental Fig. S3, available at www.jneurosci.org as supplemental material).

Discussion

In the present study, we show that activation of PI-linked D_1-like receptor promotes the number of the nigral DA neurons both *in vitro* and *in vivo* by enhancing biosynthesis and release of FGF-2 in astrocytes. We further demonstrated that activation of PLC/IP3/Ca^{2+} via PI-linked D_1-like receptor is the underlined signal pathways mediating this action. Our data provide clear evidence that astrocytic PI-linked D_1-like receptor signal pathway plays a crucial role in astrocytic function regulation.

The modulation of astrocytic FGF-2 expression by SKF83959 is mediated by PI-linked D_1-like receptor signal pathway

Although accumulating evidences demonstrated that classical DA receptor subtypes D_1–D_5 are expressed in astrocytes, the expression and functional implication of PI-linked D_1-like receptor in astrocytes remains elusive. Our data demonstrated SKF83959, a recently identified selective agonist for PI-linked D_1-like receptor (Panchalingam and Undie, 2001; Jin et al., 2003; Makihara et al., 2007), promotes DA neurons survival via stimulating astrocytic FGF-2 production. SKF83959-induced FGF-2 expression was blocked by IP3 inhibitors and D_1 receptor antagonist SCH23390 but was independent of PKA, MAPK, and PI-kinase pathways. This clearly indicates that stimulation of IP3-mediated intracellular calcium release is associated with SKF83959-mediated astrocytic FGF-2 expression and increased TH^+ neurons (Zhen et al., 2004, 2005).

It is noted that the calcium dynamics induced by SKF83959 in astrocytes exhibited distinct pattern from that seen in hippocampal neurons (Ming et al., 2006). The initial spike of cytosol calcium, as showed in Figures 4*B* and 5*A*, resulted from intracellular calcium release, whereas the late components of calcium fluctuations were extracellular calcium influx via IP3-mediated emptying of the intracellular Ca^{2+} store. In agreement with our data, the Ca^{2+} influx into the cytosol is reported to be triggered by the emptying of the IP3-sensitive calcium stores (Putney, 1990) which induces entry of Ca^{2+} directly into the store, instead of into the cytosol, through a plasma membrane transient receptor potential channel (Chakrabarti and Chakrabarti, 2006). Thus, in the presence of IP3, the store is being emptied and directly refilled alternatively, giving rise to a transient phase of quantal oscillatory Ca^{2+} release. The present data demonstrated that the initial spike stimulated by

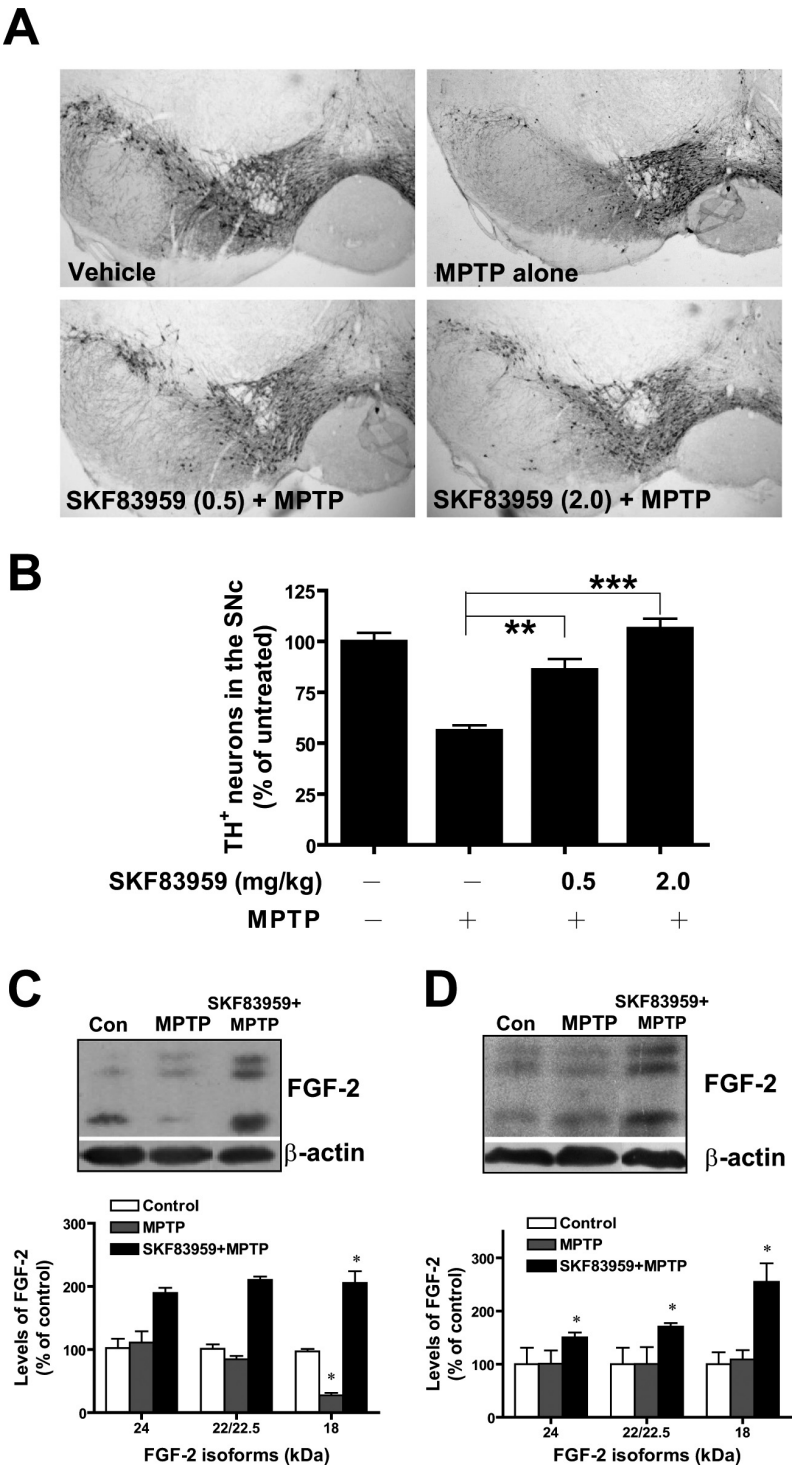

Figure 8. *In vivo* administration of SKF83959 protects dopaminergic neurons in the SNc from MPTP-induced lesion. ***A***, Immunostaining using TH antibodies. Animals received vehicle, MPTP alone, 0.5 mg/kg SKF83959 plus MPTP, or 2.0 mg/kg SKF83959 plus MPTP, respectively. ***B***, Quantitative data of number of TH^+ neurons in the SNc as shown in ***A***. Both 0.5 and 2.0 mg/kg SKF83959 significantly increase the number of TH^+ neurons compared with MPTP-treated mice. $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with the single MPTP-treated animals. ***C***, ***D***, Western blot analysis shows the elevation of FGF-2 protein in the striatum (***C***) and VM (***D***) after the treatment with MPTP either alone or in combination with SKF83959. Tissue lysates of the striatum and VM are assayed using Western blotting on day 4 after exposure to MPTP. Data represent mean ± SEM ($n = 3$); $^{***}p < 0.001$, compared with control. Con, Control.

SKF83959 was the result of intracellular calcium release triggered by IP3, which is necessary and sufficient to trigger the late components of $[Ca^{2+}]_i$ elevation via Ca^{2+} influx because chelation of intracellular calcium depressed all the calcium fluctuations (Fig. 5*C*).

Physiological implications of regulation of FGF-2 expression in astrocytes

FGF-2 is a potent growth factor that is essential for cell proliferation and growth of a variety of cell types in the CNS. Stringent regulation of its biosynthesis and release is thus required for the development and maintenance of the CNS. However, how FGF-2 expression is regulated in astrocytes remains mainly unknown. Our previous studies showed that stimulation of astrocytic D_1 and D_2 receptors leads to activation of cAMP/PKA and mitogen-activated protein kinase kinase (MEK)/MAPK signaling pathways and results in increased biosynthesis and release of FGF-2 (Li et al., 2006). This suggests that neurotransmitter dopamine may modulate FGF-2 expression in astrocytes in physiological conditions. Notably, SKF83959-evoked induction of FGF-2 in astrocytes may represent a distinct mechanism from that of dopamine regulating astrocytic FGF-2 expression. We found remarkable modulatory effect of glutamate on FGF-2 expression through induction of calcium influx, demonstrating similarity with action of SKF83959. Thus implies another interesting function of glutamate in astrocyte activity. Glutamate serves as a neurotransmitter that mediates excitatory neurotransmission and contributes to glutamate–glutamine cycle, which is essential for replenishing the neurotransmitter pool of glutamate. Meanwhile, it also modulates FGF-2 expression in astrocytes via evoking calcium oscillation. The integration of glutamate–glutamine cycle between neurons and astrocytes may be important for the maintenance of FGF expression in astrocytes. Thus, together with previous findings of DA receptor-evoked cAMP/PKA and MEK/MAPK signaling cascades in astrocytes (Li et al., 2006), we may conclude that FGF-2 expression in astrocytes is tightly regulated by multiple signaling pathways in distinct fashions (Fig. 9).

Given that D_1 and D_2 receptor agonists suppress calcium influx in astrocytes, whereas glutamate exhibits the opposite effect (Fig. 4) (van den Pol et al., 1996), we speculate that both neurotransmitters DA and glutamate are required to maintain FGF-2 expression in astrocytes within normal range in physiological condition (Fig. 9). It has been shown that in PD levels of FGF-2 in the substantia nigra are reduced (Tooyama et al., 1993, 1994); we speculate that loss of neurotransmitter DA and PI-linked D_1-like receptor ligand(s) may contribute to reduction of FGF-2 levels in the SN leading to malnutrition of DA neurons.

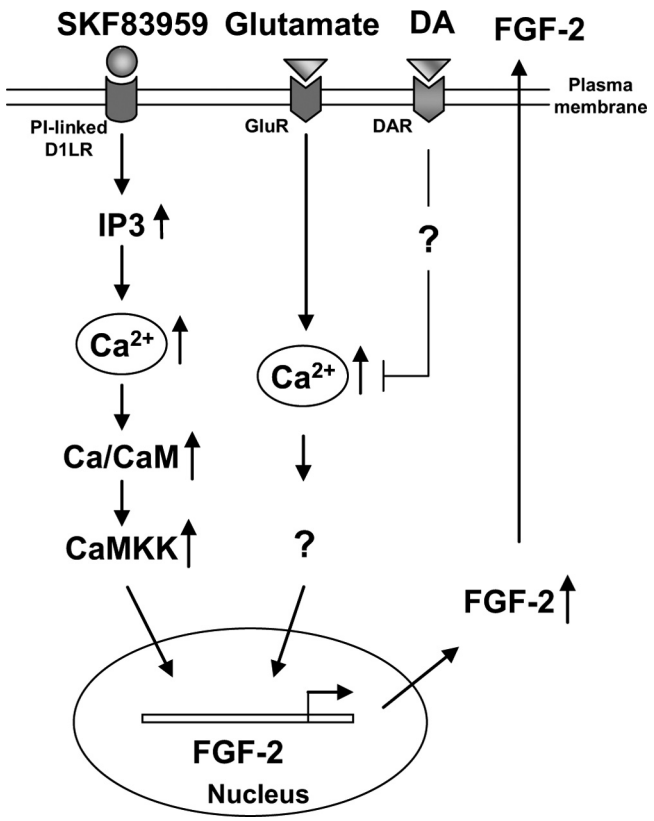

Figure 9. Schematic diagram showing involvement of signal transduction pathway PLC/IP3/Ca^{2+}/CaMK in SKF83959-modulated biosynthesis of FGF-2 in striatal astrocytes. Based on the data shown in the present study and others, it is hypothesized that neurotransmitter dopamine, glutamate, and PI-linked D_1-like receptor (PI-linked D_1LR) ligand(s) may work together to tightly regulate the basic level of FGF-2 in astrocytes via either elevating or suppressing cytosol calcium levels.

SKF83959 increases the number of TH^+ neurons *in vitro* and *in vivo*

Previous studies have shown that SKF83959 is a potent antiparkinsonian agent in nonhuman primates and rodent models of PD (Gnanalingham et al., 1995a,b,c,d; Andringa et al., 1999a; Zhen et al., 2005). Moreover, SKF83959 not only attenuated the dyskinesias, but also reduced the occurrence of motor fluctuation and wearing-off dyskinesia which is frequently observed in antiparkinsonian therapy (Andringa et al., 1999a; Zhang et al., 2007). The underlying mechanism for the anti-dyskinesia action of SKF83959 is believed to associate with the drug's powerful neuroprotection that interrupts the progressive pathological process of DA neuron loss. However, the exact molecular mechanisms underlying its neuroprotective effect is not clear. Although it has been recently shown that SKF83959 exerts neuroprotective effects via D_1-like receptor-dependent inhibition of glycogen synthase kinase-3 β, the receptor-dependent action only accounts for the partial neuroprotection of SKF83959 (Yu et al., 2008). The present study demonstrated, for the first time, that activation of PI-linked D_1-like dopamine receptor induced astrocytic FGF-2 biosynthesis in striatum and consequently promoted survival of DA neurons both *in vitro* and *in vivo*. There is evidence that the SN neurons are negative for FGF-2 mRNA, but there are impressive levels of fibroblast growth factor receptor (FGFR) mRNA in this area (Gonzalez et al., 1995), suggesting that astrocytes are major source of FGF-2 in the brain. Moreover, previous morphological studies of the lesioned rat SN showed increased number of glial cells expressing FGF-2 transcript and an enhanced number of FGF-2 immunoreactive astrocytes. We showed that the striatal astrocytes produced significant higher levels of FGF-2 in response to SKF83959 stimulation that, in turn, contributes to the drug's powerful neuroprotection. This neuroprotection appears not to associate with the change of MPTP conversion into MPP^+, since we demonstrated that SKF83959 did not alter the conversion of MPTP into MPP^+. However, it will be interesting to see if SKF83959 affects the MPP^+ reuptake that may contribute to neuroprotective effect of SKF83959 from MPTP neurotoxicity. Regardless, the finding of activation of PI-linked signal pathway regulating astrocytic FGF-2 expression may provide a novel mechanism for the antiparkinsonian effect and attenuation on development of dyskinesia of SKF83959.

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