Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2009 | 108 | 952–961 doi: 10.1111/j.1471-4159.2008.05845.x

Crucial roles of MZF-1 in the transcriptional regulation of apomorphine-induced modulation of FGF-2 expression in astrocytic cultures

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Abstract

Apomorphine (APO), a potent D1/D2 dopamine receptor agonist, is used as an anti-parkinsonian drug. It stimulates the synthesis and release of multiple trophic factors in mesencephalic and striatal neurons, preventing the loss of dopaminergic neurons *in vitro*. Furthermore, APO enhances the biosynthesis and release of FGF-2 by activating dopamine receptors in striatal astrocytes, where cAMP/PKA and PKC/MAPK signalling cascades mediate this process. We investigate the effects of APO on the fibroblast growth factor-2 (FGF-2) promoter and its regulation in astrocytes and identify the transcription factor and *cis* element underlying these effects. In screening for *cis*-acting elements over the entire region of the FGF-2 promoter stimulated by APO in the astrocytes, a sequence located in the –785/–745 region was found to serve as the *cis* element. This element was recognized by the human myeloid zinc finger protein 1 (MZF-1) transcription factor. Introducing human MZF-1 plasmid and human MZF-1-specific siRNA has different effects on the FGF-2 promoter. Furthermore, it increases FGF-2 protein expression in HeLa cells and primary astrocytes, indicating that APO stimulates the FGF-2 promoter via the MZF-1 transcription factor. These data suggest that APO can enhance the biosynthesis and release of FGF-2 through the activation of the MZF-1 transcription factor in striatal astrocytes.

Keywords: apomorphine, astrocyte, FGF-2 promoter, gene regulation, MZF-1.

J. Neurochem. (2009) **108**, 952–961.

Parkinson's disease (PD) is a degenerative disorder of the CNS that often impairs motor skills and speech as well as other functions (Jankovic 2008). The symptoms of PD result from the loss of pigmented dopamine-secreting (dopaminergic) cells in the pars compacta region of the substantia nigra (literally 'black substance') (Vila *et al.* 2001). These neurons project to the striatum, and their loss leads to alterations in the activity of the neural circuits within the basal ganglia that regulate movement, where this, essentially, results in inhibition of the direct pathway and excitation of the indirect pathway (Obeso *et al.* 2008).

There is presently no cure for PD, but medications or surgery can provide relief from the symptoms. The most widely used forms of treatment are L-dopa, dopamine receptor agonists, monoamine oxidase-B and surgery employing deep brain stimulation (Obeso *et al.* 2008). Apomorphine (APO) is a type of dopaminergic agonist. It is a morphine derivative, but does not actually contain morphine or bind to opioid receptors (Tien *et al.* 2003; Li *et al.* 2006). APO is a relatively non-selective dopamine receptor agonist (Grasby *et al.* 1993) and provides symptomatic relief by direct stimulation of post-synaptic striatal DA receptors (Li *et al.* 2006). One drawback,

Received August 25, 2008; revised manuscript received November 27, 2008; accepted November 29, 2008.

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Abbreviations used: APO, apomorphine; cAMP, cyclic adenosine monophosphate; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FBS, foetal bovine serum; FGF-2/bFGF, fibroblast growth factor-2; HIF-1α, hypoxia-induced factor-1α; MAPK, mitogen-activated protein kinase; MEK, MAP kinase or ERK kinase; MZF-1, myeloid zinc finger protein 1; PKA, protein kinase A; DTT, dithiothreitol; SDS, sodium dodecyl sulfate.

however, is that it causes gastrointestinal side effects (Kyriazis 2003).

Fibroblast growth factor-2 (FGF-2 or bFGF) acts as a critical polypeptide growth factor in neuronal survival (Timmer *et al.* 2007). Alternative translation initiations at three CUG and one AUG start codons leads to the synthesis of four isoforms of FGF-2 (18, 24, 22.5 and 22 kDa) (Meisinger and Grothe 1997). The administration of FGF-2 has been proposed as a promising therapeutic strategy for preventing and treating age-related diseases such as PD (Vila *et al.* 2001). In our previous report (Li *et al.* 2006), we showed that APO-induced activation of dopamine receptors modulates FGF-2 biosynthesis and release of FGF-2 via cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and MEK/mitogen-activated protein kinase (MAPK) signalling cascades in rat striatal astrocytes, therefore promoting the survival of dopaminergic neurons. This provides insight into the pluripotent neuroprotective effect of APO, which can easily cross the blood–brain barrier, with higher concentrations being achieved in the brain than in blood. Furthermore, APO has also been found to increase expression of neurotrophic factors in neurons and astroglia, including brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor and nerve growth factor (NGF) (Ohta *et al.* 2000).

Astrocytes are star-shaped glial cells that are present in the brain and spinal cord. They perform many functions, including biochemical support of the endothelial cells that form the blood–brain barrier (Felts and Smith 1996; Abbott *et al.* 2006), secretion or absorption of neural transmitters (Santello and Volterra 2008), the provision of nutrients to the nervous tissue, and playing a principal role in the repair and scarring processes in the brain (Holley *et al.* 2003). They comprise the largest cell population in the CNS and play multiple roles in maintaining homeostasis in the neural extracellular environment. It has previously been thought that the neuronal network was the only important component and astrocytes were viewed as gap fillers. Recently, however, they have been shown to play a number of active roles in the brain.

A number of studies have demonstrated distinct signalling pathways that account for the biological activities of FGF-2. DNA sequences of various responsive elements located in the promoter that contribute to the FGF-2 transcriptional regulation have recently been determined. Moffett *et al.* (Moffett *et al.* 1998) defined a unique −555/−513 bp growth factor-responsive element and a separate region (−624/−556 bp) that are essential for high basal promoter activity and PKC or cAMP stimulation, respectively. Hypoxia-induced factor-1α (HIF-1α) may up-regulate FGF-2 promoter activity through binding to hypoxia response element within the proximal 103 bp of the FGF-2 promoter (Black *et al.* 2008). Conversely, homocysteine (Hcy) inhibits FGF-2 by transcriptional repression of the gene promoter (−100/−34 bp) encompassed in a CpG dinucleotide-rich island (Chang *et al.* 2008). Even the polymorphisms within the promoter region of FGF-2 could interfere with transcription factor binding sites or create new ones, eventually affecting gene expression. A cross-sectional case-controlled study demonstrated the association between genetic polymorphisms (−553T/A, −834T/A, −921C/G) in the FGF-2 promoter and type 2 diabetes (Petrovic *et al.* 2008). The −553T/A region lies within the NK2 transcription factor-related locus 5 (NKX25) binding site and −553A produces new binding sites for transcription factors GATA binding protein 1 (GATA1) and LMO2COM (Nakazawa *et al.* 2006). Higher concentrations of FGF-2 in serum was detected in diabetics with the AT genotype compared with the TT genotype (Petrovic *et al.* 2008). The substitution of −834T/A generates binding sites for SRY and SOX5, and −921C/G has no effect on any of the known transcription factors (Beranek *et al.* 2003). The downstream responsive elements involved in FGF-2 transcriptional regulations, however, have not been clearly identified.

Given the activation of dopamine receptors on FGF-2 modulation in astrocytes, the process between the activated signalling cascades by APO and FGF-2 gene regulation is not well known. The aim of this study was to investigate which of the transcriptional DNA elements are responsible for APO-modulated FGF-2 biosynthesis. To understand the regulatory mechanisms involved, we focus on the characterization of the proximal promoters of the FGF-2 promoter and on the transcription factors bound to these control elements in rat primary striatal astrocyte cultures. We attempt to identify the boundary of the minimal promoter of FGF-2 and *cis*-acting elements involved in the regulation of FGF-2 expression. Promoter binding of the transcription factor prediction showed that myeloid zinc finger protein-1 (MZF-1), a transcription factor belonging to the Kruppel family of zinc finger proteins, could bind to FGF-2 promoter. We next investigated whether FGF-2 biosynthesis is controlled by MZF-1 and whether APO plays a fundamental role in transcriptional regulation in striatal astrocytes.

Materials and methods

Chemicals

R(–)-apomorphine (referred to here as 'APO'), forskolin, and the dopamine receptor agonist quinpirole, the dopamine receptor antagonists haloperidol and SCH-23390, MAP kinase inhibitor PD98059 and PKA inhibitor KT5720 were purchased from Sigma, St Louis, MO, USA. The antibodies used included an anti-GFP antibody, an anti-FGF-2 antibody and an anti-actin antibody. These antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-D2 antibody was from Chemicon (Cat. No. AB5084P, Temecula, CA, USA). The anti-MZF-1 antibody was a kind gift of Dr D. Tuan.

Cell cultures

Astrocytes were prepared from the brains of 1–2-day-old neonatal Sprague-Dawley rat pups as previously described (Menet *et al.* 2001). Briefly, the striata were trypsinized, dissociated by gentle triturating, and plated at a density of 5×10^7 cells per 75-cm^2 flask (Corning, Corning, CT, USA) in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1 : 1 v/v; Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated foetal bovine serum (FBS). Cells were maintained in complete culture medium for 5–7 days. Between the sixth and eighth days, cultures were shaken to remove the top layer of cells sitting over the astroglial monolayer to yield primarily type-1 astrocytes with a flat morphology. Astrocyte cultures were then used for transfection experiments. To investigate the effects of APO and other compounds on FGF-2 transcription, astrocytes were maintained in serum-free DMEM/F12 and treated with various drug concentrations for the indicated incubation time.

HeLa cell lines were plated at a density of 5×10^5 cells per 10-cm^2 plate (Corning) in DMEM/F12 containing 10% heat-inactivated FBS.

siRNA-based inhibition

HeLa cell lines were transfected using the Nucleofector™ (Amaxa, Cologne, Germany) with 100 nM siMZF1 or siControl (Santa Cruz Biotechnology) according to the manufacturer's instructions. After culturing the cells in antibiotic-free medium for 48 h, total RNA was extracted and analysed by RT-PCR and western blot analysis.

Plasmid construction

The rat FGF-2 promoter −1058/+54 was a gift of Peter A. Cattini (Jimenez *et al.* 2004). The fragments −922/+54, −785/+54, −651/+54, −555/+54, −151/+54, −765/+54, −745/+54, −725/+54, −705/+54, −685/+54 and −665/+54 were inserted at the *Xho* I/*Hin*d III and followed by a luciferase gene in pGL3-Basic (Promega, Madison, WI, USA). The −783(−783/+54) and −785m (−785 mutant) fragments (Fig. 3b) were inserted at the *Xho* I/*Hin*d III and followed by a luciferase gene in pGL3-Basic.

Human MZF-1 (GenBank accession number BC007777) expression plasmids were purchased from Fenlene Gene Ltd. (Guangzhou, China). Human MZF-1 full-length cDNA was followed by a GFP tag in the CMV promoter vector.

FGF-2 promoter luciferase assay

Cells were washed with culture medium containing 10% FBS and resuspended in the nucleofector solution V at a concentration of 2×10^6 cells/sample. The cells were transfected with 5 μg of each test construct by AMAXA nucleofector. When the MZF-1 plasmid was expressed, cells were co-transfected with 5 μg of the reporter plasmid and 5–20 μg of the hMZF-1 plasmid. A plasmid carrying the *Renilla* luciferase gene under the control of the human CMV promoter was introduced to normalize transfection and cell lysis efficiency. After transfection for 24 h, the cultures were maintained in serum-free DMEM/F12 medium for another 24 h and treated with APO (2 μm) for 10 min. Cells were then harvested and washed with phosphate-buffered saline (pH 7.4). Cell lysis and determination of luciferase activity were conducted using a Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions. Luminescence was measured with a Luminometer (Berthold, Postfach, Germany).

Nuclear extract preparation

Primary astrocyte cells or HeLa cells were washed with ice-cold phosphate-buffered saline and resuspended in ice-cold buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1% sodium deoxycholate and protease inhibitor cocktail). The cells were incubated on ice for 10 min and 0.5% Nonidet P-40 (Sangon, Shanghai, China) for an additional 15 min. After centrifuging at 6000 *g* for 1 min, the pellet was resuspended in extract buffer (20 mM HEPES (pH 7.9), 400 mM KCl, 4.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1% sodium deoxycholate and protease inhibitor cocktail) and incubated on ice for 1 h. The lysate was centrifuged at 10 000 *g* for 10 min. After the addition of 15% glycerol, the supernatant was stored at −80°C until use (Le Mee *et al.* 2005).

EMSA

Double-stranded DNA was prepared for a probe by annealing synthetic oligonucleotides (Supporting information Table S2) (Invitrogen, Shanghai, China). Three non-labelled (minimal probe, mutant A and mutant B), double-stranded oligonucleotides were similarly prepared to use as competitors. Their nucleotide sequences are detailed in Supporting information Table S2.

Three micrograms of nuclear extract and 0.1 pmol of DNA radiolabelled probe were incubated at 25°C with 1–25 pmol of competitors in an electrophoretic mobility shift assay (EMSA) binding buffer (Beyotime, Shanghai, China). After 20 min of incubation, the mixtures were analysed by electrophoresis in a 4% polyacrylamide gel at 120 V for 2–3 h in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA). The radiosignals were recorded by STORM 860 (Amersham Pharmacia Biotech, NJ, USA).

DNA pull-down assay

A DNA pull-down assay was performed as described previously (Hu *et al.* 2008). Oligonucleotide duplexes corresponding to the sequence of the minimal probe, mutant A and mutant B (Supporting information Table S2) in the FGF-2 promoter, were covalently linked to a biotin moiety at their 5′ ends. Streptavidin Dynabeads (Dynal A.S. Lake Success, NY, USA) were washed three times in buffer A (5 mM Tris (pH 8.0), 0.5 mM EDTA, 1 M NaCl). Annealed oligonucleotides were incubated with 200 pmol/mg of beads for 15 min at 25°C in buffer A. Beads were then washed twice with 500 μL of buffer A and three times with 500 μL of buffer C (20 mM Tris (pH 8.0), 1 mM EDTA, 10% glycerol, 1 mM DTT, 50 mM NaCl). A total of 600 g of nuclear extract from the primary striatal astrocyte cultures in buffer C were incubated with 1 mg of streptavidin Dynabeads bound to the appropriate oligonucleotides. After 15 min of incubation at 25°C, beads were washed three times with buffer C and were added to a 2× loading buffer. Finally, the samples were analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by a western blot with the anti-MZF-1 antibody.

Experimental treatments

R(−)-APO (referred to here as 'APO'), forskolin and the dopamine receptor agonist quinpirole were added to cultures starting the day after plating out the culture (day 0). The dopamine receptor antagonists haloperidol and SCH-23390, MAP kinase inhibitor PD98059 and PKA inhibitor KT5720 were applied to cultures at least 2 h before APO was added.

Western blot assay

Briefly, total proteins were collected in ristocetin-induced platelet agglutination buffer (RIPA, 20 mM Tris–HCL (pH 7.4), 0.1% SDS, 1% NP-40, 1% sodium deoxycholate and protease inhibitor cocktail) from cell samples. Immunoblots probed with peroxidase-linked secondary antibodies were visualized by the SuperSignal West Pico Chemilluminescent substrate (Pierce, Rockford, IL, USA).

RT-PCR analysis

Total RNA was isolated from the primary striatal cultures or brain tissues. PCR of the cDNA was performed for 25 cycles to amplify a 140-bp MZF-1 fragment using rTaq (Takara, Tokyo, Japan). β-actin was used as an internal control gene. Primers for MZF-1 are as follows: forward, 5′-AGCTTGATGGACCTCGGA-3′, reverse: 5′-CTGAAGCTTGACTTGAGGTCT-3′; β-actin: forward, 5′-TTGC-GTTACACCCTTTCTTGACA-3′, reverse, 5′-TCACCTTCACCG-TTCCAGTTTT-3′. The sequences for primer sets used in the amplification of dopamine D1–D5 receptor cDNA fragments are shown in Supporting information Table S1.

Statistical analysis

Results are expressed as mean ± SEM. All analyses were performed using PRISM v4.0 (GraphPad Software, Inc). A one-way or two-way ANOVA was employed followed by a Student–Newman–Keul's test. A Student's *t*-test was also used. A p-value < 0.05 was considered significant.

Results

Characterization of dopamine receptor subtypes D1–D5 in primary cultured striatal astrocytes

We first examined the purity of astrocyte cultures derived from the striatum of postnatal rodents. Immunocytochemistry in combination with Hoechst staining revealed that all cells in the cultures were Glial Fibrillary Acidic Protein (GFAP)-positive (Fig. 1a) without detectable contamination of other cell types in cultures. RT-PCR analysis showed that in the cultured astrocytes, the expression of all dopamine receptor

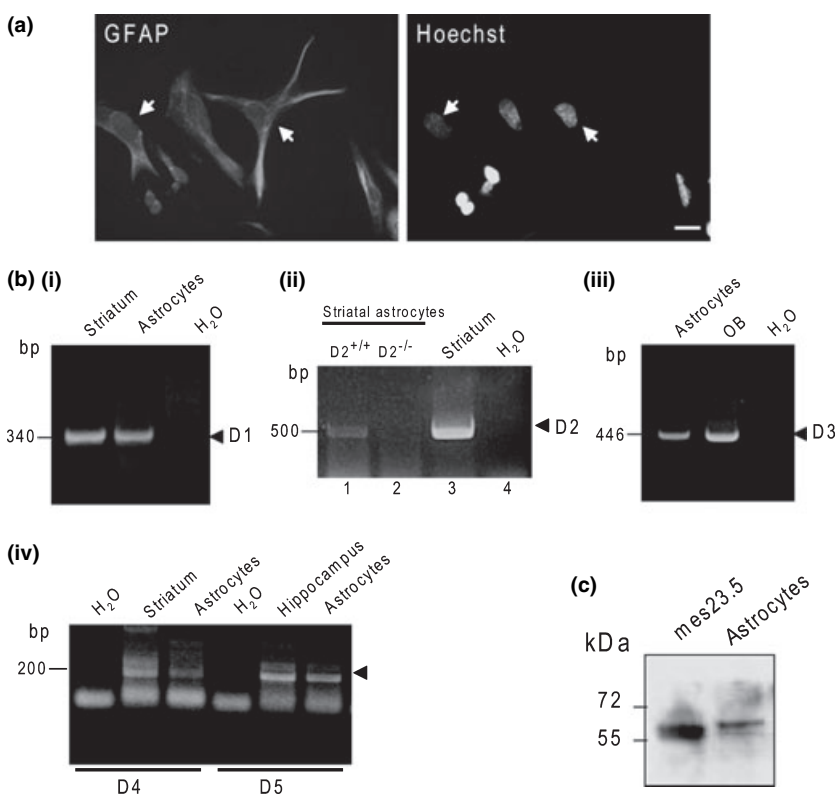

Fig. 1 Expression of dopamine receptor subtypes D1–D5 in primary cultured striatal astrocytes. (a) The primary astrocyte cultures from postnatal striatum were immunostained using GFAP antibody followed by Hoechst staining. Scale bar, 10 μm. (b) RT-PCR analysis of dopamine D1–D5 receptor mRNA in primary cultured astrocytes from the striatum. (b-i) D1 receptor mRNA expression. (b-ii) D2 receptor mRNA expression. Bands (indicated with arrows) representing a D2 mRNA fragment are detected in the cultured striatal astrocytes of wild-type mice (lane 1), but not in D2 receptor-null mice (lane 2) (Kelly *et al.* 1997). (b-iii and b-iv) Detection of D3 (b-iii), D4 and D5 (b-iv) receptor mRNA in the striatal astrocyte cultures. The striatal (b-i–iii), olfactory bulb (OB in b-iii) and hippocampal tissues serve as positive controls. H_2O serves as a negative control. (c) Western blot analysis shows that D2 receptor is expressed in the cultured striatal astrocytes and dopaminergic cell lines Mes23.5 cells.

mRNA (D1–D5) was detected (Fig. 1b). Moreover, in western blot analysis, striatal astrocytes showed protein expression of dopamine D2 receptor (Fig. 1c). These results are highly consistent with previous reports (Bal *et al.* 1994; Zanassi *et al.* 1999; Miyazaki *et al.* 2004), demonstrating that dopamine D1–D5 receptors are not only expressed in neurons but also in astrocytes from the striatum.

Identification of transcription factors binding to the minimal FGF-2 promoter in the primary astrocyte culture following APO stimulation

To define the minimal promoter of FGF-2, the six-flanking region (−1058/+54, −922/+54, −785/+54, −651/+54, −555/+54, −151/+54) was cloned into the firefly luciferase vector pGL3basic. Each resulting recombinant plasmid was then transiently transfected into the primary astrocyte cultures 24 h after the initial transfection. The cultures were maintained in serum-free DMEM/F12 medium for another 24 h and then treated with APO (2 μm) for 10 min. The activity of luciferase was then recorded. The *in vitro* −1058/+54 region was found to be sufficient to drive the expression of FGF-2 and respond to APO stimulation (Fig. 2a). In mutants, the activity of luciferase in the −785/+54 construct was comparable with those of the −1058/+54 construct. Further deletion to −651/+54 strongly reduced the activity of luciferase under the tested conditions. These results show that the −785/−651 sequence contains the core promoter (*cis* element) for the FGF-2 gene that is stimulated by APO in primary astrocyte cultures.

The other six-flanking regions (−765/+54, −745/+54, −725/+54, −705/+54, −685/+54, −665/+54) were then cloned into the firefly luciferase vector pGL3basic. Each resulting recombinant plasmid was then transiently transfected into the primary astrocyte culture 24 h after transfection. The cultures were maintained in serum-free DMEM/F12 medium for another 24 h and treated with APO (2 μm) for 10 min. The activity of luciferase was then recorded (Fig. 2b). Results indicated that the activity of luciferase *in vitro* in the −785/+54 construct was comparable with those of the−1058/+54 construct. Further deletion to −765/+54 strongly reduced the activity of luciferase under the tested conditions. These results demonstrated that the −785/−765 sequence contains the core promoter (*cis* element) for the FGF-2 gene that is stimulated by APO.

Identification of transcription factors binding to the *cis* element of the FGF-2 promoter in the primary astrocyte culture following APO stimulation

To define the *cis* element in the −785/−765 sequence, the −785/+54 sequence was analysed. Based on our data, we were able to predict the *cis* element (Fig. 3a). We found that the sequence contains two MZF-1-binding motif-like sequences (the consensus MZF-1-binding motif is

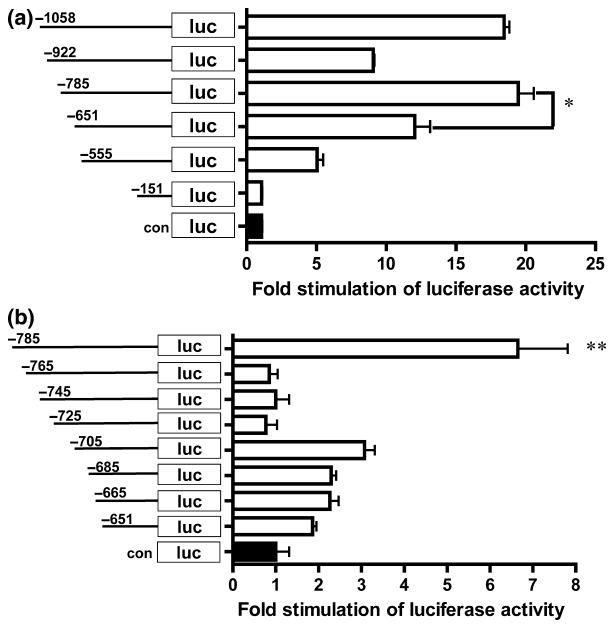

Fig. 2 Identification of the minimal promoter of the FGF-2 gene responded to APO stimulation. (a) Identification of the minimal promoter of the FGF-2 gene was in the −785 to −651 construct. The primary astrocyte cultures were co-transfected with a series of 5′-deletion mutants of FGF-2 promoter linked to luciferase and pCMV-renilla vector. Subsequent to 24 h post-transfection, the cultures were maintained in serum-free DMEM/F12 medium for another 24 h and treated with APO (2 μM) for 10 min. Cells were harvested and washed with PBS (pH 7.4). Cell lysis and determination of the luciferase activity were conducted. *$p < 0.0001$, one-way ANOVA followed by the Student–Newman–Keul's test. (b) Identification of the minimal promoter of FGF-2 gene was in the −785 to −651 region. The cultures were treated as above. Luciferase activity was normalized to level of *renilla*. *$p < 0.0001$, one-way ANOVA followed by a Student–Newman–Keul's test. Values represent mean ± SEM ($n = 3$).

5′-NGNGGGGA-3′, –785-GAGGGGGA- –778 and –750-GAGGGGGA- –743 were similar to the consensus motif). To validate the prediction, two mutant reporter plasmids were constructed by substituting three nucleotides (−785 m) or by deleting two nucleotides (−783) in this region followed by the luciferase gene (Fig. 3b). Each resulting recombinant plasmid was then transiently transfected into the primary astrocyte cultures 24 h after the initial transfection. The cultures were maintained in serum-free DMEM/F12 medium for another 24 h and treated with APO (2 μm) for 10 min and the activity of luciferase was recorded (Fig. 3c). We found that the two mutants (–785 m and –783) eliminated the activity of luciferase under the tested conditions *in vitro*.

Identification of the binding factor

Using EMSA, we next analysed the factor binding to the region. The primary astrocyte culture was maintained in serum-free DMEM/F12 medium for another 24 h and treated

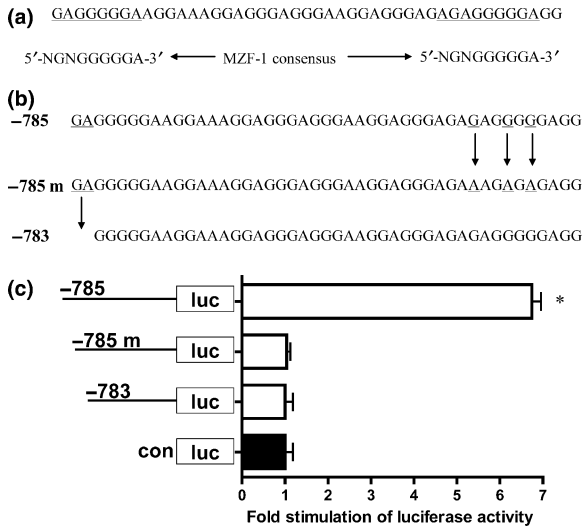

Fig. 3 Prediction of the *cis* elements and validation. (a) For prediction of the *cis* elements the sequences were compared to the MZF-1 consensus. (b) Sequences of two mutants derived from the wild-type sequence are shown. (c) −785 and two mutants were transfected into the primary culture, and the medium was replaced with DMEM/F-12 without serum after 24 h. After another 24 h, the medium was replaced with DMEM/F-12 additive 2 μM APO for 10 min. Cell lysis and determination of the luciferase activities were conducted. *$p < 0.0001$, one-way ANOVA followed by a Student–Newman–Keul's test. Values represent mean ± SEM ($n = 3$).

with 2 μm APO for 10 min. A nuclear extract prepared from that culture was added to a dsDNA probe including these two elements (Supporting information Table S2, the minimal probe). This subsequently caused the appearance of one shifted band (lane 2 in Fig. 4a). The band denoted by an arrow in Fig. 4a disappeared with the addition of a self-competitor in a dose-dependent manner (lanes 3–5). This observation demonstrates the sequence-specific binding between the probe DNA and the nuclear protein. Furthermore, the competitive effect was significantly reduced when a mutant-type competitor (i.e. mutants A and B, Supporting information Table S2), with two deleted bases or three substituted bases in the MZF-1-binding motif-like sequence, was used (lanes 6–11). This indicates that the nuclear protein specifically recognized the MZF-1-binding motif-like elements.

We then analysed the factor binding to the region by EMSA in the HeLa cell lines. The addition of the nuclear extract prepared from the HeLa cell lines to a dsDNA probe including these two elements (i.e. the minimal probe) caused the appearance of one shifted band (lane 2 in Fig. 4b), while it was not affected by the addition of a non-specific competitor (lane 12). Furthermore, the competitive effect was significantly reduced when a mutant-type competitor (mutants A and B, Supporting information Table S2), with two deleted bases or three substituted bases in the MZF-1-binding motif-like sequence, was used (lanes 6–11), indicating that the nuclear protein specifically recognized the MZF-1-binding motif-like elements.

Using DNA pull-down assay, we next analysed the factor binding to the region in the primary astrocyte culture, which had been maintained in serum-free DMEM/F12 medium for another 24 h and treated with or without 2 μm APO for 10 min. Oligonucleotide duplexes corresponding to the sequence of the minimal probe mutant A or mutant B (Supporting information Table S2) were then covalently linked to a biotin moiety at their 5′-ends. These oligonucleotides and their nuclear-binding proteins were recovered using magnetic streptavidin Dynabeads. Nuclear-binding proteins were prepared from the primary astrocyte cultures before and after APO treatment. Beads were collected and washed and the bound proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analysed by western blot. As shown in Fig. 5 (lanes 3 and 4), MZF-1 binds to the MZF-1 site in the FGF-2 promoter, regardless of APO treatment. When treated with APO, the MZF-1 enriched by the FGF-2 promoter was left untreated (Fig. 5, lanes 3 and 4). Moreover, binding was sequence-specific, as the presence of a 2-bp deletion (mutant A) or 3-bp mutation (mutant B) in the MZF-1 site removed the recognition of MZF-1 (Fig. 5, lanes 5 and 6).

It can therefore be stated that the minimal probe can bind to both the primary astrocyte cultures and the HeLa cell lines, and that the mutant probes do not alter this binding. The MZF-1 protein can also bind to the minimal probe (which includes two MZF-1 binding sites) as well as the protein enriched by the probe when treated with APO. This binding is inhibited by the mutation in the MZF-1-binding site. The results suggest that the *in vitro* minimal probe has *cis* elements, that the mutation of the MZF-1-binding sites in this probe does not alter this binding and that the MZF-1 protein can bind to the minimal probe.

Over-expression or knockdown of MZF-1 changes FGF-2 expression

We next examined whether the binding factor recognizing the element was indeed MZF-1 and whether MZF-1 stimulated FGF-2 gene expression. For this purpose, human MZF-1 expression plasmid was introduced into the primary astrocyte cultures with reporter plasmids (–785, –783 and –785 m). The over-expression of MZF-1 increased the activity of luciferase in the –785 construct, but it had no effect on the luciferase activity in the –783 and –785 m constructs (Fig. 6a). Furthermore, human MZF-1 plasmid and MZF-1-specific siRNA were introduced into HeLa cells to quantify the protein for the FGF-2 via western blot analysis (Fig. 6c). First, we used RT-PCR to determine the efficiency of knockdown (Fig. 6b). We then used western blot analysis to determine the over-expression of human MZF-1 (Fig. 6c) and determined the protein FGF-2 level. The over-expression of MZF-1 increased the amount of FGF-

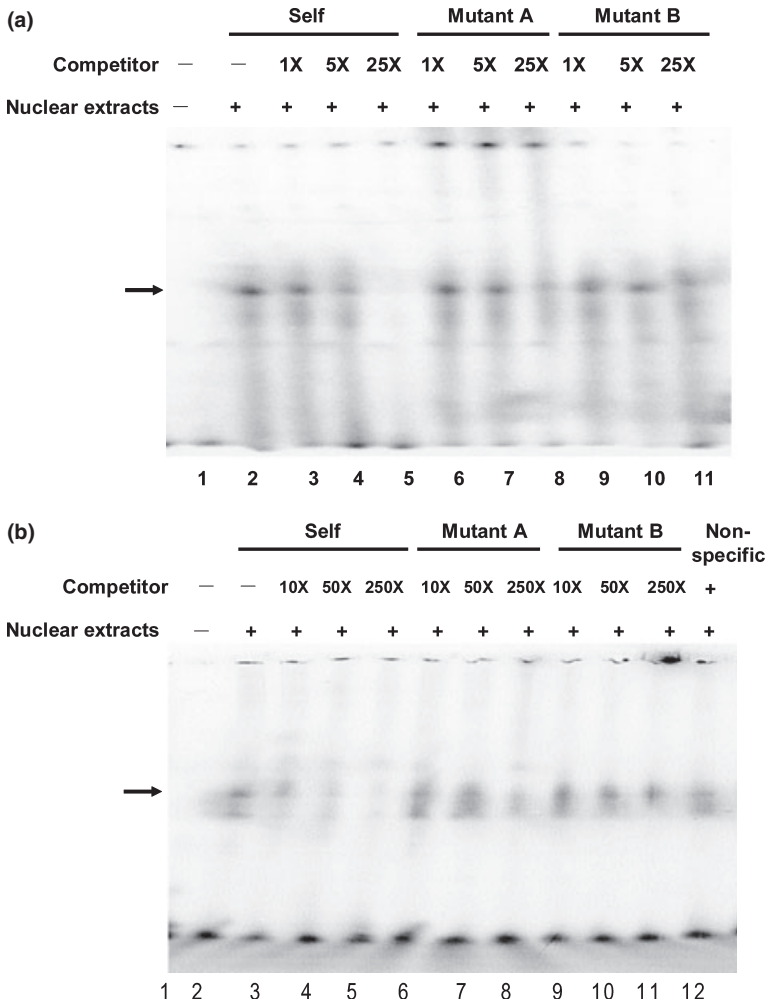

Fig. 4 Analysis of a transcription factor binding to the element by EMSA. A radiolabelled oligonucleotide (the minimal probe in Supporting information Table S2) was prepared as a probe for EMSA. Nuclear extract from primary astrocyte cultures (a) or the HeLa cells (b) was added to the probe. (a) A competitive binding assay was performed with 1×, 5×, and 25× of unlabelled oligonucleotides (self, mutant A and mutant B). (b) A competitive binding assay was performed with 5×, 25×, and 125× of unlabelled oligonucleotides (self, mutant A, mutant B, and non-specific).

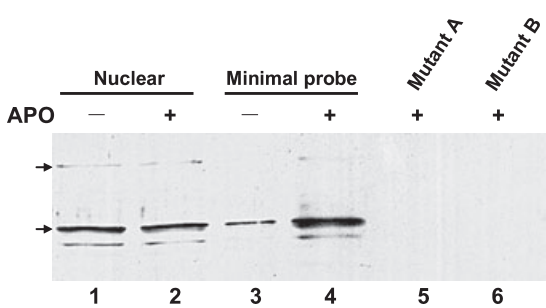

Fig. 5 MZF-1 mediates the regulation of FGF-2. MZF-1 binds directly to the MZF-1 site in the FGF-2 promoter as measured by DNA pull-down assay and Western blot. Nuclear extracts were obtained from primary cultured striatal astrocytes with or without APO treatment. A total of 600 μg of nuclear extract was incubated with 1 mg of Dynabeads Streptavidin-bound to the appropriate oligonucleotides. After 15 min of incubation, beads were washed three times. Eluted proteins were then analysed by SDS-PAGE followed by western blot with anti-MZF-1 antibodies. Arrows indicate MZF-1 proteins.

2 protein by 32%, and co-transfection with MZF-1 and its RNAi eliminated this increase (91% of control values). The transfection of MZF-1-specific siRNA, however, reduced the amount of FGF-2 protein by 65%. These results led us to the conclusion that MZF-1 stimulates FGF-2 gene expression via a *cis*-element in the FGF-2 promoter.

Activation of the dopamine receptor is responsible for APO-modulated FGF-2 biosynthesis and modulation via cAMP and PKC

We next wanted to determine whether dopamine receptor activity was required for the APO-mediated induction of the FGF-2 promoter. Pre-incubation with the D1 receptor antagonist SCH23390 (10 μM) and the D2 receptor antagonist haloperidol (1 μM) significantly blocked the APO-stimulated luciferase activity in the –785 construct (Fig. 7). To determine the mechanism underlying the dopamine receptor-mediated FGF-2 induction, we blocked key components of several signal transduction pathways while stimulating receptors with both D1- and D2-selective agonists. Three intracellular signalling pathways, i.e. the cAMP/

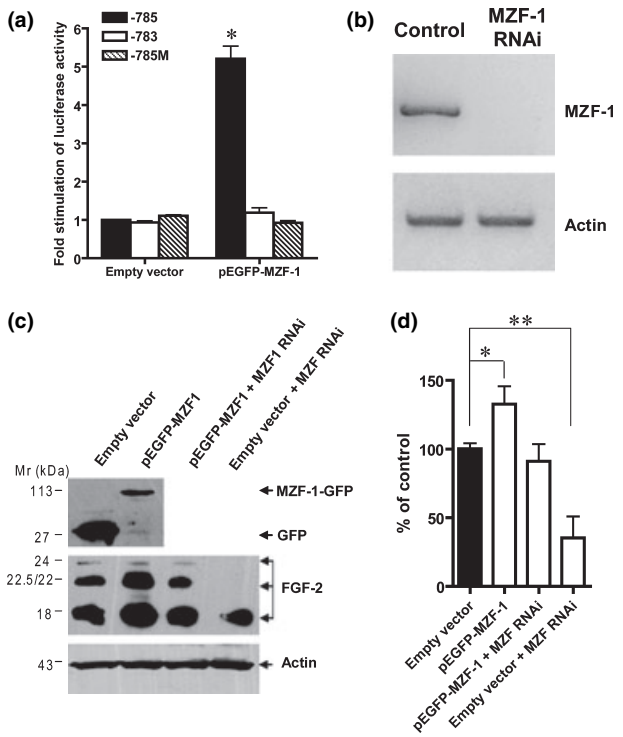

Fig. 6 Over-expression or knockdown of MZF-1 changes FGF-2 expression. (a) GFP-MZF-1 co-transfected with –785, –783 or –785 m in the primary astrocyte cultures and treated as in Figure 1. *$p < 0.05$ compared to the others ($p = 0.0003$,, –785 column compare to –783; $p = 0.0002$, one-way ANOVA followed by the Student–Newman–Keul's test,, –785 column compare to –785 m. (b) RT-PCR analysis showed the knockdown of MZF-1 mRNA expression using siRNA. (c) The changing of the MZF-1 protein levels altered the levels of FGF-2 protein expression. The plasmid or the MZF-1-specific siRNA were transfected into HeLa cells and then the protein levels of MZF-1 and FGF-2 were evaluated by western blot. (d) Quantification of data shown in (c). $p = 0.0005$, one-way ANOVA followed by a Student–Newman–Keul's test. *$p = 0.0367$, the HeLa cell line transfected with pEGFP-MZF-1 compared with the primary astrocyte transfected with empty vector; *$p = 0.3990$, one-way ANOVA followed by the Student–Newman–Keul's test, the primary astrocyte co-transfected with pEGFP-MZF1 and MZF1 RNAi compared with the primary astrocyte cultures transfected with empty vector; **$p = 0.0009$, the primary astrocyte transfected with MZF-1 RNAi compared with the primary astrocyte transfected with empty vector.

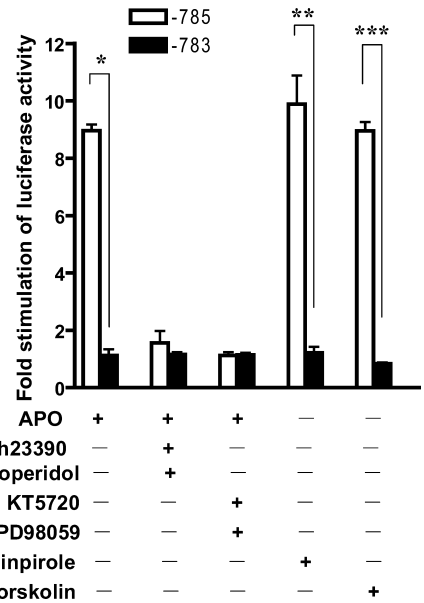

Fig. 7 Activation of the dopamine receptor is responsible for APO-modulated FGF-2 biosynthesis and the modulation via cAMP and PKC. The primary astrocyte cultures (transfected with –785 or –783) were plated into 24-well plates and the medium was switched to DMEM/F-12 without serum 24 h after plating h. After another 24 h the cultures were exposed to the D1 receptor antagonist SCH23390 (10 μM), D2 receptor antagonist haloperidol (1 μM), PKA antagonist KT5720 (50 μM) and the MAPK antagonist PD98059 (50 μM) for 2 h and followed by APO for 10 min. Striatal astrocytes were treated with only forskolin (5 μM, cAMP activator) and quinpirole (50 μM, D2 receptor agonist) for 2 h followed by APO for 10 min. The activity of luciferase was recorded and evaluated. The –785 column was compared with the –783 column (*$p < 0.0001$, Student's *t*-test, comparing the primary astrocyte transfected with 1.67 μg –785 plasmid/well to the primary cultured astrocytes transfected with 1.67 μg –783 plasmid/well; **$p = 0.0011$, Student's *t*-test, comparing the primary cultured astrocyte transfected with 1.67 μg –785 plasmid/well to those transfected with 1.67 μg –783 plasmid/well. ***$p < 0.0001$, Student's *t*-test, comparing the primary astrocyte transfected with 1.67 μg –785 plasmid/well to the cells transfected with 1.67 μg –783 plasmid/well. Values represent mean ± SEM ($n = 3$).

cAMP-dependent PKA, protein kinase C (PKC) and MAPK pathways, have been reported to mediate D1 and D2 receptor downstream cell signalling. We therefore examined whether the activation of these pathways is associated with APO-stimulated FGF-2 expression. Pre-incubation with either the PKA antagonist KT5720 (50 μM) or the MAPK antagonist PD98059 (50 μM) significantly blocked the APO-stimulated luciferase activity of the –785 construct (Fig. 7). Exposure of astrocytic cultures to the D2 receptor agonist quinpirole (50 μM) resulted in a 9.9-fold increase in –785 luciferase activity (Fig. 7). Furthermore, exposure of astrocytic cultures to the cAMP agonist forskolin (5 μM) resulted in an 8.9-fold increase in –785 luciferase activity (Fig. 7). Neither of these two agonists, however, increased the luciferase activity of the –783 construct. These results show that the activation of the dopamine receptor is responsible for APO-stimulated FGF-2 biosynthesis, that the modulation occurs via cAMP and MAPK and not the PI3K pathway and that the modulation requires the MZF-1-binding site.

Discussion

In the present study, we demonstrate that APO increases the expression of FGF-2 via the transcription factor human

MZF-1 (ZFP98) in primary striatal astrocyte cultures. First, we identified the minimal FGF-2 promoter in the primary astrocyte culture and validated the minimal FGF-2 promoter by EMSA and DNA pull-down assays. We next altered the level of MZF-1 protein expression in HeLa cell lines and found that over-expression of MZF-1 can up-regulate the FGF-2 protein level while knockdown of MZF-1 can down-regulate its expression. Furthermore, we found that APO-stimulated modulation of FGF-2 luciferase activity involves the activation of the dopamine D1 and D2 receptors, leading to the activation of cAMP/PKA and PKC/MAPK signalling pathways, respectively. We also found that blocking these two pathways could block luciferase activity, and that the mutation in MZF-1-binding site can also inhibit this activity.

APO induces the FGF-2 promoter by MZF-1 via two MZF-1 *cis* elements

A major finding of the present study is that APO induces the FGF-2 promoter by the transcription factor MZF-1 via two MZF-1 *cis* elements (–785/–745). The promoter had a 74-bp domain between nucleotides –793 and –720 and was more than 97% A/G-rich. It has been shown that the activity of the promoter increased approximately three-fold in the presence of the AGGG-repeat sequences of this domain (r) (Detillieux *et al.* 1998). This effect was neither tissue- nor species-specific, since promoter activity had been increased in this domain approximately 11-fold in both rat and human glial tumour cells. Here, we found two *cis* elements in this domain: –785 to –777 and –752 to –742. In the previous study, this domain had an AGGG-repeat sequence, and the sequences showed some similarity to those in a previously reported phenylephrine-responsive element (5′-gGGGAGGG-3′) in the rat atrial natriuretic factor promoter. We found that this domain has two sequences — –785 -GAGGGGGA- –777 and –752 -GA-GAGGGGGA- –742— and that these two sequences show some similarity to the previously reported MZF-1-binding motif (NGNGGGGGA). We used a combination mutation of the motif and changed the level of MZF-1 protein expression to validate both of these motifs and the transcription factor.

PKA and MAPK pathways can regulate MZF-1

We also found that APO stimulation modulates FGF-2 luciferase activity and involves the activation of the dopamine D1 and D2 receptors, leading to an activation of the cAMP/PKA and PKC/MAPK signalling pathways, respectively. Blocking these two pathways resulted in the blockade of luciferase activity, and the mutations in the MZF-1-binding site can also inhibit this activity. This suggests that MZF-1 was located downstream of these two pathways, and that MZF-1 is also downstream of PI3K, the other pathway that is downstream of D2 receptor. In our previous study, APO stimulated cAMP/PKA and PKC/MAPK, but not the PI3K pathway (Li *et al.* 2006). It is therefore possible to block the promoter using PKA and MAPK agonists.

APO stimulation modulates FGF-2 biosynthesis that is not dependent on FGF-2 feedback

It has been shown that FGF-2 can stimulate its own expression in ovine pulmonary-arterial smooth muscle cells via the NADPH oxidase-mediated activation of reactive oxygen species (ROS)-sensitive transcription factors, including HIF-1α (i.e. its sensitive region within the proximal 103 bp of the FGF-2 promoter) (Black *et al.* 2008). We over-expressed either the high molecular weight forms of FGF-2 (22.5/22 and 24 kDa) or the low molecular weight form of FGF-2 (18 kDa), both of which are capable of activating the FGF-2 promoter. Its sensitive region was not, however, found in the –785 to –742 sequence.

In summary, APO stimulation modulates FGF-2 luciferase activity, as APO involves the activation of the dopamine D1 and D2 receptors. This leads to the activation of the cAMP/PKA and PKC/MAPK pathways, but not the PI3K signalling pathway. Furthermore, the transcription factor was downstream of these two pathways. We also found that FGF-2 has feedback, but that this feedback is not dependent on the MZF-1 transcription factor.

Acknowledgments

We thank Dr P. Cattini, Minitoba, Canada for providing rat FGF-2 promoter; Dr Dorothy Tuan, MCG, USA, for MZF-1 antibody. This work was supported by grants from the Chinese Academy of Sciences, the Chinese Ministry of Science & Technology (No. 2006AA02Z184), Shanghai Metropolitan Fund for Research and Development (Nos. 07DJ14005, 06DZ22032), Natural Science Foundation of China (Nos. 30525041, 30621130075), State Key Program for Basic Research of China (No. 2006CB500704).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Sequences of primer sets used in RT-PCR.

Table S2 Oligonucleotide and primer sequences.

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References

Abbott N. J., Ronnback L. and Hansson E. (2006) Astrocyte-endothelial interactions at the blood-brain barrier. *Nat. Rev. Neurosci.* **7**, 41–53.

Bal A., Bachelot T., Savasta M., Manier M., Verna J. M., Benabid A. L. and Feuerstein C. (1994) Evidence for dopamine D2 receptor mRNA expression by striatal astrocytes in culture: in situ hybrid-

ization and polymerase chain reaction studies. *Brain Res. Mol. Brain Res.* **23**, 204–212.

Beranek M., Tschoplova S., Kankova K., Kuhrova V. and Vacha J. (2003) Genetic variation in the promoter region of the basic fibroblast growth factor gene. *Hum. Immunol.* **64**, 374–377.

Black S. M., DeVol J. M. and Wedgwood S. (2008) Regulation of fibroblast growth factor-2 expression in pulmonary arterial smooth muscle cells involves increased reactive oxygen species generation. *Am. J. Physiol. Cell Physiol.* **294**, C345–C354.

Chang P. Y., Lu S. C., Lee C. M. *et al.* (2008) Homocysteine inhibits arterial endothelial cell growth through transcriptional downregulation of fibroblast growth factor-2 involving G protein and DNA methylation. *Circ. Res.*, **102**, 933–941.

Detillieux K. A., Meyers A. F., Meij J. T. and Cattini P. A. (1998) An A/G-rich motif in the rat fibroblast growth factor-2 gene confers enhancer activity on a heterologous promoter in neonatal rat cardiac myocytes. *Mol. Cell. Biochem.* **188**, 169–176.

Felts P. A. and Smith K. J. (1996) Blood-brain barrier permeability in astrocyte-free regions of the central nervous system remyelinated by Schwann cells. *Neuroscience* **75**, 643–655.

Grasby P. M., Friston K. J., Bench C. J., Cowen P. J., Frith C. D., Liddle P. F., Frackowiak R. S. and Dolan R. J. (1993) The effect of the dopamine agonist, apomorphine, on regional cerebral blood flow in normal volunteers. *Psychol. Med.* **23**, 605–612.

Holley J. E., Gveric D., Newcombe J., Cuzner M. L. and Gutowski N. J. (2003) Astrocyte characterization in the multiple sclerosis glial scar. *Neuropathol. Appl. Neurobiol.* **29**, 434–444.

Hu Y., Lund I. V., Gravielle M. C., Farb D. H., Brooks-Kayal A. R. and Russek S. J. (2008) Surface expression of GABAA receptors is transcriptionally controlled by the interplay of cAMP-response element-binding protein and its binding partner inducible cAMP early repressor. *J. Biol. Chem.*, **283**, 9328–9340.

Jankovic J. (2008) Parkinson's disease: clinical features and diagnosis. *J. Neurol. Neurosurg. Psychiatry* **79**, 368–376.

Jimenez S. K., Sheikh F., Jin Y., Detillieux K. A., Dhaliwal J., Kardami E. and Cattini P. A. (2004) Transcriptional regulation of FGF-2 gene expression in cardiac myocytes. *Cardiovasc. Res.* **62**, 548–557.

Kelly M. A., Rubinstein M., Asa S. L. *et al.* (1997) Pituitary lactotroph hyperplasia and chronic hyperprolactinemia in dopamine D2 receptor-deficient mice. *Neuron* **19**, 103–113.

Kyriazis M. (2003) Neuroprotective, anti-apoptotic effects of apomorphine. *J. Anti Aging Med.* **6**, 21–28.

Le Mee S., Fromigue O. and Marie P. J. (2005) Sp1/Sp3 and the myeloid zinc finger gene MZF1 regulate the human N-cadherin promoter in osteoblasts. *Exp. Cell Res.*, **302**, 129–142.

Li A., Guo H., Luo X., Sheng J., Yang S., Yin Y., Zhou J. and Zhou J. (2006) Apomorphine-induced activation of dopamine receptors modulates FGF-2 expression in astrocytic cultures and promotes survival of dopaminergic neurons. *FASEB J.* **20**, 1263–1265.

Meisinger C. and Grothe C. (1997) Differential expression of FGF-2 isoforms in the rat adrenal medulla during postnatal development in vivo. *Brain Res.* **757**, 291–294.

Menet V., Gimenez y Ribotta M., Chauvet N., Drian M. J., Lannoy J., Colucci-Guyon E. and Privat A. (2001) Inactivation of the glial fibrillary acidic protein gene, but not that of vimentin, improves neuronal survival and neurite growth by modifying adhesion molecule expression. *J. Neurosci.* **21**, 6147–6158.

Miyazaki I., Asanuma M., Diaz-Corrales F. J., Miyoshi K. and Ogawa N. (2004) Direct evidence for expression of dopamine receptors in astrocytes from basal ganglia. *Brain Res.* **1029**, 120–123.

Moffett J., Kratz E., Myers J., Stachowiak E. K., Florkiewicz R. Z. and Stachowiak M. K. (1998) Transcriptional regulation of fibroblast growth factor-2 expression in human astrocytes: implications for cell plasticity. *Mol. Biol. Cell*, **9**, 2269–2285.

Nakazawa F., Nagai H., Shin M. and Sheng G. (2006) Negative regulation of primitive hematopoiesis by the FGF signaling pathway. *Blood* **108**, 3335–3343.

Obeso J. A., Rodriguez-Oroz M. C., Benitez-Temino B., Blesa F. J., Guridi J., Marin C. and Rodriguez M. (2008) Functional organization of the basal ganglia: therapeutic implications for Parkinson's disease. *Mov. Disord.* **23**(Suppl 3), S548–S559.

Ohta M., Mizuta I., Ohta K., Nishimura M., Mizuta E., Hayashi K. and Kuno S. (2000) Apomorphine up-regulates NGF and GDNF synthesis in cultured mouse astrocytes. *Biochem. Biophys. Res. Commun.* **272**, 18–22.

Petrovic M. G., Krkovic M., Osredkar J., Hawlina M. and Petrovic D. (2008) Polymorphisms in the promoter region of the basic fibroblast growth factor gene and proliferative diabetic retinopathy in Caucasians with type 2 diabetes. *Clin. Exp. Ophthalmol.* **36**, 168–172.

Santello M. and Volterra A. (2008) Synaptic modulation by astrocytes via Ca(2+)-dependent glutamate release. *Neuroscience* [Epub ahead of print] doi: 10.1016/j.neuroscience.2008.03.039.

Tien L. T., Park Y., Fan L. W., Ma T., Loh H. H. and Ho I. K. (2003) Increased dopamine D2 receptor binding and enhanced apomorphine-induced locomotor activity in mu-opioid receptor knockout mice. *Brain Res. Bull.* **61**, 109–115.

Timmer M., Cesnulevicius K., Winkler C., Kolb J., Lipokatic-Takacs E., Jungnickel J. and Grothe C. (2007) Fibroblast growth factor (FGF)-2 and FGF receptor 3 are required for the development of the substantia nigra, and FGF-2 plays a crucial role for the rescue of dopaminergic neurons after 6-hydroxydopamine lesion. *J. Neurosci.* **27**, 459–471.

Vila M., Jackson-Lewis V., Guegan C., Wu D. C., Teismann P., Choi D. K., Tieu K. and Przedborski S. (2001) The role of glial cells in Parkinson's disease. *Curr. Opin. Neurol.* **14**, 483–489.

Zanassi P., Paolillo M., Montecucco A., Avvedimento E. V. and Schinelli S. (1999) Pharmacological and molecular evidence for dopamine D(1) receptor expression by striatal astrocytes in culture. *J. Neurosci. Res.* **58**, 544–552.

