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# Fibroblast growth factor-20 protects against dopamine neuron loss *in vitro* and provides functional protection in the 6-hydroxydopamine-lesioned rat model of Parkinson's disease

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ABSTRACT

Fibroblast growth factor-20 (FGF-20) has been shown to protect dopaminergic neurons against a range of toxic insults *in vitro*, through activation of fibroblast growth factor receptor 1 (FGFR1). This study set out to examine whether FGF-20 also displayed protective efficacy in the unilateral, 6-hydroxydopamine (6-OHDA) lesion rat model of Parkinson's disease. Initial studies demonstrated that, in embryonic ventral mesencephalic (VM) cultures, FGFR1 was expressed on tyrosine hydroxylase (TH)-positive neurons and that, in line with previous data, FGF-20 (100 and 500 ng/ml) almost completely protected these TH-positive neurons against 6-OHDA-induced toxicity. Co-localisation of FGFR1 and TH staining was also demonstrated in the substantia nigra pars compacta (SNpc) of naïve adult rat brain. In animals subject to 6-OHDA lesion of the nigrostriatal tract, supra-nigral infusion of FGF-20 (2.5 μg/day) for 6 days post-lesion gave significant protection (~40%) against the loss of TH-positive cells in the SNpc and the loss of striatal TH immunoreactivity. This protection of the nigrostriatal tract was accompanied by a significant preservation of gross locomotion and fine motor movements and reversal of apomorphine-induced contraversive rotations, although forelimb akinesia, assessed using cylinder test reaching, was not improved. These results support a role for FGF-20 in preserving dopamine neuron integrity and some aspects of motor function in a rodent model of Parkinson's disease (PD) and imply a potential neuroprotective role for FGF-20 in this disease.



## 1. Introduction

In Parkinson's disease (PD), degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) underlies the key motor symptoms of tremor, rigidity, bradykinesia and postural instability. Early in the course of the disease, symptomatic improvement can be achieved by restoring dopaminergic transmission with either levodopa (L-DOPA) or dopamine receptor agonists such as apomorphine, pergolide or ropinirole. These agents do not alter the progression of the disease, however, and so their symptomatic benefit lessens over time and the elevated doses lead to side-effects, such as L-DOPA-induced dyskinesia, on-off fluctuations and psychosis (Fahn, 1974; Marsden, 1994; Jankovic, 2000). It follows that agents which can provide neuroprotection or repair in PD will not only offer significant advantages over the existing treatments, but may also help extend their period of effectiveness.

The mechanisms underlying degeneration of dopaminergic neurons in PD are still not fully elucidated but oxidative stress, mitochondrial dysfunction, proteasome dysfunction, inflammation and excitotoxicity have all been implicated in the path towards apoptotic cell death (see, for example, Hirsch, 2007; Levy et al., 2009). To date, pharmacological interference with these various mechanisms individually has not yielded any neuroprotective agents that have proven successful in clinical trials (Löhle and Reichmann, 2010). However, neurotrophic factors may lead to a breakthrough given their ability to promote cell survival through activation of multiple intracellular pathways that oppose apoptosis (Dawbarn and Allen, 2003). A number has already been shown to protect dopaminergic neurons or enhance their regeneration in the 6-hydroxydopamine (6-OHDA) rat model of PD. For example, intrastriatal administration of glial cell-derived neurotrophic factor (GDNF), cerebral dopamine neurotrophic factor (CDNF) and neurturin (NTN) have all been shown to protect

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against 6-OHDA administration (Tomac et al., 1995; Rosenblad et al., 1999; Lindholm et al., 2007; Voutilainen et al., 2011). Direct or viral vector-mediated gene delivery of GDNF and NTN into the striatum or SNpc has also been shown to prevent neurodegeneration and provide functional recovery in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated primate model of PD (Gash et al., 1996; Kordower et al., 2000, 2006; Grondin et al., 2002, 2008; Li et al., 2003). To date, clinical trials with GDNF and NTN have produced mixed results in patients with Parkinson's disease, with intra-putaminal GDNF infusion producing improved motor symptoms in early small scale open-label trials (Gill et al., 2003; Slevin et al., 2005), but failing, as did viral vector-mediated gene delivery of NTN into the putamen, in double-blind, randomised trials (Lang et al., 2006; Marks et al., 2010). Nevertheless, PD remains an attractive candidate for neurotrophic factor therapy and the outcome of future trials will be eagerly monitored.

One growth factor which has received less attention to date is fibroblast growth factor 20 (FGF-20). FGF-20 is a member of the FGF-9 sub-family of FGFs that, in the adult, shows preferential expression in the brain, with little or no expression in other major organs (Kirikoshi et al., 2000; Ohmachi et al., 2000; Jeffers et al., 2001). Within the brain, FGF-20 mRNA shows further regional selectivity, with the cerebellum and SNpc showing highest levels (Ohmachi et al., 2000; Jeffers et al., 2001). The presence in the SNpc is especially interesting given that certain FGF-20 single nucleotide polymorphisms or haplotypes are associated with an increased risk of developing PD in some (van der Walt et al., 2004; Noureddine et al., 2005; Satake et al., 2007), but not all (Clarimon et al., 2005; Wider et al., 2010) patient studies.

In support of a potential neuroprotective role in PD, FGF-20 has been shown to protect primary cultures of dopaminergic neurons *in vitro* from cell death following either serum withdrawal or incubation with the dopaminergic neurotoxin, 6-OHDA (Ohmachi et al., 2000, 2003; Murase and McKay, 2006). These effects are believed to arise through activation of FGF receptor-1 (FGFR1), the exact cellular location of which remains to be established (Ohmachi et al., 2003; Murase and McKay, 2006).

Given that dopaminergic neurons in the human SNpc also express FGFR1 and continue to do so in PD patients (Walker et al., 1998), FGF-20 could offer an effective means of providing neuroprotection in early PD. To date the efficacy of FGF-20 in providing neuroprotection in an animal model of PD has not been investigated. However, Huang et al. (2009), recently showed that 3 days' supra-nigral injection of FGF-9, the most closely related member of the FGF family (Kirikoshi et al., 2000), partially protected nigral dopaminergic neurons against 1-methyl-4-phenylpyridinium ($MPP^+$) toxicity in mice.

The aims of the present study were two-fold; firstly to confirm the single published study to date showing that FGF-20 could protect dopaminergic VM cultures from 6-OHDA toxicity *in vitro* and, secondly, to examine for the first time whether FGF-20 could provide functional neuroprotection in the 6-OHDA rat model of PD.

## 2. Materials and methods

### 2.1. Chemicals, reagents and antibodies

Human recombinant FGF-20 (purity > 90%) was purchased from PeproTech (Rocky Hill, New Jersey. U.S.A.). Rabbit anti-tyrosine hydroxylase (TH) and mouse anti-TH antibodies were obtained from Chemicon, Millipore (Watford, UK), whilst rabbit anti-FGFR1 primary antibody (F5421) was obtained from Sigma–Aldrich (Somerset, UK). Fluorescent secondary antibodies were purchased from Molecular Probes, Invitrogen Ltd. (Paisley, UK), whilst all other secondary antibodies came from Dako Ltd., (Ely, UK). All other reagents, unless otherwise stated were purchased from Sigma–Aldrich (Somerset, UK).

### 2.2. Primary ventral mesencephalic (VM) cell cultures

Pregnant time-mated Sprague–Dawley rats (Charles River, UK) were killed at embryonic day 15 (E15, where E0 was day of mating) with $CO_2$ and the foetuses removed. Ventral mesencephalic brain tissue was dissected out, pooled and incubated in 0.25% trypsin in Dulbecco's PBS (DPBS) at 37 °C for 10 min. Tissues were then placed into culture medium (Dulbecco's modified Eagle's medium (DMEM) Glutamax containing 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin–neomycin) and the suspension centrifuged for 2 min at room temperature (RT). The supernatant was discarded, 1 ml fresh medium added and the cell suspension triturated using a flame polished Pasteur pipette. Viable cell density was estimated using trypan blue exclusion and cells were plated onto sterile 13 mm diameter poly-D-lysine pre-coated glass coverslips at a density of 300 000 cells per coverslip. Cultures were maintained in 10% FBS-containing medium at 37 °C in a humidified atmosphere of 5% $CO_2$ and 95% air.

#### 2.2.1. Co-localisation of fibroblast growth factor receptor-1 (FGFR1) with tyrosine hydroxylase (TH), the neuronal marker HuCD and glial fibrillary acidic protein (GFAP) in VM cultures

Naïve VM cultures, prepared as described above, were fixed in ice-cold 4% PFA on day 6 *in vitro* (DIV6) then processed for immunofluorocytochemistry. Briefly, all at RT, coverslips were incubated for 10 min in blocking buffer (1% bovine serum albumin [BSA] and 10% sodium azide dissolved in 0.5 M Tris-buffered saline [TBS]) before overnight incubation with rabbit anti-FGFR1 (1:50). Cells were than washed in TBS before subsequent incubation for 1 h with either mouse monoclonal anti-TH primary antibody (1:1000), anti-GFAP (1:1000) or anti-HuCD (1:1000). Thereafter, cultures were incubated for 2 h with AlexaFluor-488 goat anti-mouse (1:1000) and AlexaFluor-594 donkey anti-rabbit (1:1000) secondary antibody solution containing the nuclear stain Hoechst (1:10 000). Images were acquired at 63× magnification using a Zeiss Apotome fluorescent microscope (Carl Zeiss Ltd., Hertfordshire, UK) and Axiovision image analysis software.

#### 2.2.2. FGF-20 protection study in VM cultures

On DIV6, FGF-20 (10, 100 or 500 ng/ml) or equivalent vehicle (serum free medium containing 10 ng/ml rat serum albumin) was added to naïve VM cultures for 24 h. On DIV7, FGF-20 was removed and cells were exposed to 6-OHDA (40 or 60 μM, depending on culture sensitivity) in order to achieve around 60–80% cell loss in controls, or equivalent vehicle (0.02% ascorbic acid in serum-free medium) for 4 h. Cells were then replaced into serum-containing medium before fixing on DIV8 in ice-cold 4% paraformaldehyde (PFA) in preparation for TH immunocytochemistry. Fixed cells were incubated for 10 min in 3% hydrogen peroxide in $dH_2O$ to inactivate natural peroxidase activity then incubated for 10 min in blocking/permeabilisation buffer (1% BSA and 0.1% Tween20 and 10% sodium azide in 0.5 M TBS). Cells were incubated overnight at RT with primary rabbit anti-TH antibody (1:1000) then for 2 h with the secondary biotinylated goat anti-rabbit antibody (1:200). Horse radish peroxidase was conjugated to the secondary antibody using a Vectastain ABC kit (Vector Laboratories, Peterborough, UK) and the signal developed in diaminobenzidine (DAB) solution (0.05% DAB and 0.03% $H_2O_2$ dissolved in 0.1 M TBS) for 10 min. Images of the entire stained coverslips were taken at 10× magnification and the total number of TH-positive neurons on each coverslip blind-counted. Three to five coverslips were used per treatment group on each of the three independent runs.

### 2.3. Neuroprotective effects of FGF-20 in vivo

#### 2.3.1. Animals

Male Sprague–Dawley rats (Charles River, UK; body weight 250–300 g) were used. Food and water were provided *ad libitum*. Animals were housed in a temperature- and humidity-controlled environment which alternated between light and dark every 12 h. All the experiments were carried out in accordance with the guidelines set out in the UK Animals (Scientific Procedures) Act, 1986 and every effort was made to minimise the number of animals used and their suffering.

#### 2.3.2. Co-localisation of FGFR1 with TH and GFAP in the rat SNpc

Before proceeding into *in vivo* studies, we next determined whether FGFR1 is co-localised on TH positive cells in the rat SNpc, Brains were removed from terminally anaesthetised naïve rats following intra-cardiac perfusion of 4% PFA, fixed overnight in 4% PFA at 4 °C, then transferred into 30% sucrose in 0.1 M PBS. Following embedding in 20% gelatine (in 0.1 M PBS), 30 μm vibrotome slices were taken from the SNpc 5.3 mm posterior to Bregma (Paxinos and Watson, 1997). Sections were incubated at RT overnight with rabbit anti-FGFR1 (1:50) primary antibodies. Sections were washed in TBS before subsequent incubation for 1 h with either mouse monoclonal anti-TH primary antibody (1:1000) or anti-GFAP (1:1000) then for 2 h with AlexaFluor-488 goat anti-mouse (1:1000) and AlexaFluor-594 donkey anti-rabbit (1:1000) secondary antibody solution containing the nuclear stain Hoechst (1:10 000). Images were acquired at 63× magnification using the Zeiss apotome microscope and recorded using Axiovision LE software.