Neurobiology of Disease 88 (2016) 1–15

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Growth/differentiation factor-15 deficiency compromises dopaminergic neuron survival and microglial response in the 6-hydroxydopamine mouse model of Parkinson's disease

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ARTICLE INFO

Article history:
Received 26 August 2015
Revised 21 December 2015
Accepted 25 December 2015
Available online 28 December 2015

Keywords:
Gdf-15
6-OHDA
Dopaminergic neurons
Microglia
Neurotrophic factors
Neuroinflammation
Parkinson's disease
Nigrostriatal system

ABSTRACT

Growth/differentiation factor-15 (Gdf-15) is a member of the TGF-β superfamily and a pleiotropic, widely distributed cytokine, which has been shown to play roles in various pathologies, including inflammation. Analysis of *Gdf-15*$^{-/-}$ mice has revealed that it serves the postnatal maintenance of spinal cord motor neurons and sensory neurons. In a previous study, exogenous Gdf-15 rescued 6-hydroxydopamine (6-OHDA) lesioned *Gdf-15*$^{+/+}$ nigrostriatal dopaminergic (DAergic) neurons in vitro and in vivo. Whether endogenous Gdf-15 serves the physiological maintenance of nigrostriatal DAergic neurons in health and disease is not known and was addressed in the present study. Stereotactic injection of 6-OHDA into the medial forebrain bundle (MFB) led to a significant decline in the numbers of DAergic neurons in both *Gdf-15*$^{+/+}$ and *Gdf-15*$^{-/-}$ mice over a time-period of 14 days. However, this decrease was exacerbated in the *Gdf-15*$^{-/-}$ mice, with only 5.5% surviving neurons as compared to 24% in the *Gdf-15*$^{+/+}$ mice. Furthermore, the microglial response to the 6-OHDA lesion was reduced in *Gdf-15*$^{-/-}$ mice, with significantly lower numbers of total and activated microglia and a differential cytokine expression as compared to the *Gdf-15*$^{+/+}$ mice. Using in vitro models, we could demonstrate the importance of endogenous Gdf-15 in promoting DAergic neuron survival thus highlighting its relevance in a direct neurotrophic supportive role. Taken together, these results indicate the importance of Gdf-15 in promoting survival of DAergic neurons and regulating the inflammatory response post 6-OHDA lesion.

Abbreviations: 6-OHDA, 6-hydroxydopamine; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BSA, bovine serum albumin; CNS, central nervous system; CPu, caudate putamen; CXCL12, chemokine (C–X–C motif) ligand 12; CXCR4, chemokine (C–X–C motif) Receptor 4; DA, dopamine; DAB, 3,3′-diaminobenzidine; DAergic, dopaminergic; DAPI, 4′,6-diamidino-2-phenylindole; DMEM/F-12, Dulbecco's modified Eagle's medium/Ham's F12; DRG, dorsal root ganglia; E14, embryonic day 14; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GDF-15/Gdf-15, growth/differentiation factor-15; GFAP, glial fibrillary acidic protein; HS, horse serum; Iba1, ionized calcium binding adapter molecule 1; IFN-γ/Ifn-γ, interferon-gamma; IL, interleukin; iNOS/iNos, inducible nitric oxide synthase; Ir, immunoreactive; LD_{50}, median lethal dose; LPS, lipopolysaccharide; MFB, medial forebrain bundle; MHC-II, major histocompatibility complex class-II; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NeuN, neuronal nuclei; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; P0, postnatal day 0; PBS, phosphate buffered saline; PD, Parkinson's disease; PFA, paraformaldehyde; PMA, phorbol 12-myristate 13-acetate; RT, room temperature; SN, substantia nigra; TGF-β/Tgf-β, transforming growth factor-beta; Th, tyrosine hydroxylase; TNF-α/Tnf-α, tumor necrosis factor-α.

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Available online on ScienceDirect (www.sciencedirect.com).

http://dx.doi.org/10.1016/j.nbd.2015.12.016
0969-9961/

1. Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder affecting more than 7 million people worldwide (Antony et al., 2013). Pathological features include the selective degeneration of dopaminergic (DAergic) neurons in the substantia nigra (SN), loss of projecting fibers, and a concomitant depletion of the neurotransmitter DA in the caudate putamen (CPu) (Hirsch et al., 1988). The neurotoxin 6-hydroxydopamine (6-OHDA) leads to the degeneration of catecholaminergic neurons (Ungerstedt, 1968) due to a preferential uptake of 6-OHDA by the DAergic and noradrenergic transporters (Luthman et al., 1989). Evidence from both, PD patients and animal models, suggest that neuroinflammation plays a role in the pathophysiology of PD (Hirsch and Hunot, 2009). Activated microglia, T-lymphocytes and an increased expression of pro-inflammatory cytokines as well as growth factors have been observed in the SN of PD patients, post-mortem (McGeer et al., 1988; Mogi et al., 1994a, 1994b). Moreover, in vivo imaging studies in PD patients show a progressive microglial activation associated with a decrease in DAergic neurons (Gerhard et al., 2006; Ouchi et al., 2009), as well as increased cytokine levels in the serum and the cerebrospinal fluid of PD patients (Dobbs et al., 1999; Müller et al., 1998; Nagatsu et al., 2000a; Rentzos et al., 2007; Stypuła et al., 1996). 6-OHDA PD animal models also show a strong glial reaction involving microglia and astrocytes, as well as peripheral lymphocyte infiltration post 6-OHDA administration, indicating that inflammatory processes may accompany neuron death, but are unlikely its primary cause (Harms et al., 2011; Marinova-Mutafchieva et al., 2009; Stott and Barker, 2014).

Growth/differentiation factor-15 (Gdf-15) is a divergent member of the transforming growth factor-β) TGF-β superfamily (see Unsicker et al., 2013 for a review). It was discovered by its role as an autocrine regulator of macrophage activation and a target of several cytokines, including IL-1β, TNF-α, IL-2, and TGF-β (Bootcov et al., 1997), thus limiting the later phases of macrophage activation. Systemically, Gdf-15 has broad anti-inflammatory and immunosuppressive properties as demonstrated in models of myocardial infarction, atherosclerosis and rheumatoid arthritis (Breit et al., 2011; Unsicker et al., 2013).

Gdf-15 is ubiquitously expressed in rat and mouse CNS, in isolated astrocytes and Schwann cells (Strelau et al., 2000b, 2009). We have previously shown that Gdf-15 is a potent neurotrophic factor for central and peripheral neurons, including DAergic neurons (Strelau et al., 2000b, 2009; Subramaniam et al., 2003), and that it promotes the survival of cultured DAergic neurons (Strelau et al., 2000b). Unilateral injection of Gdf-15 into the SN and into the left ventricle immediately before a 6-OHDA injection into the medial forebrain bundle (MFB) prevented pathological rotational motor behavior and significantly reduced the loss of DAergic neurons in the SN. Together, these data are consistent with the notion that Gdf-15 is a potent neurotrophic factor for cultured and in vivo lesioned midbrain DAergic neurons, with an emerging potential for therapeutic administration. Analysis of *Gdf-15*$^{-/-}$ mice revealed its endogenous importance, as its loss causes a substantial postnatal numerical decline of spinal cord motor and dorsal root ganglia (DRG) neurons (Strelau et al., 2009). Whether other neuron populations, including midbrain DAergic neurons are physiologically dependent on Gdf-15 is not known.

The prominent effects of Gdf-15 in the protection of lesioned DAergic neurons in conjunction with its role in regulating inflammation make it an interesting candidate to explore the functions of endogenous Gdf-15 in the maintenance of unlesioned and lesioned midbrain DAergic neurons, and its putative roles in regulating the inflammatory response to a nigrostriatal lesion. In this study, we show that lack of Gdf-15 aggravates the loss of 6-OHDA lesioned DAergic neurons in the SN. Using in vitro models, we could demonstrate the direct neuroprotective effect of Gdf-15 on lesioned DAergic neurons. Moreover, *Gdf-15*$^{-/-}$ mice also exhibited an attenuated lesion-induced microglial response in the nigrostriatal system.

2. Materials and methods

2.1. Animals

All animal work was performed in strict compliance with the German federal animal welfare law and after approval through the official animal welfare officer of the Center for Experimental Models and Transgenic Services (CEMT) at the University of Freiburg. The Gdf-15$^{lacZ/lacZ}$ mouse colony was established via embryo-transfer from Heidelberg, Germany (Strelau et al., 2009). The animals were housed at 22 ± 2 °C under a 12 h light/dark cycle with free access to food and water.

2.2. Generation of hemiparkinsonian mice

All animal-related procedures were approved by the local authorities and conducted in accordance with the German legislation on protection of animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Age-matched 10–12 week old C57BL/6 wild type mice (Charles River, Germany) and *Gdf-15*$^{-/-}$ mice were used for the 6-OHDA lesions.

Mice were deeply anesthetized by an intraperitoneal injection of ketamine (75 mg/kg) and Xylazine-HCl (5.8 mg/kg; Rompun, Bayer, Germany), mounted in a mouse adaptor (Stoelting Co., Wood Dale, USA) and fixed in a rat stereotaxic apparatus (Kopf, Tujunga, USA). The skull was opened with a dental drill and animals were unilaterally lesioned by an injection of 6-OHDA (6-OHDA-HCl, Sigma) into the right MFB via a 26 G Hamilton syringe (5 μg 6-OHDA/2 μl, dissolved in 0.9% saline, containing 0.2% ascorbic acid (Merck, Germany)) with coordinates referring to bregma: AP − 1.2, ML − 1.1, V − 5 (Paxinos and Franklin, 2012). The solution was injected over a time period of 4 min, and after further 3 min, the syringe was pulled off slowly and the skin was sutured. Then, the animals received supplementary baby food (cereals and fruit mash) and chocolate for the following 14 days to prevent heavy weight loss. Samples were harvested at 2 days, 6.5 days and 14 days post 6-OHDA lesion.

2.3. Tissue processing

Mice were injected with an overdose of pentobarbital (60 mg/kg) and transcardially perfused with ice cold 0.9% sodium chloride (10 ml), followed by 50 ml of 3.7% paraformaldehyde (PFA) (Merck, Germany). Brains were immediately removed from the skull, post fixed in 3.7% PFA overnight, and transferred into 15% sucrose followed by 30% sucrose solution (overnight, 4 °C). The cryo-protected brains were embedded in Tissue Tek (Leica, Germany), snap frozen in liquid nitrogen (− 196 °C) and stored at − 80 °C. Brains were cut with a cryostat (Leica, Germany) at 30 μm thickness and serial sections were collected on super-frost slides (Thermo Scientific, USA) and stored at − 80 °C until further processing.

2.4. Cell culture

2.4.1. Primary microglia cultures

Whole brains obtained from P0 pups (*Gdf-15*$^{+/+}$ or *Gdf-15*$^{-/-}$), were washed with ice-cold Hank's BSS (PAA, Germany) and the vessels and meninges were removed from the surface. Cleaned brains were collected in a tube with ice-cold Hank's BSS and enzymatically dissociated using 0.25% Trypsin (PAA, Germany) for 10 min at 37 °C. Trypsinization was blocked by adding an equal volume of Fetal Calf Serum (FCS) (PAA, Germany) and 0.05 mg/ml of DNase (Roche, Germany). Cells were dissociated using pipettes of decreasing bore-diameter until no fragments were visible. Dissociated cells were centrifuged, collected and resuspended in DMEM/F-12 (PAA, Germany), 10% FCS and 1% Penicillin–Streptomycin (PAA, Germany). Cells were seeded on poly-D-lysine-coated flasks with a density of 1.5 brains/75 cm^2 flask. Cultures were maintained in a humidified 5% CO_2 environment at 37 °C with a medium