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Research Paper

Long-term protective effects of AAV9-mesencephalic astrocyte-derived neurotrophic factor gene transfer in parkinsonian rats

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ABSTRACT

Intrastriatal injection of mesencephalic astrocyte-derived neurotrophic factor (MANF) protein has been shown to provide neuroprotective and neurorestorative effects in a 6-hydroxydopamine (6-OHDA) - lesioned rat model of Parkinson's disease. Here, we used an adeno-associated virus serotype 9 (AAV9) vector to deliver the *human MANF* (*hMANF*) gene into the rat striatum 10 days after a 6-OHDA lesion to examine long-term effects of hMANF on nigral dopaminergic neurons and mechanisms underlying MANF neuroprotection. Intrastriatal injection of AAV9-hMANF vectors led to a robust and widespread expression of the *hMANF* gene in the injected striatum up to 24 weeks. Increased levels of hMANF protein were also detected in the ipsilateral substantia nigra. The *hMANF* gene transfer promoted the survival of nigral dopaminergic neurons, regeneration of striatal dopaminergic fibers and an upregulation of striatal dopamine levels, resulting in a long-term improvement of rotational behavior up to 16 weeks after viral injections. By using SH-SY5Y cells, we found that intra- and extracellular application of MANF protected cells against 6-OHDA-induced toxicity via inhibiting the endoplasmic reticulum stress and activating the PI3K/Akt/mTOR pathway. Our results suggest that AAV9-mediated *hMANF* gene delivery into the striatum exerts long-term neuroprotective and neuroregenerative effects on the nigrostriatal dopaminergic system in parkinsonian rats, and provide insights into mechanisms responsible for MANF neuroprotection.

1. Introduction

Mesencephalic astrocyte-derived neurotrophic factor (MANF) is a secreted protein which has more selective neuroprotective effects on dopaminergic (DA) neurons than glial cell line-derived neurotrophic factor (GDNF) (Petrova et al., 2003). MANF is first identified from the culture medium of a rat mesencephalic type-1 astrocyte cell line (Petrova et al., 2003) and is a neurotrophic factor in cerebral dopamine neurotrophic factor (CDNF)/MANF family (Lindholm and Saarma, 2010). MANF has been observed to be present in various parts of the developing and adult brain including the striatum and midbrain (Lindholm et al., 2008; Wang et al., 2014). Importantly, MANF was

Abbreviations: 6-OHDA, 6-hydroxydopamine; AAV, adeno-associated virus; ATF4, activating transcription factor 4; ATF6α, activating transcription factor 6α; Bip, binding immunoglobulin protein; CDNF, cerebral dopamine neurotrophic factor; CR3, complement receptor 3; CHOP, C/EBP-homologous protein; DA, dopaminergic; DOPAC, dihydroxyphenylacetic acid; ER, endoplasmic reticulum; eIF2α, eukaryotic initiation factor 2α; GFAP, glial fibrillary acidic protein; GDNF, glial cell line-derived neurotrophic factor; GFP, green fluorescent protein; HVA, homovanillic acid; hEPO, human erythropoietin; HRP, horseradish peroxidase; IRE1, inositol-requiring enzyme 1; IR, immunoreactive; LGP, lateral globus pallidus; MANF, mesencephalic astrocyte-derived neurotrophic factor; MHC, major histocompatibility antigen; mTOR, mammalian target of rapamycin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease; PI3K, phosphoinositide-3-kinase; PDI, protein disulfide isomerase; PERK, protein kinase-like ER kinase; SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulata; TH, tyrosine hydroxylase; UPR, unfolded protein response; XBP1s, spliced x-box binding protein 1.

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found to co-localize with DA neurons in the substantia nigra (SN) (Lindholm et al., 2008), and the mutation of *MANF* gene caused degeneration of DA axons in *Drosophila melanogaster* (Palgi et al., 2009), suggesting important roles of MANF in the development and function of DA neurons. It has been reported that endogenous MANF is up-regulated by endoplasmic reticulum (ER) stress inducers *in vitro* (Apostolou et al., 2008; Mizobuchi et al., 2007; Tadimalla et al., 2008; Yu et al., 2010) and by cerebral ischemia, myocardial ischemia or status epilepticus *in vivo* (Lindholm et al., 2008; Tadimalla et al., 2008; Yu et al., 2010).

Although exact mechanisms responsible for MANF effects are still elusive, accumulating evidence has suggested that MANF plays an important role in regulating the ER stress and unfolded protein response (UPR) (Apostolou et al., 2008; Glembotski et al., 2012; Henderson et al., 2013; Lindahl et al., 2014; Parkash et al., 2009). In addition to secretion from cells like other neurotrophic factors, MANF has also been found to remain inside the cells and localize in the ER (Apostolou et al., 2008; Glembotski et al., 2012; Henderson et al., 2013; Matlik et al., 2015). MANF therefore possesses intra- and extracellular dual modes of action.

Intrastriatal administration of MANF protein has been shown to protect nigral DA neurons and restore the motor behavior in a 6-hydroxydopamine (6-OHDA) - lesioned rat model of Parkinson's disease (PD) (Voutilainen et al., 2009). Since direct intracranial administration of protein is not feasible to develop into a long-term treatment, viral gene transfer of *MANF* into the brain has been examined in parkinsonian rats. By using lentiviral vectors, a combination of *MANF* and *CDNF* genes into the SN protected nigral DA neurons and improved the rotational behavior in 6-OHDA lesioned rats (Cordero-Llana et al., 2014). By using adeno-associated virus serotype 2 (AAV2) vectors, delivery of *CDNF* gene into the striatum provided neuroprotective and neurorestorative effects in the same rat model of PD (Back et al., 2013; Ren et al., 2013). In a previous study, we developed an efficient AAV serotype 9 (AAV9) - mediated gene delivery system which primarily transduced neuronal cells in the brain (Xue et al., 2010). By using this system, we showed that AAV9-mediated robust and stable expression of *human erythropoietin* (*hEPO*) gene in the striatum protected nigral DA neurons from 6-OHDA-induced neurodegeneration and led to behavioral improvement in parkinsonian rats (Xue et al., 2010). In the present study, we generated an AAV9-mediated *human MANF* (*hMANF*) gene delivery system to apply in a rat model of PD. We attempted to address: 1) whether intrastriatal administration of AAV9-hMANF vectors could result in robust and stable expression of the *hMANF* gene; 2) whether intrastriatal overexpression of the *hMANF* gene led to a long-term protection of nigral DA neurons and functional recovery in 6-OHDA lesioned rats; 3) mechanisms responsible for MANF neuroprotection. In addition, we used a DA cell line-SH-SY5Y cells and examined whether intra- or extracellular application of MANF protected cells against 6-OHDA-induced toxicity via inhibiting the ER stress and activating the PI3K/Akt/mTOR pathway.

2. Materials and methods

2.1. In vivo study

2.1.1. Animals

Adult female Sprague-Dawley rats (8–10 weeks old, 225–250 g) were obtained and housed under a 12 h light/dark cycle with ad libitum access to food and water in the Animal Core Facility of Capital Medical University (CMU), Beijing, China. All experiments associated with rats were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Use and Care Committee of CMU. The number of animals used was the minimum required for statistical analysis, and all precautions were taken to minimize animal suffering.

2.1.2. AAV9 vector production

The hMANF DNA was obtained from pCR3.1-hMANF vectors (kindly provided by Dr. Mart Saarma) and subcloned into an AAV expression plasmid. The expression of the *hMANF* gene was driven by the hybrid cytomegalovirus immediate early enhancer/chicken β-actin promoter. AAV9 vectors carrying either *the hMANF* or *green fluorescent protein* (*GFP*) gene were produced as previously described (Xue et al., 2010). Titers of AAV9-hMANF and AAV9-GFP vectors were 1.02×10^{13} vg/ml and 1.0×10^{13} vg/ml, respectively.

2.1.3. Intrastriatal injection of 6-OHDA and viral vectors

Anesthetized rats (equithesin, 3 ml/kg, i.p.) received an injection of 15 μg of 6-OHDA (5 μg/μl dissolved in 0.1% ascorbic acid, Sigma-Aldrich, St. Louis, MO, USA) into the right striatum according to the following stereotaxic coordinates: AP, +0.5 mm; ML, −2.8 mm; DV, −4.8 mm at a rate of 1 μl/min. The cannula was left in place for another 5 min before withdrawal. D-amphetamine (Sigma-Aldrich) - induced rotational asymmetry was performed 8 days later to select rats with >5 full body turns per min. One group of rats (denoted as 6-OHDA alone group) was sacrificed 10 days after 6-OHDA lesions (0 week), serving as a reference showing the extent of DA neuron degeneration at the time point of viral injections. Remaining rats were assigned into three groups based on rotational scores. Group 1 (6-OHDA-hMANF group): rats received an injection of 2 μl of AAV9-hMANF vectors (10^{13} vg/ml) into the right striatum (AP, 0 mm; ML, −2.8 mm; DV, −5 mm) at a rate of 0.2 μl/min; Group 2 (6-OHDA-GFP group) and Group 3 (6-OHDA-Saline group): rats received an injection of 2 μl of either AAV9-GFP vectors (10^{13} vg/ml) or saline as the same coordinates as Group 1. *D*-amphetamine-induced rotational behavior was tested 2, 4, 6, 8, 12 and 16 weeks after viral injection. Rats were sacrificed 6 weeks and 16 weeks after viral injection respectively and brain tissues were prepared for immunohistochemistry, Western blot, real-time quantitative PCR and high-performance liquid chromatography (HPLC) analysis. Another study was performed in which naïve rats were injected with 2 μl of AAV9-hMANF vectors (hMANF group), AAV9-GFP vectors (GFP group) or saline (Saline group) into the right striatum to observe whether intrastriatal long-term overexpression of the *hMANF* gene affects the nigrostriatal DA pathway up to 24 weeks. The number of rats used in each part of the study was summarized in Table 1.

2.1.4. Behavioral analysis

Rotational behavior was examined as previous description (Chen et al., 2014; Xue et al., 2010). Briefly, rats were injected intraperitoneally with *d*-amphetamine (2.5 mg/kg, Sigma-Aldrich) followed by recording for 90 min using automated rotometer bowls (TSE systems, Chesterfield, MO, USA). Net rotational asymmetry score was expressed as the number of 360° turns per min. Rotation towards the lesioned side was considered to be positive.

2.1.5. Immunohistochemistry

Free floating brain sections containing the striatum (from AP + 1.6 mm to AP − 0.92 mm) and SN (from AP − 4.8 mm to AP − 6.04 mm) from each rat were prepared for immunohistochemistry as previously described (Chen et al., 2014; Xue et al., 2010). The avidin-biotin complex immunoperoxidase technique was used to visualize tyrosine hydroxylase (TH), MANF, major histocompatibility antigen (MHC) class II, complement receptor 3 (CR3) and glial fibrillary acidic protein (GFAP) immunoreactivity. Primary antibodies were rabbit *anti*-TH (1:300, sc-14007; Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), rabbit *anti*-MANF (1:1000, ABN306; Millipore, Bedford, MA, USA), mouse *anti*-MHC class II (1:100, MCA46GA; AbD Serotec, Oxford, UK), mouse *anti*-CR3 (1:100, MCA275GA; AbD Serotec) and mouse *anti*-GFAP (1:400, MAB360; Millipore). Secondary antibodies were biotinylated goat *anti*-rabbit or mouse IgG (1:200, Vector Laboratories, Burlingame, CA, USA). For double- or triple-label immunofluorescent staining, primary antibodies were mouse *anti*-TH (1:400, MAB318;