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Developing therapeutically more efficient Neurturin variants for treatment of Parkinson's disease

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ABSTRACT

In Parkinson's disease midbrain dopaminergic neurons degenerate and die. Oral medications and deep brain stimulation can relieve the initial symptoms, but the disease continues to progress. Growth factors that might support the survival, enhance the activity, or even regenerate degenerating dopamine neurons have been tried with mixed results in patients. As growth factors do not pass the blood-brain barrier, they have to be delivered intracranially. Therefore their efficient diffusion in brain tissue is of crucial importance. To improve the diffusion of the growth factor neurturin (NRTN), we modified its capacity to attach to heparan sulfates in the extracellular matrix. We present four new, biologically fully active variants with reduced heparin binding. Two of these variants are more stable than WT NRTN *in vitro* and diffuse better in rat brains. We also show that one of the NRTN variants diffuses better than its close homolog GDNF in monkey brains. The variant with the highest stability and widest diffusion regenerates dopamine fibers and improves the conditions of rats in a 6-hydroxydopamine model of Parkinson's disease more potently than GDNF, which previously showed modest efficacy in clinical trials. The new NRTN variants may help solve the major problem of inadequate distribution of NRTN in human brain tissue.

1. Introduction

Parkinson's disease (PD) is a neurological disease in which midbrain dopaminergic neurons are lost. While the symptoms initially can be relieved by oral medications and later by deep brain stimulation, the dopaminergic neurons continue to degenerate in this chronic, progressive disease (Kordower and Björklund, 2013). Based on animal models, growth factors that support the survival or activity, or even restore the function of dopaminergic neurons have been regarded as candidates for a novel type of disease-modifying treatment. The Glial cell line-Derived Neurotrophic Factor (GDNF) (Lin et al., 1993) showed a strong potential in animal trials. However, although GDNF seemed beneficial in two open label clinical studies (Gill et al., 2003; Slevin et al., 2005), it failed in a double-blind study (Lang et al., 2006). More recently also neurturin (NRTN), a close homolog of GDNF (Kotzbauer et al., 1996) showed only limited effects in gene therapy-based Phase 2a and 2b clinical trials (Marks et al., 2010; Bartus and Johnson, 2016a; Bartus and Johnson, 2016b; Ceregene, Press release 21.5.2013). The reason why GDNF and NRTN work better in animal models than in the clinical trials is unclear (Bartus et al., 2013). GDNF and NRTN do not pass the blood-

Abbreviations: GDNF, Glial cell line-Derived Neurotrophic Factor; IHC, immunohistochemical; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFRα1, GDNF family receptor α1; GFRα2, GDNF family receptor α2; IP, immunoprecipitated; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NRTN, neurturin; NV1–4, V5-tagged NRTN variants N1-4; N1–4, untagged NRTN variants; OHDA, hydroxydopamine; PD, Parkinson's disease; pgsA 745 cells, CHO cells deficient in heparan sulfate from ATCC; P-TYR, phosphotyrosine; RET, Rearranged during Transfection (a transmembrane tyrosine kinase receptor); SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; WB, Western blotting.

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brain barrier and are delivered intraparenchymally. However, in the brain tissue their diffusion is hindered by their strong binding to extracellular matrix and cell surface heparan sulfated proteoglycans (Hamilton et al., 2001; Bespalov et al., 2011). Therefore promising results with these ligands in small rat brains (2 g), may not necessarily be directly applicable to the much larger human brain (1300 g). In primates an efficient intracranial diffusion of the growth factors is critically important. Monkeys with the largest distribution of GDNF recovered best from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesions (Gash et al., 2005). Monkey studies with identical catheters and flow rates as in the unsuccessful clinical trial, suggested that the trial failed due to a poor and variable distribution of GDNF (Salvatore et al., 2006). Autopsies on patients receiving NRTN gene therapy showed that NRTN diffused <2 mm around the injection site (Bartus et al., 2011), and that an enigmatically sparse tyrosine hydroxylase (TH) response was achieved after the virus vector-driven NRTN delivery (Bartus and Johnson, 2016a). It is worth to keep in mind that NRTN easily aggregates at high concentration, therefore its limited diffusion cannot necessarily be compensated by a substantial increase of the dose. Here we developed biologically active NRTN variants with reduced binding to heparin and improved intracranial diffusion for treatment of PD.

2. Materials and methods

2.1. Modeling and cloning of NRTN and its variants

The 3D structure of NRTN was modeled based on the crystal structure of GDNF (Eigenbrot and Gerber, 1997; Parkash et al., 2008) using PyMol software (DeLano Scientific). For expression in mammalian cells, human NRTN (OpenBiosystems, BC137399) was subcloned (excluding the endogenous signal and prosequence) with an IgG signal sequence followed by the sequence of mature NRTN as previously described (Fjord-Larsen et al., 2005), except that pAAV-MCS (Stratagene) was used as a backbone. The variants were created by inverse PCR mutagenesis (primer sequences in the supplement) and all inserts were sequenced.

The V5-tag (GKPIPNPLLGLDST) was added to the N-terminus with the residues (AR) between the signal sequence and the V5 tag and (SG) between the V5-tag and the mature NRTN sequence. The cloning (primer sequences in the supplement) was done as above. The V5-tagged variants of human NRTN were named NV1-NV4. The cloning of NRTN for up-scaled expression is described below.

2.2. Expression, purification and characterization of NRTN

CHO cells were transiently transfected with GFP or the V5-tagged NRTN variants NV1-NV4 using Turbofect (Thermo Scientific). Two days later the media (DMEM, Sigma-Aldrich; 10% FBS, HyClone; 100 U/ml penicillin, Gibco; 100 μg/ml streptomycin, Gibco) were collected, boiled with reducing Laemmli buffer and analyzed by Western blotting with V5-antibodies (R960-25, Invitrogen).

NRTN production was up-scaled in CHO cells using constructs without the prosequence and tag-sequences with the proprietary QMCF Technology at Icosagen Ltd. (European Patent EP1851319). The NRTN variants were purified from the media by heparin affinity chromatography, GFRα2 affinity chromatography and gel filtration. The proteins were stored in 150 mM NaCl, 10 mM Na-citrate pH 5.0 at −20 °C (purification protocol in the Supplemental material). The N-terminal sequence of the purified NRTN variants was determined by Edman degradation using a Procise 494A HT Sequencer (Perkin Elmer/Applied Biosystems, Foster City, CA). The molecular mass of the proteins was determined using MALDI-TOF mass spectrometry (Ultraflex TOF/TOF, Bruker Daltonics). To increase the detection sensitivity in Western blots, untagged NRTN was analyzed under non-reducing conditions, without boiling with antibodies to NRTN (AF477, R&D Systems). Commercial NRTN and GDNF (from *E.coli*) were from Peprotech Inc. and ProSpec Ltd., respectively.

2.3. Heparin affinity chromatography

For initial screening, media from transiently transfected CHO cells were diluted with 10 mM HEPES, pH 7.2 and analyzed using heparin columns (Supplemental material). To characterize the affinity of the purified NRTN variants to heparin, we used a SMART System chromatograph (Pharmacia Biotech) equipped with a self-packed column (2 × 20 mm) with heparin Sepharose from HiTrap Heparin HP columns (GE Healthcare). The flow rate was 50 μl/min. After loading 2 μg of each purified NRTN variant to the column, the samples were eluted with a linear NaCl gradient (10 mM HEPES, pH 7.2, with NaCl up to 2 M). The elution was monitored by A214 nm and the corresponding salt concentration by measuring conductivity.

2.4. RET-phosphorylation and cell-based binding assays

Both assays were done as described in Supplemental material and (Virtanen et al., 2005). When purified NRTN variants, commercial NRTN or GDNF were used in RET-phosphorylation assays, they were diluted in DMEM. In cell-based binding assays competition of the ^{125}I-NRTN variants was performed with the unlabeled NRTN variants in DMEM with 0.5% BSA and 0.2% dry milk (containing soluble heparan sulfates). Monitoring of ^{125}I-NRTN was done either from cell lysates using a gamma counter (Perkin Elmer Wizard, 1480 automatic) or from intact cells using LigandTracer Grey (Ridgeview Instruments AB). In both cases IC_{50} was calculated based on 4–9 parallel data points with GraphPad Prism. Thereafter the mean IC_{50} was determined based on 2–5 independent measurements. As the concentration of ^{125}I-NRTN (50 pM) was much lower than the IC_{50} values obtained, the IC_{50} values correspond approximately to K_d (dissociation constant) values.

2.5. In vitro survival assay on embryonic dopaminergic neurons

Dissociated midbrain cultures from E13.5 NMRI mice were prepared (Planken et al., 2010), fixed at 5DIV and stained with anti-TH antibody (AB 1542, Millipore) and Cy3 affinity pure donkey anti-sheep IgG (713-165-147, Jackson). Quantification of TH-positive cells was done using images analyzed with Image-Pro Plus 5.1.2.59, with constant intensity and size range criteria throughout an experiment. The number of TH-positive cells for each data point was the average of at least 2 replicate micro islands. For inter-experiment normalization, this average was divided by the mean of all of the data points within the corresponding experiment. The resulting relative number of TH-positive cells in each experiment was used to calculate the average of all of the experiments (n = 3–8), finally expressed as per cent of the number of TH-positive cells surviving without added growth factors.

2.6. In vitro organ culture of kidney explants

E11.5 mouse urogenital blocks were dissected and cultured on Transwell filters (Fisher) in a Trowell-type system (Sainio et al., 1997) in DMEM medium (10% FCS, 1% Glutamax and 1% penicillin/streptomycin), supplemented with 500 ng/ml WT, NRTN variants N2 or N4. After 48 h, the explants were stained for calbindin D28K (sc-7691, Santa Cruz).

2.7. Immunocytochemical staining

NRTN variants were transiently expressed (2 days) in CHO cells. The media were stored at +4 °C. A second set of CHO cells was plated on uncoated cover-slips (2 days), transfected with GFRα2 (24 h) and exposed to the stored media containing the different NRTN variants. The cells were incubated with media for 10 min, rinsed with DMEM, fixed with