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Regular Article

# GDNF signaling implemented by GM1 ganglioside; failure in Parkinson's disease and GM1-deficient murine model

Piotr Hadaczek [a,1], Gusheng Wu [b,1], Nitasha Sharma [a], Agnieszka Ciesielska [a], Krystof Bankiewicz [a], Amy L. Davidow [c], Zi-Hua Lu [b], John Forsayeth [a,*], Robert W. Ledeen [b,**]

[a] Department of Neurological Surgery, University of California San Francisco, San Francisco, CA 94103-0555, USA
[b] Department of Neurology and Neurosciences MSB-H506, The State University of New Jersey, 185 South Orange Ave., Newark, NJ 07103, USA
[c] Department of Biostatistics/Epidemiology, New Jersey Medical School, Rutgers, The State University of New Jersey, 185 South Orange Ave., Newark, NJ 07103, USA

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ABSTRACT

GDNF is indispensible for adult catecholaminergic neuron survival, and failure of GDNF signaling has been linked to loss of dopaminergic neurons in Parkinson's disease (PD). This study demonstrates attenuated GDNF signaling in neurons deficient in ganglio-series gangliosides, and restoration of such signaling with LIGA20, a membrane permeable analog of GM1. GM1 is shown to associate in situ with GFRα1 and RET, the protein components of the GDNF receptor, this being necessary for assembly of the tripartite receptor complex. Mice wholly or partially deficient in GM1 due to disruption of the *B4galnt1 gene* developed PD symptoms based on behavioral and neuropathological criteria which were largely ameliorated by gene therapy with AAV2-GDNF and also with LIGA20 treatment. The nigral neurons of PD subjects that were severely deficient in GM1 showed subnormal levels of tyrosine phosphorylated RET. Also in PD brain, GM1 levels in the occipital cortex, a region of limited PD pathology, were significantly below age-matched controls, suggesting the possibility of systemic GM1 deficiency as a risk factor in PD. This would accord with our finding that mice with partial GM1 deficiency represent a faithful recapitulation of the human disease. Together with the previously demonstrated age-related decline of GM1 in human brain, this points to gradual development of subthreshold levels of GM1 in the brain of PD subjects below that required for effective GDNF signaling. This hypothesis offers a dramatically different explanation for the etiology of sporadic PD as a manifestation of acquired resistance to GDNF.



## Introduction

Parkinson's disease (PD) is characterized by gradual loss of dopaminergic (DA) and other catecholaminergic neurons, progressing from peripheral to central neuronal systems over periods of many years (Braak et al., 2003). Unlike familial forms, sporadic PD has no cogent theory that reconciles the disparate data into a compelling narrative regarding its etiology. The vulnerability of these neurons has drawn attention to glial cell line-derived neurotrophic factor (GDNF), considered indispensable for adult catecholaminergic neuron survival. It belongs to a family of neurotrophic factor ligands related to TGFβ that includes neurturin, persephin, and artemin, all of which signal via multicomponent receptors consisting of the RET receptor tyrosine kinase plus a GPI-linked co-receptor (GFRα) that gives binding specificity. GDNF acts molecularly by high affinity binding to GFRα1, causing redistribution of RET into raft microdomains (Tansey et al., 2000). Interaction of these GDNF receptor components transmits survival signals to the cell commencing with homodimerization and autophosphorylation of RET (pRET). Successful results with GDNF-based therapies in animal studies led to clinical trials in which some PD patients experienced therapeutic benefit after putaminal infusions of GDNF (Gill et al., 2003; Slevin et al., 2005; Patel et al., 2005). However, a randomized, placebo-controlled trial that showed limited benefit did not achieve efficacy end-points (Lang et al., 2006) for reasons that may relate to adequate distribution of infused GDNF within the target putamen (Gimenez et al., 2011). In light of delivery-related problems, use of gene therapy for delivery of GDNF to the nigrostriatal system is now under consideration (Johnston et al., 2009; Kells et al., 2012). Another possible explanation for the variable results is inadequate GM1 in the affected

*Abbreviations:* aSyn, alpha synuclein; BDNF, brain-derived neurotrophic factor; BSS, basic salt solution; CtxB, B subunit of cholera toxin; DA, dopamine or dopaminergic; GDNF, glial cell line-derived neurotrophic factor; HPTLC, high performance thin-layer chromatography; HT, heterozygote; KO, knockout; LIGA20, membrane permeable analog of GM1; NGF, nerve growth factor; PBST, PBS with Tween-20; PD, Parkinson's disease; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; WT, wild type.

* Correspondence to: J. Forsayeth, Department of Neurological Surgery, University of California San Francisco, San Francisco, CA 94103-0555, USA.
** Correspondence to: R. W. Ledeen, Department of Neurology & Neurosciences, MSB-H506, New Jersey Medical School, Rutgers, The State University of New Jersey, 185 South Orange Ave., Newark, NJ 07103, USA.
*E-mail addresses:* john.forsayeth@ucsf.edu (J. Forsayeth), ledeenro@njms.rutgers.edu (R.W. Ledeen).
[1] These authors contributed equally.

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neurons. Association of this ganglioside with the RET/GFRα1 receptor is required for optimal GDNF neuroprotection, as revealed in the present study. This is consonant with previous studies revealing Parkinsonian degeneration in genetically altered mice deficient in GM1 (Wu et al., 2011a). Interestingly, heterozygous (HT) mice with only partial deficiency of GM1 showed a similar pattern of neurodegeneration as the homozygous (knockout, KO) mice, albeit less severely progressive (Wu et al., 2012). These PD manifestations were alleviated by peripheral administration of LIGA20, a membrane-permeable analog of GM1 that penetrates the blood brain barrier (Wu et al., 2005) and accesses both membrane and intracellular loci of neurons (Wu et al., 2001a, 2001b) where it mediates the multiple functions of GM1. GM1 itself, despite limited brain penetration, was nevertheless able at high doses and frequent application to improve motor symptoms and reduce the rate of symptom progression in PD subjects (Schneider et al., 2013).

In view of the demonstrated requirement of GM1 for functional efficacy of other neurotrophic factors, such as NGF (Mutoh et al., 1995) and BDNF (Pitto et al., 1998), we considered the possibility that deficiency of this ganglioside might cause impaired GDNF signaling. The present study supports that hypothesis, based on evidence for deficient GDNF receptor activation in the above GM1-deficient mice as well as PD subjects. We have also performed in vitro experiments in cells that express RET and GFRα1 as well as GM1 and its metabolic precursor, GD1a. In each of these systems, reduced expression of GM1 (and GD1a) resulted in attenuated formation of phosphorylated RET (pRET), the first step in GDNF-induced signaling, and in downstream formation of pMAPK. This signaling deficit was largely alleviated with LIGA20. Coordinate activity of GM1 and GDNF was further indicated in the virtual elimination of alpha-synuclein (aSyn) aggregation both by LIGA20 (intraperitoneal) and GDNF application via AAV2 vector transmission into the striatum. The latter treatment also improved motor performance with a pole test and restored lost tyrosine hydroxylase (TH) immunoreactivity, similar to that previously shown for LIGA20 with the KO and HT mice (Wu et al., 2011a, 2012). These results demonstrate the ability of upregulated GDNF to override the GDNF resistance acquired with depressed GM1. That a similar mechanism based on GM1 insufficiency may be operating in PD itself was suggested by the earlier observation of depressed levels of GM1 in DA neurons of the substantia nigra pars compacta (SNpc) (Wu et al., 2012). We now report significantly diminished pRET formation in similar neurons of PD subjects, correlated with diminished GM1. Our concurrent finding of significant GM1 deficit in the occipital cortices of PD subjects further suggests the possibility of systemically low levels of GM1 in PD subjects which, with the progressive diminution of GM1 known to occur with aging, eventually falls below the level needed to maintain effective GDNF signaling. In this respect, our hypothesis offers a dramatically different explanation for the etiology of sporadic PD as a manifestation of acquired resistance to GDNF in a manner somewhat reminiscent of the insulin resistance seen in Type 2 diabetes mellitus.

## Materials and methods

### *Generation of Neuro2a (α1-GA) cells with knockdown of B4galnt1 (GM2/GD2 synthase)*

A Neuro2a cell line, previously engineered to over-express human GFRα1 (Crowder et al., 2004), (α1), was received from Dr. Jeffrey Milbrandt (Washington University, St. Louis, MO). We used a commercially available lentivirus vector encoding shRNA (3 to 5 expressing constructs) specific for mouse B4galnt1 mRNA (Santa Cruz Biotechnology, Inc.; sc-77390-V) to suppress GM2/GD2 synthase expression. To generate cells with a stable suppression of the enzyme (α1-GA), we followed a detailed protocol available on manufacturer's website (http://www.scbt.com/protocols.html?protocol=shrna_lentiviral_particles_transduction). The concentration of puromycin used for selection of clones was 2 μg/ml (puromycin death curve was prepared prior to lentiviral infection). Control shRNA lentivirus (a scrambled shRNA sequence) was used as a negative control (sc-108080).

The effect of lenti-shRNA transfection on GM2/GD2 synthase expression was analyzed by quantitative RT-PCR. RNA was isolated with QIAzol Lysis Reagent and RNAeasy MinElute columns from Qiagen. One microgram of RNA was used for the RT reaction (iScript™ cDNA synthesis Kit from Bio-Rad). One microliter of cDNA (1:10 dilution) was subsequently used for real-time PCR (TaqMan® Gene Expression Assay for mouse B4galnt1; cat. no. 4351372 and mouse Hprt1 used as a house-keeping control cat. no. 4453320, Applied Biosystems). The reaction was performed and analyzed in ViiA™ 7 Real-Time PCR System (Applied Biosystems). Threshold Ct was set to 0.1. Relative change of GM2/GD2 synthase expression was determined by subtraction of the ΔCt value for the control cell line from the ΔCt value for the B4gallnt1 shRNA-transfected cells (ΔΔCt value). Fold change was subsequently calculated with the formula $2^{-\Delta\Delta Ct}$, defined as the expression of GM2/GD2 synthase relative to HPRT mRNA.

### *Measurement of MAPK activation by GDNF in Neuro2a (α1) and Neuro2a (α1-GA) cells; dose response study (Fig. 1B)*

To measure the activation of MAPK1/2 in α1 and α1-GA cells by GDNF, a dose–response curve was generated by means of a custom in-cell ELISA. Cells were seeded at a density of 30,000 cells/well in a 96-well plate, Optilux™, black/clear bottom (Fisher Scientific). After overnight incubation at 37 °C, the RPMI-1640 medium with 10% FBS penicillin/streptomycin was removed and cells were starved in serum-free medium for 6 h. Following starvation, serum-free medium with various concentrations of GDNF (range: 0.78–100 ng/ml) was added to cells (6 replicates per concentration) and incubated for 10 min at 37 °C. Later, the medium was removed and cells were fixed immediately with cold methanol (200 μl/well) for 20 min. After 2 washes in PBS with 0.1% Tween-20 (PBST), the cells were blocked with 5% chicken serum in PBST for 1 h at room temperature. A rabbit monoclonal antibody against phospho-p44/p42 MAPK (Cell Signaling Technology) was then added at a dilution of 1:200 in PBST with 2% chicken serum and incubated at 4 °C overnight. After 3 rinses in PBST, a chicken anti-rabbit IgG (1:500) labeled with Alexa488 (Invitrogen) was added to each well and incubated at room temperature for 1 h. After 2 rinses in PBST containing 0.2 μg/ml DAPI (4′,6-diamidino-2-phenylindole), cells were rinsed in PBS and the plate was read in BioTek FL-800 fluorescence microplate reader. Fluorescence was determined via two pairs of filters: 488/515 green channel (for phospho-p44/p42 MAPK) and 360/420 blue channel (DAPI for nuclear staining). The Alexa 488 fluorescence was then divided by the DAPI fluorescence to normalize for cell count per well. The curve of MAPK1/2 activation (RFU, relative fluorescence units, $R_{Alexa488/DAPI}$) was plotted against GDNF concentration. The difference between the two curves was obtained by regression analysis of RFU values with each dose treated as either a categorical variable or continuous variable; in both models the effect of the cell group was statistically significant ($p < 0.0001$). Individual GDNF doses were analyzed by two-way ANOVA with Bonferroni post-hoc test.

### *Measurement of RET and MAPK activation by GDNF in SH-SY5Y, Neuro2a (α1), and Neuro2a (α1-GA) cells; restoration of lost signaling by LIGA20 (Figs. 1C, 2C)*

These cells were cultured in DMEM-10% FBS in 96-well Nunclon Delta black microwell plates (Nunc 137101) containing 30,000 cells/100 μL/well. Some SH-SY5Y cells were differentiated with retinoic acid (40 μM) for 6 days and some of these were treated with d-threo-PDMP (10 μM; Matreya, Pleasant Gap, PA) during the last 24 h. Portions of the PDMP-treated SH-SY5Y cells and the above Neuro2a (α1-GA) cells were treated with LIGA20 (gift of Fidia Pharmaceuticals, Abano Terme, Italy) (10 μM) for 2 h. For signaling measurements, all cell types were replaced to DMEM without serum for 4 h, then stimulated