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Research paper

Overexpression of α-synuclein simultaneously increases glutamate NMDA receptor phosphorylation and reduces glucocerebrosidase activity

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HIGHLIGHTS

- Overexpression of α-syn in mice and cells phosphorylates NMDA subunits.
- Overexpression of α-syn in mice and cells reduces GCase levels and activity.
- Cells overexpressing α-syn are more sensitive to neurotoxicity of 6-OHDA and NMDA.
- NMDA reduces lysosomal GCase activity in cells.

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ABSTRACT

Progressive accumulation of α-synuclein (α-syn)-containing protein aggregates throughout the nervous system is a pathological hallmark of Parkinson's disease (PD). The mechanisms whereby α-syn exerts neurodegeneration remain to be fully understood. Here we show that overexpression of α-syn in transgenic mice leads to increased phosphorylation of glutamate NMDA receptor (NMDAR) subunits NR1 and NR2B in substantia nigra and striatum as well as reduced glucocerebrosidase (GCase) levels. Similarly, molecular studies performed in mouse N2A cells stably overexpressing human α-syn ($^{\alpha\text{-syn}}$N2A) showed that phosphorylation states of the same NMDAR subunits were increased, whereas GCase levels and lysosomal GCase activity were reduced. $^{\alpha\text{-syn}}$N2A cells showed an increased sensitivity to neurotoxicity towards 6-hydroxydopamine and NMDA. However, wildtype N2A, but not $^{\alpha\text{-syn}}$N2A cells, showed a further reduction in viability when co-incubated with 6-hydroxydopamine and the lysosomal inhibitors NH_4Cl and leupeptin, suggesting that α-syn per se perturbs lysosomal functions. NMDA treatment reduced lysosomal GCase activity to the same extent in $^{\alpha\text{-syn}}$N2A cells as in wildtype N2A cells, indicating that the α-syn-dependent difference in NMDA neurotoxicity is unrelated to an altered GCase activity. Nevertheless, these data provide molecular evidence that overexpression of α-syn simultaneously induces two potential neurotoxic hits by increasing glutamate NMDA receptor phosphorylation, consistent with increased NMDA receptors functionality, and reducing GCase activity.

1. Introduction

Parkinson's disease (PD) is pathologically characterized by the progressive appearance of intracytoplasmic protein inclusions, called Lewy bodies, in dopamine neurons of the substantia nigra. The major component of Lewy bodies is aggregated α-synuclein (α-syn) [1]. Native α-syn is mainly located in presynaptic nerve

Abbreviations: α-syn, α-synuclein; CMA, chaperone-mediated autophagy; GBA, glucosidase beta acid; GCase, glucocerebrosidase; GFP, green fluorescent protein; NMDAR, *N*-methyl-D-aspartate receptor; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; TH, tyrosine hydroxylase.

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terminals, associated with the SNARE complex close to synaptic vesicles [2]. The pathophysiological involvement of α-syn in PD is further supported by genetic evidence. Increases in copy number or point mutations of the α-syn gene cause PD [3]. Biochemical studies have shown that overexpression of α-syn is cytotoxic in vitro and in vivo in experimental models of Parkinsonism [4–6]. Nonetheless, the downstream mechanisms whereby α-syn exerts its cytotoxic effects are not entirely understood. Since there is a strong interaction between dopamine and glutamate neurotransmission [7], it is plausible that glutamate receptors are altered by α-syn overexpression in dopamine neurons. This could occur indirectly via altered levels of dopamine influencing the phosphorylation states of glutamate receptor NR1 and/or NR2B subunits located in dopamine nerve terminals [8] or directly via protein–protein interactions between α-syn and glutamate receptors. It has, indeed, been reported that α-syn directly binds to NR2B subunits of NMDA receptors [9]. Overactivation of NMDA receptors cause excitotoxicity via enhanced calcium-dependent enzymatic processes, including production of free radicals and nitric oxide [10]. There is much evidence that α-syn is accumulated because of impaired clearance. α-Syn is degraded both by the ubiquitin-proteasomal system and by lysosomes via chaperone-mediated autophagy (CMA) [11,12]. In particular, several studies have shown that impaired functions of proteins involved in CMA, including LIMP-2 [13], cysteine cathepsins [14] and glucocerebrosidase (GCase) [15], result in α-syn accumulation. This is potentially clinically significant since the most common risk factor for development of PD is mutations in the glucosidase beta acid (GBA) gene encoding GCase [16,17]. Both heterozygotic and homozygotic GBA mutation carriers have a 6–10-fold increased risk to develop PD [16,17]. At the molecular level, α-syn has been shown to interact with GCase under acidic conditions, resembling the lysosomal environment, and reduce GCase enzyme activity [18]. Moreover, Mazzulli et al. [19] demonstrated a pathogenic feedback mechanism of α-syn and GCase depletion in the lysosome, where accumulation of glycosylceramide due to downregulation of GCase activity leads to accumulation of α-syn and the stabilization of α-syn oligomers with consequent neurotoxic effects. Accumulation of α-syn blocked the ER-Golgi trafficking of GCase and led to decreased levels of GCase in the lysosome, further advancing glycosylceramide accumulation and stabilization of soluble α-syn oligomers in the lysosome.

Using transgenic mice and mouse neuroblastoma N2A cells overexpressing human α-syn ($^{\alpha\text{-syn}}$N2A), we examined the effect of increased gene dosage of α-syn on NMDAR subunit levels along with their phosphorylation states and on GCase levels and activity to elucidate mechanisms of α-syn-mediated toxicity with relevance to PD.

2. Materials and methods

2.1. Animals

Adult six months old C57BL/6J mice overexpressing wildtype human α-synuclein under the tyrosine hydroxylase promotor and corresponding age-matched wildtype mice were used ([20] available from Jackson lab (C57BL/6J-Tg(Th-SNCA) 5Eric/J)). A cohort consisting of 24 WT mice (7 males and 17 females) and 21 α-syn overexpressing mice (Tg, 7 males and 14 females) were used. To preserve protein phosphorylation, the mice were sacrificed by focused microwave irradiation (4.5–5 kW for 1.4 s), using a small animal microwave (Muromachi Kikai, Tokyo, Japan) [21]. Striata and substantia nigra were dissected out and stored at −80 °C until further analysis. Experiments were approved by the local ethical committee at Karolinska Institute and followed the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Cell culture, α-syn transfection and stable cell line selection

N2A cells are commonly used for studies relevant to neurodegenerative disorders (e.g., [22–25]). N2A cells were grown in Dulbecco's modified essential medium (DMEM) (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Sigma–Aldrich, Stockholm, Sweden) and a penicillin (100 U/ml)/streptomycin (100 μg/ml) mix (Invitrogen) at 37 °C and 5% CO_2. For α-syn transfection, pcDNA3.1 plasmids containing a human α-syn-green fluorescent protein (GFP) insert were used. Exogenously expressed α-syn-GFP fusion proteins have previously been reported to retain the subcellular distribution of native synuclein [26,27]. α-Syn-carrying plasmids or empty vector as control, were introduced into cultured N2A cells by transfection with 1% Lipofectamine2000 (Invitrogen). Stable colonies were selected by culturing with 300 μg/ml geneticin (G418; Invitrogen).

2.3. Immunoblotting

Frozen cells or tissue samples were sonicated in 1% SDS and boiled for 10 min. Protein concentration of homogenates was determined using the BCA™ protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein (25 μg) were separated on 12% SDS polyacrylamide gels and transferred to Immobilon®-P Polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated for 1 h at room temperature with 5% (w/v) dry milk in TBS-Tween20. Immunoblotting was performed with phosphorylation state-specific antibodies against Ser^{896}-NR1 (UBI,Hauppauge, NY, USA), Ser^{897}-NR1 (Cell signalling, Piscataway, NJ, USA), Ser^{1303}-NR2B (Upstate), or Tyr^{1472}-NR2B (Millipore). Immunoblotting was also carried out using antibodies against total NR1 (UBI), NR2A (Millipore), NR2B (Millipore), α-syn (Santa Cruz Biotechnology, Heidelberg, Germany), β-actin (Sigma–Aldrich) and GCase (Sigma–Aldrich), in 5% dry milk, TBS-Tween20. Membranes were washed with TBS–Tween20 and incubated with secondary HRP goat anti-rabbit antibody (Dako, Agilent technologies, CA, USA) for 1 h at room temperature. Membranes were then washed with TBS-Tween20, and the immunoreactive bands were detected by chemiluminescence using ECL Plus substrate (PerkinElmer, Waltham, MA, US) and Biomax XAR film (Kodak, Rochester, NY, USA). The autoradiograms were digitised using a Dia-Scanner (Epson, Nagano, Japan) and quantified using the ImageJ software (NIH Bethesda, MA, USA). The levels of phosphorylated proteins were normalized to total levels of the same protein. The levels of total protein were normalized to β-actin.

2.4. MTT cell viability assay

Cells were plated in 96-well plates containing 140 μL of supplemented DMEM. Cell counting was performed using an automated cell counter TC10™ (Bio-Rad Laboratories, Sundbyberg, Sweden) and the same number of cells were seeded into each well. After overnight incubation to allow cell attachment, the medium was replaced with 140 μL of supplemented DMEM along with vehicle, 6-hydroxydopamine (6-OHDA) (30 μM, Sigma–Aldrich), lysosomal inhibitors; NH_4CL (20 mM)/leupeptin (50 μM; Sigma–Aldrich), NMDA (500 μM, Sigma–Aldrich), NMDA receptor antagonist; MK-801 (100 μM, Sigma–Aldrich), alone or in combination, and incubated for 24 h at 37 °C. All experiments were performed at least in triplicate.

MTT assays were used to determine the viability of wild type (WT) N2A cells or N2A cells stably expressing α-syn ($^{\alpha\text{-syn}}$N2A). Cells were cultured in 96-well plates to 50–80% confluence in a final