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High-content analysis of α-synuclein aggregation and cell death in a cellular model of Parkinson's disease

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HIGHLIGHTS

- We describe a high-content assay to measure oxidative stress-induced α-synuclein aggregation and cell death in human neuronal cell lines.
- The presented method allows the quantification of both α-synuclein inclusion formation and apoptosis in an automated and reproducible manner.
- The high-content assay can contribute to the identification of compounds and genes that might alter α-synuclein aggregation and toxicity.

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ABSTRACT

Background: Alpha-synuclein (α-SYN) aggregates represent a key feature of Parkinson's disease, but the exact relationship between α-SYN aggregation and neurodegeneration remains incompletely understood. Therefore, the availability of a cellular assay that allows medium-throughput analysis of α-SYN-linked pathology will be of great value for studying the aggregation process and for advancing α-SYN-based therapies.
New method: Here we describe a high-content neuronal cell assay that simultaneously measures oxidative stress-induced α-SYN aggregation and apoptosis.
Results: We optimized an automated and reproducible assay to quantify both α-SYN aggregation and cell death in human SH-SY5Y neuroblastoma cells.
Comparison with existing methods: Quantification of α-SYN aggregates in cells has typically relied on manual imaging and counting or cell-free assays, which are time consuming and do not allow a concurrent analysis of cell viability. Our high-content analysis method for quantification of α-SYN aggregation allows simultaneous measurements of multiple cell parameters at a single-cell level in a fast, objective and automated manner.
Conclusions: The presented analysis approach offers a rapid, objective and multiparametric approach for the screening of compounds and genes that might alter α-SYN aggregation and/or toxicity.

Abbreviations: α-SYN, alpha-synuclein; PD, Parkinson's disease; HCA, high-content analysis; Thio S, Thioflavin S; LDH, lactate dehydrogenase.

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1. Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder characterized by a loss of dopaminergic neurons in the substantia nigra pars compacta. The protein alpha-synuclein (α-SYN) is considered a key player in synucleinopathies, including PD (Deleersnijder et al., 2013). First, aggregated α-SYN is a major constituent of Lewy Bodies, i.e. eosinophilic cytoplasmic inclusions that are a pathological hallmark of PD (Spillantini et al., 1997). Second, missense mutations in the α-SYN gene (A53T, A30P, E46K, G51D and H50Q) (Appel-Cresswell et al., 2013; Kruger et al., 1998; Lesage et al., 2013; Polymeropoulos et al., 1997; Zarranz et al., 2004) and locus duplication (Chartier-Harlin et al., 2004; Ibanez et al., 2004) or triplication (Singleton et al., 2003) of the wild type (WT) gene were identified as cause of autosomal-dominant monogenetic PD. Finally, overexpression of WT and mutant forms of α-SYN in C. *elegans*, Drosophila, mice, rats and non-human primates leads to neuronal inclusions and/or behavioral symptoms very similar to those observed in PD patients. (Eslamboli et al., 2007; Feany and Bender, 2000; Kirik et al., 2003, 2002; Lakso et al., 2003; Lauwers et al., 2003; Oliveras-Salva et al., 2013; Van der Perren et al., 2015).

Although research on PD has accelerated in the last decade due to increased insight in the pathogenic mechanisms (Gasser, 2009; Lubbe and Morris, 2014; Mizuno et al., 2008; Olanow and Brundin, 2013), there is still a lack of efficient and robust cell culture models. Such assays could speed up the discovery of new candidate inhibitors of PD pathology and in particular of α-SYN aggregation.We developed an oxidative stress-induced cellular model of α-SYN aggregation in human SH-SY5Y neuroblastoma cells, followed by high-content image-based analysis to simultaneously quantify α-SYN aggregate formation and cell death (Aelvoet et al., 2014; Deleersnijder et al., 2011; Gerard et al., 2010; Oliveras-Salvá et al., 2014; Van der Veken et al., 2012). High-content analysis (HCA) is the process of extracting complex information from images of cells and tissues using a combination of automated microscopy and automated image analysis (Denner et al., 2008; Dragunow, 2008; Rausch, 2005). HCA has already been successfully applied to determine several aspects of the brain's complexity, such as neurite outgrowth (Radio, 2012; Vallotton et al., 2007; Wang et al., 2010), neurogenesis (Blackmore et al., 2010), cell count (Narayan et al., 2007), inclusion formation (Boyd et al., 2014; Scotter et al., 2008; Teboul et al., 2007), apoptosis (Dragunow et al., 2006; Fennell et al., 2006), cell migration (Schmandke et al., 2013) and signal transduction (Grimsey et al., 2008). To our knowledge, this technology has not been used yet to study α-SYN aggregation, while it has several advantages over manual methods of α-SYN aggregate analysis. Most cellular studies thus far have been based on estimating the intensity of Thioflavin S (Thio S) staining in cells or on manual counting of α-SYN inclusion formation, which are both slow, subjective and laborious methods. Moreover, manual methods do not allow to discriminate between changes in aggregate size or density and changes in aggregate number. Furthermore, although biochemical and biophysical techniques (e.g. gel filtration, low speed or analytical centrifugation, transmission electron microscopy, structural stability, protease resistance, turbidity, circular dichroism and immunological investigations) can provide useful information on the size and the nature of the α-SYN aggregates, they lack cellular resolution and/or concurrent analysis of cell health or simply do not allow for bigger throughput (Harper and Lansbury, 1997; Lundvig et al., 2005). Additionally, routine application of most of the aforementioned techniques in large-scale assays for therapeutically relevant interactions or promising drug therapies is not feasible. Our HCA method for quantifying α-SYN aggregation circumvents the caveats listed above as it allows simultaneous measurements of multiple cell characteristics at a single-cell level in a fast, objective and automated manner. The presented analysis platform offers a rapid, objective and multiparametric approach for the validation of compounds and genes that might alter α-SYN aggregation as well as the concurrent measurement of changes in cell viability. This assay allows identification of both inhibitors and stimulators of α-SYN aggregation and can also be used to study α-SYN aggregation itself.

2. Materials and methods

2.1. Cell culture

Human SH-SY5Y neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Life Technologies, Cat No. 31966047), 50 mg/ml gentamycin (Gibco-BRL, Life Technologies, Cat No. 15750045) and 1% non essential amino acids (Gibco-BRL, Life Technologies, Cat No. 11140068) (referred to herein as DMEM-complete) at 37 °C and 5% CO_2 in a humidified atmosphere.

2.2. Antibodies and other reagents

DAPI (4′,6-diamidino-2-phenylindole, Cat No. D9592), propidium iodide (PI, Cat No. P4170), staurosporine (Cat No. S6942) and FK506 (tacrolimus, Cat No. F4679) were purchased from Sigma-Aldrich (Bornem, Belgium). FTI-277 was a kind gift from Janssen Pharmaceutica (Beerse, Belgium). 1 mg/ml stock solutions of PI (40×) and DAPI (10,000×) were made in H_2O. Staurosporine and FTI-277 were dissolved in DMSO (Sigma-Aldrich, Cat No. 276855) in a stock solution of 1 mM. FK506 was dissolved in DMSO in a stock solution of 10 mM (1000×). For Western blot the anti-human anti-α-SYN antibody 15G7 (1:100, Enzo Life Sciences, Cat No. ALX-804-258) was used and a mouse monoclonal anti-α-tubulin antibody (1:50,000, Sigma, Cat No. T5168) was used as internal loading control. The rabbit polyclonal antibody against cleaved caspase-3 was purchased from Cell Signaling Technology (Danvers, MA, USA Cat No. 9661s), and used at 1:500 and 1:1000 for immunocytochemistry (ICC) and western blotting, respectively. The cytotoxicity detection kit was purchased from Roche (Cat No. 11644793001) and the lactate dehydrogenase activity was determined according to the manufacturer's instructions.

2.3. Generation of polyclonal stable overexpression cell lines using lentiviral vectors

cDNA encoding α-SYN was cloned into the pCHMWS-IRES-puro lentiviral (LV) transfer plasmid. LV vectors encoding α-SYN under control of the cytomegalovirus (CMV) promoter were prepared in house as previously described using the triple transfection method with a transfer plasmid (Geraerts et al., 2005), an envelope plasmid encoding VSV-G and a packaging plasmid (p8.91). The LV constructs allowed co-expression of the puromycin resistance gene from the same transcript as the α-SYN cDNA via an internal ribosome entry site (IRES). The LV titer was determined via measurement of p24 antigen content by an enzyme linked immunosorbent assay (HIV-1 p24 ELISA kit, XpressBio Europe Cat No. XB-1000). Prior to transduction, 1.5×10^5 SH-SY5Y cells were plated in a 24-well plate and grown in DMEM-complete. The following day, vector was applied to the cells for 2 days, after which the vector-containing medium was replaced by DMEM-complete with 1 μg/ml puromycin (Invitrogen, Cat No. ANT-PR-1). Overexpression of α-SYN was controlled via western blot.

2.4. Western blotting

Cells were plated in 6-well plates (2×10^6 cells/well). After 1 day, cells were washed with PBS (Lonza, Cat No. BE15-512D) and