MODELING OF MOLECULAR PROCESSES UNDERLYING PARKINSON’S DISEASE IN CELLS OF METHYLOTROPHIC YEAST HANSENULA POLYMORPHA

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ormal oligomerisation and aggregation of the protein called alpha-synuclein (α-syn) are the key events in the pathogenesis of Parkinson’s disease (PD). Recent discoveries revealed cellular pathways that potentially relate neurodegenerative disease (ND) to abnormal functioning of mitochondria or anomalous glucose metabolism. In this study we describe for the first time strains of the thermotolerant methylotrophic yeast Hansenula polymorpha that produce human GFP-tagged α-syn as a new model of molecular processes leading to PD. We observed thatNCYC495-SNCA wild-type strain did not form visible α-syn amyloid-like aggregates but exhibited plasma membrane perforations and cytoplasm leakage. gcr1-2-SNCA mutant strain deficient in catabolite repression and glucose transport exhibited enhanced aggregation of fluorescently tagged α-syn. However, the observed differences did not result from the impaired glucose metabolism as were observed in both α-syn-producing strains grown on glycerol. Production of α-syn was detrimental for both strains and decreased their growth rate on alternative carbon sources. Our data suggests that H. polymorpha may serve as an informative new yeast model for deciphering molecular mechanisms of PD that regulate amyloid formation and degradation under the influence of various extra- and intracellular factors.

**Keywords:** methylotrophic yeast, α-synuclein, amyloids, neurodegeneration.

**INTRODUCTION**

Human neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, dementia with Lewy bodies, and Huntington’s disease belong to the superfamily of pathologies known as protein misfolding disorders [27]. It is now known that these disorders have some common cellular and molecular mechanisms including protein misfolding and aggregation and formation of inclusion bodies. These aggregates usually consist of fibers containing misfolded proteins with β-sheet conformation, termed amyloid [21]. The composition of the aggregates, as well as their localization in organs and tissues, is specific for each disease. For example, in PD there is abnormal accumulation of synuclein in neuronal cell bodies, axons, and synapses [11]. This disease represents
a group called synucleinopathies. Pathological brain inclusions composed of filamentous, mainly insoluble $\alpha$-syn protein that affect substantia nigra are called Lewy bodies (LB).

Human $\alpha$-syn is a small heat-stable cytoplasmic protein of 140 amino acids found primarily at presynaptic terminals of the central nervous system (CNS) [17, 29]. Although the exact role of $\alpha$-syn in normal cell functioning remains to be fully defined, $\alpha$-syn is assumed to be involved in the regulation of synaptic-vesicle release and trafficking, fatty acid-binding and stabilising complexes of SNARE family proteins, therefore affecting membrane stability and neuronal plasticity and survival [29, 31]. Ample evidence suggests that overproduction of $\alpha$-syn interrupts normal cell functions resulting in decreased neurite outgrowth and cell adhesion [7].

Abnormal interactions and misfolding of synaptic proteins in the nervous system are being extensively explored as important pathogenic events resulting in neurodegeneration in various neurological disorders. A natively unfolded protein, $\alpha$-syn, can adopt different conformational states and different aggregated morphologies, including small aggregates, oligomers, spherical and linear protofibrils, and fibrils [31]. At present it is widely accepted that abnormal oligomerisation and aggregation of $\alpha$-syn are the key events in PD pathogenesis [5, 6]. There is ongoing intense debate regarding the nature of the aggregates and protein forms that are toxic in neurodegenerative disorders. Some studies suggest that earlier aggregation intermediates, such as oligomers, are more toxic than larger inclusions and that the smaller nonfibrillar species of $\alpha$-syn are the ones that may primarily exert the neurotoxic effect [6]. The formation of such oligomers in vitro led to the increase in leakiness of synthetic lipid vesicles [30]. Direct in vivo data supporting the “toxic oligomeric $\alpha$-syn hypothesis” are still relatively limited and most of the evidence is circumstantial. The conclusion about the role of large insoluble $\alpha$-syn aggregates, the hallmark of PD, inside or outside cells has also been switching between the toxic and cytoprotective properties during the last decade. However, it has been recently demonstrated that $\alpha$-syn fibrillar assemblies exhibit a level of cytotoxicity at least 1,000-fold higher than that of their precursor oligomers at identical particles concentrations, the toxicity being associated with their ability to bind and permeabilize the cell membranes [20].

Despite the fact that a primary cause for PD is yet to be identified, a number of risk factors are known. Although there is the occasional case of the disease being developed in a young adult, it generally manifests itself in the middle to late years of life and the risk continues to increase with age. Various trigger factors, either genetic such as point mutations in $\alpha$-syn SNCA gene or environmental, such as low pH, high temperature, metal cations, pesticides, heparin and other glycosaminoglycans have also been shown to play a role in conversion of $\alpha$-syn into its $\beta$-sheet conformation [7]. Several recent discoveries have highlighted common cellular pathways that potentially relate neurodegenerative processes with abnormal functions of mitochondria and anomalous glucose metabolism [23, 26, 32]. It has been reported that diabetes mellitus type II is associated with increased PD risk [26, 32]. PD has also been associated with reduced insulin-mediated glucose uptake, inhibition of early insulin secretion and long-term hyperinsulinaemia and hyperglycaemia after glucose loading [1].

Intense research has also been conducted to generate artificial models that closely resemble PD, including animal and yeast models [9]. Importantly, yeast and human cells share basic fundamental aspects of eukaryotic cell biology. This allows a number of key processes, which are of particular interest to PD pathology, to be efficiently investigated in the well-understood yeast model. These include the mechanisms of protein folding,
quality control and degradation, the components involved in the secretory pathway and vesicular trafficking, the study of mitochondrial dysfunction and oxidative stress, the mechanisms of cell death and survival [9].

Bakers’ yeast *Saccharomyces cerevisiae* has traditionally been the most popular model yeast for PD studies [18, 27]. However, it is now recognized that its physiology and genetic apparatus, for instance involved in glucose consumption, are highly specialized to support fermentative growth [24]. Here we attempted to evaluate methylotrophic yeast *Hansenula polymorpha* as an alternative yeast model for investigation of molecular mechanisms of PD. The full genome for this yeast is publicly available, regulated heterologous gene expression systems are well developed and mutants deficient in glucose transport were described for *H. polymorpha* [10, 13]. Crucially, it is the thermostolerant yeast, contrary to *S. cerevisiae*, capable of growth at 37 °C which is a physiological temperature for human organism.

In this study we addressed the question whether α-syn expression in *H. polymorpha* host impaired in glucose transport and metabolism would be more toxic relative to the wild type strain. For this, we utilized previously described by us *H. polymorpha* strain harboring mutation in the *GCR1* gene, encoding for putative glucose sensor/transporter protein [25].

**MATERIALS AND METHODS**

**Strains.** Used in this study are listed in Table 1.

*Table 1. Strains of yeast and bacteria used in this study*

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<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tr>
<td><em>Hansenula polymorpha</em></td>
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<tr>
<td>NCYC 495 <em>ura</em> 3</td>
<td><em>ura</em> 3</td>
<td></td>
</tr>
<tr>
<td>gcr 1-2 <em>ura</em> 3</td>
<td>gcr 1-2 <em>ura</em> 3</td>
<td>[25]</td>
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<tr>
<td>NCYC 495 <em>SNCA</em></td>
<td>*leu1-1 ScLUE2 <em>P</em>&lt;sub&gt;<em>MET25</em>&lt;/sub&gt;EGFP3-*SNCA-T&lt;sub&gt;<em>CYC1</em>&lt;/sub&gt;</td>
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<td>gcr 1-2 <em>SNCA</em></td>
<td>gcr 1-2 *leu1-1 ScLUE2 <em>P</em>&lt;sub&gt;<em>MET25</em>&lt;/sub&gt;EGFP3-*SNCA-T&lt;sub&gt;<em>CYC1</em>&lt;/sub&gt;</td>
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<tr>
<td><em>Escherichia coli</em></td>
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<tr>
<td>DH5α</td>
<td><em>lacZΔM15, recA1, endA1, gyrA96, thi-1, hsdR17(r&lt;sup&gt;K&lt;/sup&gt;−, m&lt;sup&gt;K&lt;/sup&gt;−), supE44, relA1, deoR, Δ(lacZYA-argF)&lt;/Delta&gt;U169</em></td>
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**Media and growth conditions.** Yeast strains were cultivated at 37 °C in rich medium YPS (1 % yeast extract, 2 % bactopeptone, 1 % sucrose) and synthetic minimal medium (SMD) which contained 0.17 % yeast nitrogen base without amino acids and ammonium sulfate (YNB), 1 % glucose or 1 % glycerol, 0.5 % ammonium sulfate, and 40–50 mg/L of auxotrophic amino acids if needed. Solid media contained agar in 2% concentration.

Biomass (in optical density units, OD<sub>600</sub>) was estimated via optical absorption (at the wavelength of 600 nm in 1 cm cuvette) of diluted suspensions using spectrophotometer “Helios-γ”.

**DNA procedures.** Standard DNA techniques were carried out essentially according to previously described [22]. Transformation of *H. polymorpha* was performed by electroporation [8]. Plasmids used in this study are listed in Table 2. Primers used in this study are listed in Table 3.
Table 2. Plasmids and constructed in used this study

<table>
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<th>Plasmid</th>
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<tr>
<td>pKF48</td>
<td>AmpR CEN ScURA3 P&lt;sub&gt;MET25&lt;/sub&gt; -GFP- 6xHis-T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
<td>[4]</td>
</tr>
<tr>
<td>pKF48-SNCA 1</td>
<td>AmpR CEN ScURA3 P&lt;sub&gt;MET25&lt;/sub&gt; -GFP- SNCA(U251MG)-6xHis-T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
<td>This work</td>
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<tr>
<td>pKF48-SNCA 2</td>
<td>AmpR CEN ScURA3 P&lt;sub&gt;MET25&lt;/sub&gt; -GFP- SNCA (HEK293)-6xHis-T&lt;sub&gt;CYC1&lt;/sub&gt;</td>
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Table 3. PCR primers utilized for amplification of SNCA ORF

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<tr>
<td>OG21s</td>
<td>5'-GCGATCGATATGGATGTATTCATGAAAGGAC-3'</td>
</tr>
<tr>
<td>OG22</td>
<td>5'-GTACTCGAGTTAGGTCTCCAGGTTCTAGTCT-3'</td>
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Fluorescent microscopy. For fluorescent microscopy yeast cells were synchronized in YPS medium. Then cells were washed three times with sterile distilled water and transferred into SMD with different carbon sources with or without methionine (100 mg/L) for regulation of MET25 promoter with OD<sub>600</sub> = 0.1 to induce GFP-SNCA expression. At 4, 24, 32 and 48 h cells were collected by centrifugation and placed on ice until observation. Images were captured on fluorescence microscope (Axio Imager A1; Carl Zeiss MicroImaging, Jena, Germany) coupled to a monochrome digital camera (Axio Cam MRM; Carl Zeiss MicroImaging) and processed using the AxioVision 4.5 (Carl Zeiss MicroImaging) and Adobe Photoshop CS5 software (Adobe Systems, Mountain View, CA).

RESULTS AND DISCUSSION

Construction of vectors for regulated expression of human SNCA ORF in yeast cells. According to the published data [2, 15] α-syn overproduction is observed in human embryonal kidney, glioblastoma, neuroblastoma and some other tissues. Therefore, we used cDNA from human glioblastoma (U251MG) and from human embryonal kidney (HEK293) to amplify SNCA gene (encoding α-syn) using standard PCR method with primers for SNCA ORF. Primers OG21s/OG22 (see Table 3) were flanked with restriction sites for Clal and Xhol, respectively. The obtained fragments had different sizes: SNCA cDNA from U251MG was approximately 0.3 kb length and SNCA cDNA from HEK293 was approximately 0.4 kb (Fig. 1). This difference could be explained by alternative splicing of SNCA transcript, which results in formation of two differently sized mRNAs of 339 and 420 bps in length. It is known that at least three isoforms of α-syn are produced via alternative splicing [3]. The major form of the protein, and the one most investigated, is the 140 aminoacids-long transcript. Other isoforms are α-syn-126, where exon 3 is lost and lacks residues 41–54; and α-syn-112 [28] which lacks residue 103–130 due to the loss of exon 5 [3].

The PCR products harboring the SNCA transcripts and the initial plasmid pKF48 [4] were digested with restriction endonucleases Xhol and Clal for subcloning of the human SNCA under control of methionine-regulated yeast P<sub>MET25</sub> promoter. As a result, the SNCA fragments were fused in-frame to the ORF encoding green fluorescent protein (yEGFP3),
producing N-terminally GFP-tagged α-syn as a final translation product (Fig. 2). $P_{MET25}$ promoter is repressed by supplementation of exogenous methionine to the growth medium and, correspondingly, the maximum expression level can be achieved in medium lacking methionine.

**Fig. 1.** PCR products obtained after amplification with a pair of primers OG21s/OG22 from templates: 1 – U251MG cDNA (human glioblastoma); 2 – HEK293 cDNA (human embryonal kidney)

The obtained plasmids were verified for the presence of SNCA transcripts placed under $MET25$ (0.34 kb or 0.42 kb length) by PCR using primers OG21s and OG22 (Table 3). Based on the PCR analysis, we obtained pKF48-SNCA1 vector containing short SNCA form from glioblastoma and pKF48-SNCA2 with long SNCA form from human embryonal kidney. The scheme of pKF48-SNCA2 (hereinafter referred to as pKF48-SNCA) that harbors full length SNCA from HEK293, which was selected for further analysis, is shown in Fig. 2.

**Isolation of H. polymorpha starins producing human α-syn.** The constructed vector pKF48-SNCA was linearized with StuI and transformed into $H. polymorpha$NCYC495 $ura3$ and $gcr1-2 ura3$ uracil auxotrophic strains. The transformants were selected on plates with synthetic minimal medium based on the restored prototrophy due to the presence of yeast $URA3$ selectable marker on the initial vector $pKF48$.

We performed fluorescent microscopy analysis of $yEGFP3-SNCA$ expression in the isolated prototrophic strains incubated for 4 hours in minimal medium lacking methionine conferring maximal induction of $MET25$ promoter versus methionine-supplemented medium. We observed green fluorescence in transformant strains cultured in the methionine-devoid medium, contrary to diminished fluorescence in methionine-supplemented medium or no fluorescence in parental strain that served as a negative control (data not shown).

**α-syn production affects growth of the yeast model strains on different carbon sources.** We analyzed whether α-syn gene expression affects growth of the obtained $H. polymorpha$ recombinant strains on alternative carbon sources. We observed that, indeed, grown on glucose or glycerol as carbon sources, the wild-type NCYC495-SNCA and glucose transport-impaired $gcr1-2$-SNCA strain both exhibited growth impairment relative to initial strains NCYC495 and $gcr1-2$ and strains expressing GFP gene only under $P_{MET25}$ promotor (Fig. 3).
Fig. 2. Plasmid pKF48-SNCA (AmpR CEN URA3 PMET25-GFP-6xHis-TCYC1). Physical map of vector: arrows indicate locations and orientations of the ORFs. The vector contains the URA3 selectable marker, and gene of ampiciline resistance (AmpR) for selection of the E. coli transformants. It also contains 6xHis-tag encoding region, allowing to detect fused protein by Western blot analysis using the corresponding primary antibodies. pKF48 bears a yEGFP3 (yeast-enhanced Green Fluorescent Protein) coding sequence under the control of the MET25 promoter which is positively regulated in medium lacking methionine. Human SNCA gene has been cloned into pKF48-SNCA using restriction enzymes XhoI, ClaI (marked on the map) so that the GFP is fused to N-terminal end of α-syn protein.

Fig. 3. Growth of H. polymorpha strains producing human α-syn (NCYC 495 SNCA and gcr 1-2 SNCA) cultivated in media with different carbon sources: A – NCYC495 SNCA on 1 % glucose and 1 % glycerol; B – gcr1-2 SNCA on 1 % glucose and 1 % glycerol.

Interestingly, whereas both α-syn-expressing strains were similarly significantly affected in the growth on glucose, impairment of glycerol utilization was more pronounced for the wild-type NCYC495-SNCA relative to the mutant strain gcr1-2-SNCA.
**gcr1-2 mutation enhances human α-syn aggregation in* H. polymorpha **cells.

Glucose (a glycolytic substrate) and glycerol (a gluconeogenic substrate) were used as alternative carbon sources to compare the possible effect of defective glucose transport/metabolism proficiency on α-syn aggregation. Fluorescent microscopy of the wild-type strain NCYC495-SNCA grown either in glucose- or glycerol-containing liquid medium revealed that there was only minor aggregate formation in cytoplasm of the cells (Fig. 4). We assumed therefore that α-syn is accumulated in cytoplasm primarily in oligomeric forms. Fluorescent microscopy of gcr1-2-SNCA cells with defect in glucose utilization indicated significantly enhanced aggregation of fluorescently tagged α-syn (Fig. 4). However, such amyloid-like aggregates were evidently formed in gcr1-2-SNCA cells in both glucose- and glycerol-containing media. Therefore, we concluded that polymerization of α-syn in gcr1-2-SNCA is not a result of disturbances in glucose-dependent metabolism but rather of other pleiotropic effects of gcr1-2 mutation [25], which we will address in further studies. It has to be also further elucidated, how the rate α-syn aggregation affects cells growth rate depending on the producer genetic background.

**Fig. 4.** Fluorescent microphotography of* H. polymorpha **strains NCYC 495 SNCA and gcr 1-2 SNCA producing human α-syn (GFP-tagged) grown on media containing glucose (1 %) and glycerol (1 %) as carbon sources. The bar represents 5 µm length

**Рис. 4.** Флюоресцентна мікроскопія штамів* H. polymorpha **NCYC 495 SNCA та gcr 1-2 SNCA, які продукують α-син (GFP-мічений), вирощених на середовищі з глюкозою (1 %) та гліцеролом (1 %) як джерелами Карбону. Відрізок на рисунку відповідає 5 мкм

**Oligomers or small aggregates of α-syn cause plasma membrane perforations and cytosol leakage in yeast cells.** Remarkably, we also observed that glucose-grown NCYC495-SNCA cells apparently exhibited perforation of plasma membrane with further cytosol leakage after 24 h of cultivation (Fig. 5). Extracellular fluorescent α-syn aggregates evidenced cytosol leakage out of yeast cells. Similar changes in the glycerol-grown cells of this strain were observed after 48 h of cultivation (data not shown). However, no such effect was observed for the mutant strain gcr1-2-SNCA.

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<tr>
<th>NCYC 495 SNCA</th>
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<tr>
<td><strong>1 % Glucose</strong></td>
<td><strong>1 % Glycerol</strong></td>
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<td>4 h</td>
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<td>24 h</td>
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<td>32 h</td>
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<td>48 h</td>
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Two mechanisms have been proposed to explain such effect of α-syn oligomers on membrane permeability [31]: 1) α-syn annular oligomers may integrate into the membrane resulting in the formation of pores or channel-like structures that could cause uncontrolled membrane permeability [14, 33], and 2) oligomers enhance the ability of ions to move through the membrane bilayer, without the formation of pores [12]. According to the Barrel model (Fig. 6) α-syn oligomers (β-sheet-rich) form circle structures with central pore. These aggregates can bind with the membrane causing its permeabilization in a concentration-dependent manner [19]. Thus we hypothesize that intercalation of α-syn oligomers into cell membrane causes cell lysis in yeast strains that produce this heterologous protein.

Formation of large α-syn aggregates (fibrils), as observed in the gcr1-2-SNCA mutant, apparently have cytoprotective effect and may prevent cell membrane perforation and cytosol leakage.

**CONCLUSIONS**

Modeling processes associated with human neurodegenerative diseases in yeasts has been already proven to be very useful to deciphering the complex mechanisms and pathways underlying these pathologies [9]. In this report we describe for the first time that methylotrophic yeast *H. polymorpha* can be an informative model for investigation of molecular mechanisms of PD. This yeast has several advantages, such as full genome sequencing, well developed platform for regulated heterologous protein production and its extreme thermostolerance [13]. The latter fact allows to study PD-related mechanisms at physiological for both human organism and this yeast temperature, 37 °C. *H. polymorpha* has also been extensively used as a model for studying autophagic protein degradation [16] known to be involved in protein aggregates clearance, like in the case of α-syn amyloids.

In this study we have constructed *H. polymorpha* strains with normal and partially defective glucose transport/metabolism, *gcr1-2* [25], wich heterologously produce human α-syn in a regulated manner. We demonstrated that α-syn expression dramatically affects physiology of the yeast cells, as is indicated by the decreased growth rate on
Fig. 6. Primary and secondary structure of α-syn [19]. Top scheme shows the three main domains of α-syn. The N-terminal (red) is an amphipathic domain that contains the three point mutations (white bars) linked to the autosomal dominant form of PD. The central region (green-pale brown) is a highly hydrophobic domain that was originally identified in patients with AD or LBD, which is the precursor of the non-amyloidogenic component of the extracellular senile plaque (NAC), which promotes the protein aggregation. The C-terminal domain (blue) has an acidic character, which possesses anti-amyloidogenic properties. According to the operational model of membrane perforation, once α-syn interacts with the plasma membrane, it should acquire a new secondary structure to form pores/perforations. The formation of α-syn pores may be explained by two different models: (A) A toroidal model involving a sequential binding of α-syn monomers to the plasma membrane that results in the formation of pores or channels with α-helical conformation; and (B) A barrel model showing that α-synuclein oligomers (β-sheet-rich) form ring structures with a central pore. The formation of α-syn oligomers enriched in β-sheet structures could occur in the extracellular space (1) or at the plasma membrane (2).
alternative carbon sources (Fig. 3). Remarkably, mutant strain with gcr1-2 mutation exhibited more efficient amyloid formation than corresponding wild-type strain, and no membrane perforations which are most probably caused by α-syn oligomers (Fig. 4). However, these effects are not a direct function of the impaired glucose metabolism as were also observed in cells grown on a gluconeogenic substrate. The question of what molecular mechanisms evoked by gcr1 mutation stimulate α-syn aggregation will be addressed in our prospective studies.

Kлючові слова: метилотрофні дріжджі, α-синуклеїн, амілоїди, нейродегенерація.

МОДЕЛИРОВАНИЕ МОЛЕКУЛЯРНЫХ ПРОЦЕССОВ, ЛЕЖАЩИХ В ОСНОВЕ БОЛЕЗНИ ПАРКИНСОНА, В КЛЕТКАХ МЕТИЛОТРОФНЫХ ДРОЖЖЕЙ HANSENULA POLYMORPHA

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Аномальная олигомеризация и агрегация белка, который называется α-синуклеин (α-syn), являются ключевыми событиями в патогенезе болезни Паркинсона (БП). Последние исследования обнаружили связь нейродегенеративных заболеваний (НЗ) с аномальным функционированием митохондрий и дефектным метаболизмом глюкозы. В этой статье мы впервые описываем штаммы термотолерантных метилотрофных дрожжей Hansenula polymorpha, продуцирующие GFP-меченый человеческий α-syn, которые могут служить моделью БП. Нами было обнаружено, что штамм дикого типа NCYC495-SNCA не формировали видимых амилодов α-syn, однако характеризовался наличием перфораций в цитоплазматической мембране и вытеканием цитозоля. Мутантному штамму gcr1-2-SNCA, имеющему поврежденную катаболитную репрессию и глюкозный транспорт, была присуща усиленная агрегация флюоресцентно меченого α-syn . Однако такое различие в способности образовывать агрегаты не является результатом повреждения метаболизма глюкозы, поскольку также наблюдалось и в случае выращивания клеток в среде с глицеролом. Продукция α-syn была вредной для обоих штаммов и подавляла их рост на альтернативных карбоновых субстратах. Полученные нами данные свидетельствуют о том, что H. polymorpha может служить новой информативной дрожжевой моделью процессов, лежащих в основе БП, для выяснения молекулярных механизмов, которые регулируют формирование и деградацию амилоидов под влиянием различных вне- и внутриклеточных факторов.

Ключевые слова: метилотрофные дрожжи, α-синуклеїн, амілоїди, нейродегенерація.

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