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OVEREXPRESSION OF HUMAN *SNCA* GENE IN *DROSOPHILA* MOTOR NEURONS CAUSES MORPHOLOGICAL AND FUNCTIONAL ABNORMALITIES IN LARVAL NEUROMUSCULAR JUNCTION

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To gain insight into the synaptic function of *SNCA* (α-synuclein) gene in Parkinson disease patients, wild type α-synuclein, its mutant forms α-synuclein A30P and α-synuclein A53T were expressed in motor neurons of *Drosophila melanogaster* larvae. It was found that targeted expression of α-synuclein in *Drosophila* larval motor neurons did not cause significant morphological changes in neuromuscular junctions (NMJs) but a dramatically decreased the NMJ area and changed the distribution of synaptotagmin 1 protein in synaptic terminals was detected. Analysis of the number and distribution of mitochondria showed that motor neurons overexpressing α-synuclein had a decrease of mitochondria in the axon and presynaptic terminals.

Keywords: Parkinson disease, SNCA, neuromuscular junctions, *Drosophila melanogaster*.

A large number of neurodegenerative diseases are characterized by inclusions of aberrant proteins and neurodegeneration. However early clinical symptoms accompany only with synaptic loss or dysfunction. These results allow us to suggest the existence of common molecular and cellular mechanisms for progress of neurodegeneration, which are directly connected to synaptic dysfunction and loss. Parkinson disease (PD) is a neurodegenerative disorder characterized by the dysfunction and loss of dopaminergic neurons (DA) in the substantia nigra and accompanied by the presence of cellular inclusions known as Lewy bodies. An important component of the Lewy bodies is the protein α-synuclein. α-synuclein is normally presented in the human brain, but is physically altered in PD. A duplication, triplication of the α-synuclein locus and autosomal-dominant mutations in the gene have been detected in cases of familial forms of PD [10, 12], supporting a role for overexpression of the wild-type protein in pathogenesis. Polymorphisms in regulatory elements of the α-synuclein gene predispose to PD [8]. Taken together, these observations suggest a causative role for α-synuclein in sporadic as well as inherited PD.

One of the most interesting approaches to research genetic forms of human diseases is their modeling on the fruit fly *D. melanogaster* [1, 7]. Targeted expression of human α-synuclein has been effectively used to recreate the pathology of PD in *D. melanogaster* [6].

In this work we analyzed the morphological and functional states of neuromuscular junctions (NMJs) of *Drosophila* larvae expressing α-synuclein wild type, its mutant forms α-synuclein A30P and α-synuclein A53T. It has been shown that α-synuclein expression induces morphological changes in neuromuscular junctions (NMJs), changing the distribution of synaptotagmin 1 protein in synaptic terminals, as well as reducing the number of mitochondria in axon presynaptic terminals.

Materials and methods

Fly Stocks. Transgenic strains: (1) P{UAS- wild-type α-synuclein}- carries human wild type α-synuclein insertion (hereinafter, syn wt), P{UAS- A30P α-synuclein} carries A30P human

mutant α-synuclein form (hereinafter, syn A30P}, P{UAS- A53T α-synuclein} – carries A53T human mutant α-synuclein form (hereinafter, syn A53T) [6]; transgene expression was carried out in the UAS-GAL4 system [2] and was induced in motor neurons of third stage larvae by tissue specific transcription activator GAL4-D42.

(2) UAS-CD8-GFP; GAL4-D42 strain (below in the text, CD8;D42) expressing fluorescent protein GFP in nerve cell membranes was used for visualization of *D. melanogaster* neuromuscular junctions and examination of their morphology.

(3) UAS-syt-GFP strain with inserted synaptotagmin 1 gene and GFP was applied for the analysis of synaptotagmin distribution in bouton) [17].

(4) UAS-mito-GFP/GAL4-D42 strain (abbreviated as mito/D42) with GFP expressed in mitochondria membrane was used for quantitation of mitochondria in axons and neuromuscular junctions. *D. melanogaster* strains were obtained from Bloomington Stock Center (United States). Flies were maintained on standard yeast medium at 25°C and a 12h light day.

Sample preparation and assay of neuromuscular junction morphology. Third stage larvae were dissected in freshly prepared HL3 solution (110 mM NaCl, 5 mM KCl, 10 mM NaHCO3, 5 mM HEPES, 30 mM sucrose, 5 mM tregalose, 10 mM MgCl2, pH 7.2) [3]. Then, larvae body wall samples were fixed with 4% formaldehyde (Sigma Aldrich, United States) for 15 min, washed with phosphate buffer (PBS), and mounted in glycerol mixed with PBS (1 : 1). Preparations were visualized under a Leica TCS SP5 laser confocal scanning microscope (Leica, Germany). The bouton number was estimated with ImageJ software. Each experiment was performed in triplicate.

Immunohistochemistry. Third-instar larvae were dissected in PBS and fixed in 4% paraformaldehyde for 20 min. Larvae were washed with PBS and blocked in blocking buffer (Visual protein, USA), washed two times with in PBS for 1 hr, followed by overnight incubation in primary antibodies in blocking buffer, three times washing in PBS, incubation with secondary antibodies in blocking buffer for 1 hr, three final washes in PBS, and equilibration in VectaShield (Vector Laboratories). The following primary antibodies were used: mouse anti-Brp (Bruchpilot) [1:200; mAb NC82; Developmental Studies Hybridoma Bank (DSHB)], Goat Cy3-conjugated secondary antibodies against mouse were obtained from Jackson Immuno Research. Antibodies obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Mitochondria assay. Larvae dissected in HL3 and fixed with 4% paraformaldehyde for 15 min were mounted in Vecta Shield (Vector Laboratories). Samples were analyzed under laser confocal microscope Leica TCS-SP5 (Leica, Germany) at 488 nm. Relative fluorescence was estimated with ImageJ software on serial confocal sections. It was analyzed 6-8 larvae of each genotype. Each experiment was performed in triplicate.

Statistical treatment. The significance of control and experiment differences was estimated with Kyplot software (one way ANOVA and Tukey–Kramer test).The difference with $P < 0.05$ was considered to be significant.

Results and discussion

We used *D. melanogaster* larval neuromuscular junctions to investigate α-synuclein synaptic functions. NMJs represent a suitable model to observe synaptic development and functions [5, 14]. In contrast with central synapses of the vertebrates every single presynaptic motor neuron and postsynaptic muscular cell are unique and easily distinguishable in this system. Moreover, this model allows to combine genetic, functional and structural research. Furthermore, NMJs is one of the most widespread models for researching neurodegenerative processes [4, 11, 16].

α-synuclein impact on NMJ junction morphology and structure was examined in transgenic strain CD8;D42 with motor neuron membrane labeled with GFP. NMJs in the fourth muscle of the third abdominal segment (see terminology in [3]) were assessed in the progeny of this strain crossed with strains expressing α-synuclein. Each muscle in *D. melanogaster* larvae is innervated by motor neurons with two types of synaptic terminals designated as 1b (large, 3–5 μm in diameter) and 1s (small, 1–1.5 μm in diameter), which correspond to certain types of motor neurons in larva brain [3].

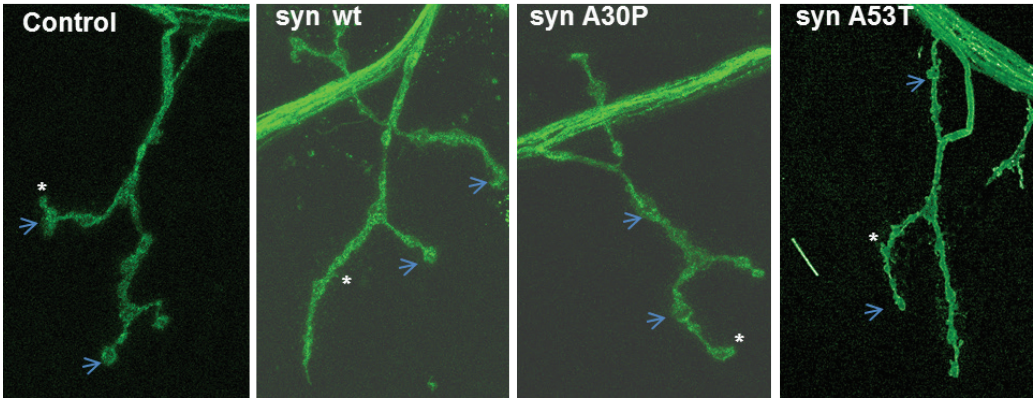

Fig. 1. Morphological assay of neuromuscular junctions in *D. melanogaster* larvae with α-synuclein expression. Neuromuscular junctions of the fourth muscle of the third abdominal segment. Strain genotypes are indicated at the top of images. *Arrows* indicate 1b boutons. *Asterisk* designates satellite boutons. Confocal microscopy. Scale bars: 25 μm.

Table 1

Morphological characteristics of larval NMJs with α-synuclein expression

Strain	Total number of boutons	Number of big boutons	Number of satellite boutons	Relative area of NMJs (absolute area of NMJs divided by area corresponding muscle)
Control	19,6±1,1	12,6±1,0	7,0±1,0	0,32±0,03*
syn WT	13,3±1,1	10,8±0,8	2,5±0,6*	0,09±0,01*
syn A30P	15,5±1,4	12,5±0,6	3,0±1,0*	0,11±0,01*
syn A53T	17,9±3,0	14,5±2,5	2,5±0,5*	0,13±0,01.*

Comment. Asterisk denotes a statistically significant difference ($P<0.05$).

It was demonstrated that all NMJs in the fourth muscle of the third abdominal segment in control strain were also found in strains with α-synuclein expression; that, is no neurodegeneration was observed. The number of synaptic boutons in NMJ of α-synuclein strains was decreased, but the decrease was not statistically significant comparing to control (Fig. 1, Table 1). However, all strains with α-synuclein expression had an increased number of satellite boutons (Fig. 1, Table 1). Satellite boutons are boutons that bud both from formed boutons and the interbouton axon ligament connecting two neighboring boutons [16].

α-synuclein expression in motor neurons also decreased area of NMJs (Fig. 1, Table 1). The greatest decrease area of NMJs was observed in strain with expression of wild type α-synuclein.

Next, we examined the distribution of synaptotagmin 1 [17], a presynaptic marker, in NMJs. Synaptotagmin 1 is integral membrane-associated glycoprotein of synaptic vesicles and is believed to be main calcium sensor, starting neurotransmitters exocytose [9, 13]. Fig. 2 shows that synaptotagmin 1 in the control strain is localized inside synaptic boutons. It should be noted

that these boutons are separated from each other and resemble a small beaded thread. This pattern of presynaptic protein distribution is common for neuromuscular junctions in *Drosophila*; proteins of synaptic vesicles are usually not found in areas connecting two adjacent boutons [3]. In control stock synaptotagmin 1 located in synaptic boutons closer to membrane, while in strains with α-synuclein overexpression synaptotagmin 1 located mostly within synaptic bouton (Fig. 2).

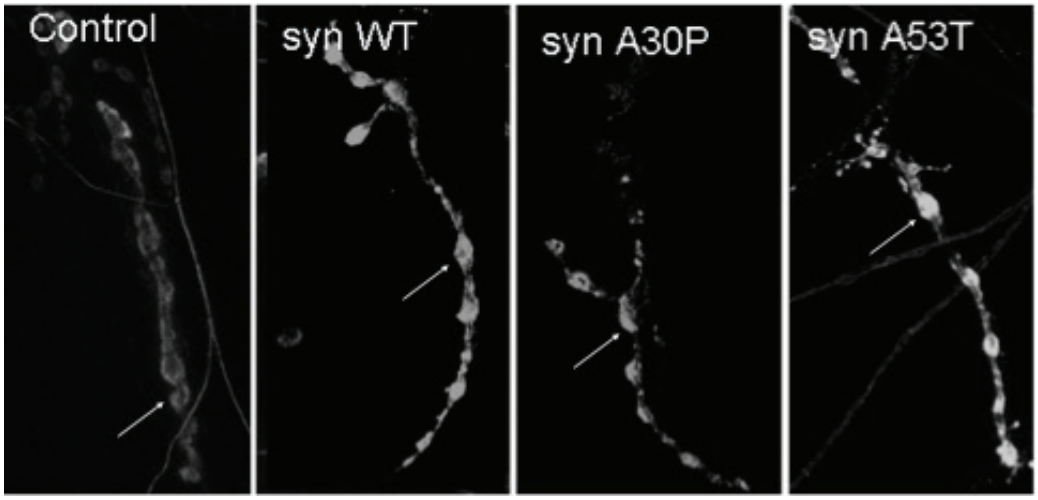

Fig. 2. Synaptotagmin 1 distribution in NMJs of *D. melanogaster* larvae with α-synuclein. *Arrows* indicate synaptotagmin 1 localization. Confocal microscopy. Scale bars: 25 μm.

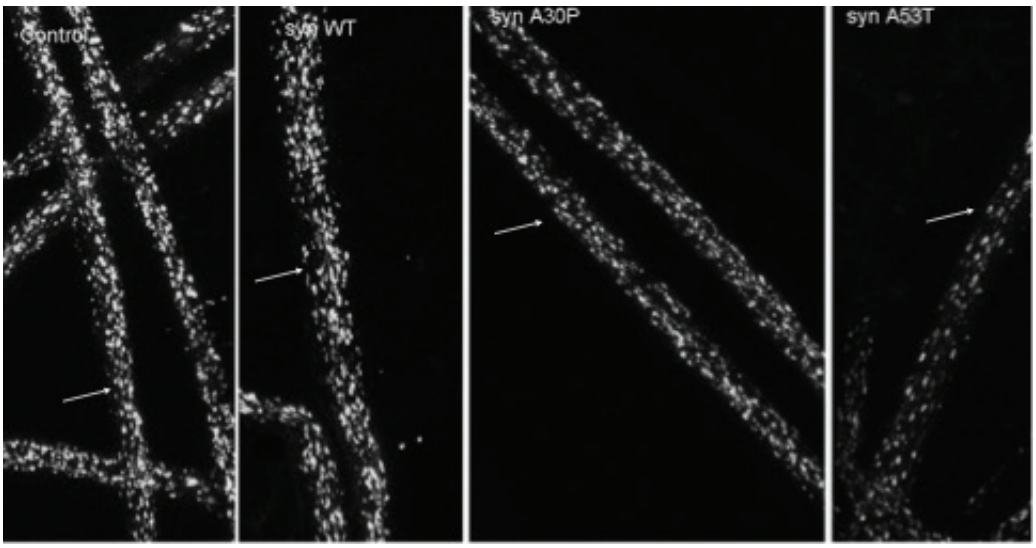

Fig. 3. Visualization of mitochondria clusters in (in green) axon of *D. melanogaster* larvae with α-synuclein expression. *Arrows* indicate mitochondrial clusters. Confocal microscopy. Scale bar is 25 μm.

As mitochondria functioning is critical for synaptic transmission, we examined localization of mitochondria in axon and NMJs. To identify mitochondria, we used transgenic strain mito/D42 having mitochondria with GFP labeled membranes. To analyze the transport of the mitochondrion in the axon we counted the number of mitochondrial cluster in five axons of every

larva through 200 μm along the axon. In control flies, large clusters of mitochondria were visible in each axon and NMJ (Fig. 3, 4), whereas, in strains with α-synuclein expression, the cluster size was diminished (Figs. 3, 4, Table 2). The number of clusters of mitochondria was measured with the Image J software. The transgenic strains with α-synuclein expression were characterized by decreasing of mitochondrial cluster in both axons and NMJs comparing with control (Table 2).

Table 2

Number of mitochondrial clusters in axon and NMJs

Strain	Number of mitochondrial clusters in axon	Number of mitochondrial clusters in axon in NMJs
Control	42,2±3,0	126±5
syn WT	32,3±2,6*	76±5*
syn A30P	31,8±1,9*	54±6*
syn A53T	41,5±4,4	93,5±0,5*

Comment. *Asterisk* denotes a statistically significant difference (P<0.05).

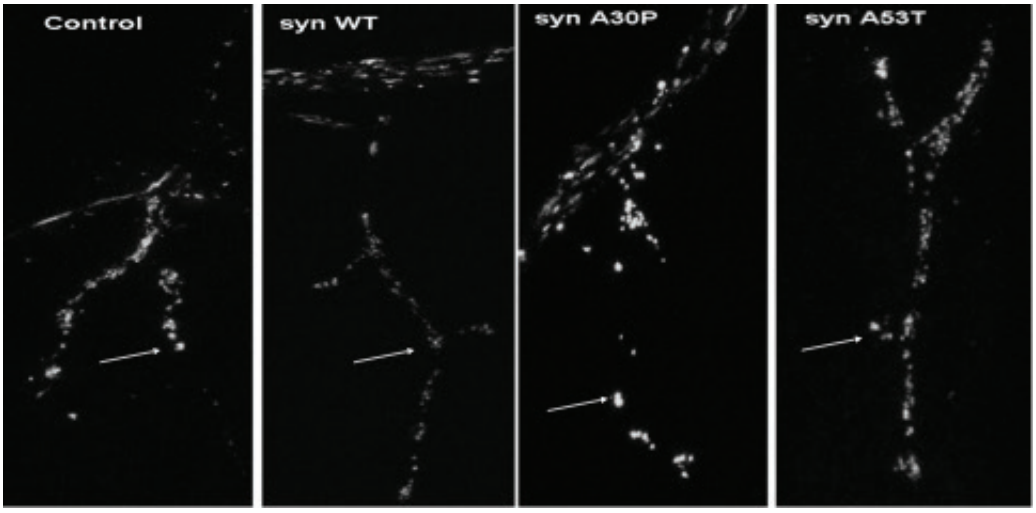

Fig. 4. Visualization of mitochondria clusters (in green) in NMJs of *D. melanogaster* larvae with α-synuclein expression. Neuromuscular junctions on the fourth muscle of the third abdominal segment. *Arrows* indicate mitochondria clusters. Confocal microscopy. Scale bar is 25 μm.

In conclusion, we demonstrated that the α-synuclein overexpression can modulates the formation and maintenance of synaptic contacts. Its altered expression or mutations may cause synaptic dysfunctions and synaptic pathology in PD patients.

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НАДЕКСПРЕСІЯ ГЕНА *SNCA* ЛЮДИНИ В МОТОРНИХ НЕЙРОНАХ *DROSOPHILA* ПРИВОДИТЬ ДО МОРФОЛОГІЧНИХ І ФУНКЦІОНАЛЬНИХ ПОРУШЕНЬ У НЕЙРОМ'ЯЗОВИХ З'ЄДНАННЯХ ЛИЧИНОК

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Для розуміння синаптичних функцій гена *SNCA* (α-синуклеїну) у хворих на хворобу Паркінсона в моторних нейронах личинок *Drosophila melanogaster* було експресовано дикий тип α-синуклеїну та його мутантні форми: α-синуклеїн A30P і

α-синуклеїн A53T. Було встановлено, що напрямлена експресія α-синуклеїну в моторних нейронах личинок *Drosophila melanogaster* не викликала значних морфологічних змін у нейронм'язових з'єднаннях, але різко знизила площу нейром'язових з'єднань і змінила розподіл білка синаптотагміну в синаптичних закінченнях аксонів. Аналіз кількості й розподілу мітохондрій показав, що в моторних нейронах при надекспресії α-синуклеїну знижено число мітохондрійних кластерів у аксонах і пресинаптичних закінченнях.

Ключові слова: хвороба Паркінсона, SNCA, нейром'язові з'єднання, *Drosophila melanogaster.*

ИПЕРЭКСПРЕССИЯ ГЕНА *SNCA* ЧЕЛОВЕКА В МОТОРНЫХ НЕЙРОНАХ *DROSOPHILA* ПРИВОДИТ К МОРФОЛОГИЧЕСКИМ И ФУНКЦИОНАЛЬНЫМ НАРУШЕНИЯМ В НЕЙРОМЫШЕЧНЫХ СОЕДИНЕНИЯХ ЛИЧИНОК

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Для понимания синаптической функции гена *SNCA* (α-синуклеина) у больных болезнью Паркинсона в моторных нейронах личинок *Drosophila melanogaster* были экспрессированы α-синуклеин дикого типа и его мутантные формы: α-синуклеин A30P и α-синуклеин A53T. Было установлено, что направленная экспрессия α-синуклеина в моторных нейронах личинок *Drosophila melanogaster* не вызвала значительных морфологических изменений в нейромышечных соединениях, но резко снизила площадь нейромышечного соединения и изменила распределение белка синаптотагмина в синаптических окончаниях аксона. Анализ количества и распределения митохондрий показал, что в моторных нейронах при гиперэкспрессии α-синуклеина снижено число митохондриальных кластеров в аксонах и пресинаптических окончаниях.

Ключевые слова: болезнь Паркинсона, SNCA, нейромышечные соединения, *Drosophila melanogaster.*