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Neuroprotective effects of a new nootropic agent Mitochondrin-2

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Since neurodegenerative diseases are incurable, it is important to study experimental neuroprotective preparation on model organisms. In this work, we studied the neuropeptide Mitochondrin-2 (M-2) effect on Drosophila neurodegenerative models of brain pathology induced by functional knockdown of swiss cheese (sws) and Superoxide dismutase 1 (Sod-1) genes in gliocytes. We found that, after using M-2, brain zone degeneration decreased and markers of oxidative stress (conjugates diene CD and thiobarbituric acid reactive substances TBARS) reached the control level. However, we did not observe any improvement in life span or locomotor activity. This suggests a certain neurotrophic and antioxidant effect of M-2, especially for glial cells, but its effect on the protection of neurons function remains unclear. Further research is needed for effect mechanism of peptide neuroprotectors.
Key words: Drosophila melanogaster; neurodegeneration; swiss cheese gene; Sod-1 gene; neuropeptides.

INTRODUCTION

There is a permanent tendency worldwide to increase the number of people suffering from neurodegenerative diseases, which are a common cause of cognitive impairment and premature death in older adults. There is no effective treatment and methods of symptoms of neurodegenerative diseases because the molecular characteristics and mechanisms of the occurrence of these diseases remain unknown. Given the complexity of conducting clinical and genetic studies in humans, there is a general understanding that for the purposes of studying the nature of neurodegenerative diseases and the search for therapeutic agents, it can be useful in many cases to use model organisms, such as *Drosophila (D.) melanogaster*. About 75% of human genes have their orthologs in the *Drosophila* genome [1]; pathological changes in the brain structure of the neurodegenerative *Drosophila* mutants are similar, by morphological, biochemical and functional characteristics, to those of people suffering neuropathy [2]. It is recognized that glial cells play a critical role in the development and function of the brain. Recently, it has been shown that glial cell dysfunction contributes to various neurological disorders, including neurodegenerations [3]. Clarifying the function of glial cells gives us a better understanding of function and dysfunction of the nervous system, and inspires the development of new therapies to treat these devastating disorders.

The search for neuroprotective drugs – compounds of different natures and origin which may delay neurodegeneration and also may be used in pathology progress prevention is one of the directions of pharmaceutical research. Among these are peptide neuroprotectors – special proteins or mixes of peptides and amino acids obtained from different animals' brain tissues. It is known that neuropeptides can move within the *central nervous system* and act outside synapses, including in the places distant from the release place [4, 5], leading to substantial increase in neuromodulation and the whole brain system functioning. A number

of similar experimental drugs were created, for example, Mitochondrin-2 (M-2) could be more effective because it was developed from brains of piggies that suffered hypoxia at the birth but had no negative consequences. First perspective data on this drug effect has already been obtained on *sws*-depending *D. melanogaster* neurodegeneration model, and it was found out that M-2 is able to retard and slow down degeneration in the brain [6]. In this work, we expanded research of M-2 drug on neurodegenerative models of *Drosophila* with functional inhibition (knockdown) of *sws* and *Sod-1* genes exclusively in glia, because of hypothetic nonspecific effect of peptide neuroprotectors that could be realized through glia.

METHODS

Flies and experimental design. To make the knockdown of genes we used UAS/GAL4 binary system. Fly stock *w*; P{UAS-sws-RNAi}3* (*UAS-sws^{RNAi}*) was obtained from Vienna Drosophila RNAi Center (Austria), *w*;*P{UAS-Sod.IR}4* (*UAS-Sod1^{RNAi}*), *Sod^{X-39} e^{1}/TM3* (*Sod^{X-39}*) were obtained from Bloomington Drosophila Stock Center (USA) and the driver-strain for glia-specific expression *Repo-Gal4/TM3* was kindly provided by Prof. Karl-Friedrich Fischbach (Freiburg University). As controls, we used heterozygote driver *Oregon/Repo-Gal4* and *Oregon/e^{1}*, respectively, which are the genetic background for the experimental flies. All flies were kept in vials in a thermostat under standard conditions (+24 to +25°C). Experimental preparation Mitohondrin-2 (M-2), kindly provided by developers [7], was added to the standard nutritional medium for larvae feeding [8]. The maximum daily dose for humans (26 μl) was recalculated per 100 ml of the fly medium. Adult flies were kept in this case in a preparation-free medium.

Calculating area of brain tissue degeneration. Morphological degenerative changes in *Drosophila* ganglia tissues were observed in histological sections that were made according to the standard method [9]. The analysis was done on "Loboval-3 Corl 2 Zeiss-Jena" microscope in ultraviolet light at ×600 magnification. The total area of vacuoles in the brain tissue was calculated by analysis of microphotographs made at a fixed magnification in the graphic editor Kompas 13 portable mini as described before [6].

Expression pattern of driver line repo-Gal4. We obtained *repo>UAS-CD8:GFP* flies by crossing, their brains were prepared by the «whole-mount» method, then immunohistochemical detection of GFP protein expression was performed according to the standard procedure [10]. Primary anti-GFP was used in concentration 1:200. The samples were analyzed on a scanning laser Nikon LSM A1 Clem Confocal Microscope.

Survival assays. 100 flies of each genotype and each examined group were collected, divided into vials of 10 flies, and moved to proper fresh medium every 2-3 days. The number of live flies and those lost to follow-up were counted every 2-3 days. Survival curves were analyzed using Kaplan-Meier plots and log-rank statistical analysis using Graph Pad Prism 7 (Graphpad Software Inc., La Jolla, CA, USA).

Behavior assays. To characterize the locomotor behavior of flies with "open field" method, observations were made on single flies in an open-field arena from glass Petri dish fitted with a clear glass lid. The arena bottom was divided into 0.5-cm squares. Above the arena, the digital video recorder was fixed. Each fly was put into the arena and had 3 min to adapt. The movement of the fly within the arena was recorded for 10 consecutive minutes after the fly adaptation. Analysis of video allowed to estimate the number of 0.5-cm squares involved whilst the fly was engaged in locomotion. Tested flies were 21-days old, at least 10 males of each genotype. The motor activity index (L) was calculated taking the total movement activity (total distance covered in cm) of control males as a unit of measurement. Statistical significance was calculated in Excel data analysis using t-

Test: Two-Sample Assuming Unequal Variances.

Oxidative stress markers. Homogenate (10% solution) was prepared in 0.1 M Tris-HCl buffer (pH 8.6). Homogenization was done at 0…+4°C, samples were centrifuged for 10 min at 4000 rpm. Protein concentration was measured using the standard protocol [11]. Content of TBA thiobarbituric acid reactive substances (TBARS) was measured using the standard protocol [12]; molar extinction was calculated using spectrophotometry at 532 nm wavelength against butanol. The content of conjugated dienes (CD) was measured using the standard protocol [12]; molar extinction was calculated using spectrophotometry at 233 nm wavelength against heptane.

RESULTS

Brain tissue neurodegeneration

Individuals with knockdowns of *sws* and *Sod-1* genes in glia, *repo>sws^{RNAi}* and *repo>Sod1^{RNAi}* respectively, show a clear neurodegenerative phenotype in the optical part of the brain (Fig. 1,B). Earlier we estimated the size of the neurodegenerative zones in the brain tissue of *repo>sws^{RNAi}* individuals before and after the use of the M-2 drug and found a significant reduction in the vacuolization area [6]. Thus, in the case of *repo>sws^{RNAi}*, the conventional area of degenerated tissue was reduced by 31.5%, but only in the case of larvae feeding [6]. In this study, we extended the model for testing and performed the same analysis of *repo>Sod1^{RNAi}* brain tissue. We found a decrease of vacuole size by 24.5% (from 141.5 ± 12.4 to 106.8 ± 14.2 mm^2, $P < 0.05$) after M-2 treatment (Fig. 1,C).

Survival

Flies with knockdown of both *sws* and *Sod-1* genes in glia had a significantly lower lifespan ($P < 0.0001$) compared to control individuals (Fig. 2). However, we did not observe any sig-

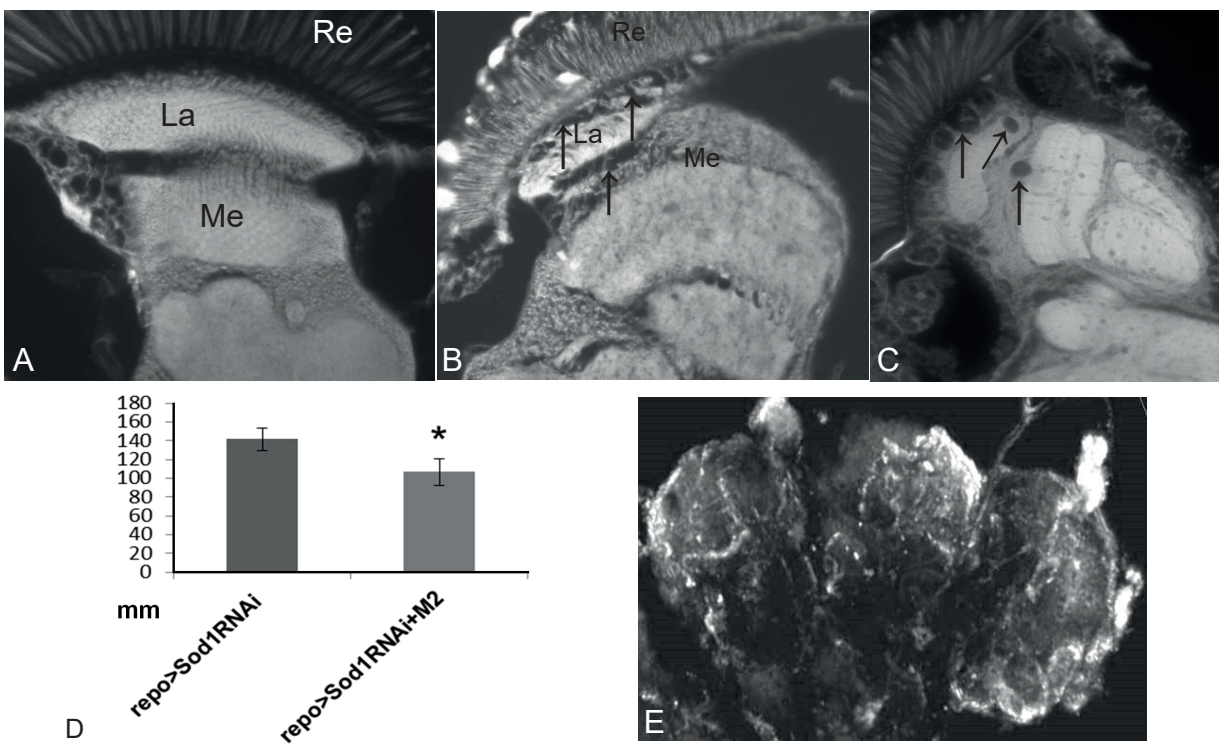

Fig. 1. Photomicrographs of brain tissues of 20-day old flies. A. Control. B. *Sod-1* knockdown in glia (*repo>Sod1^{RNAi}*). C. *sws* knockdown in glia (*repo>sws^{RNAi}*), Re - retina, La - lamina, and Me - medulla, arrows indicate manifestations of neurodegeneration processes in brain tissues (vacuolization). D. The mean total area of neurodegenerative zones before and after M-2 treatment *repo>Sod1^{RNAi}* of flies. E. Z-projections of confocal stacks of whole-mount adult *repo>UAS-CD8:GFP* (evidence of driver expression). *$P < 0.05$

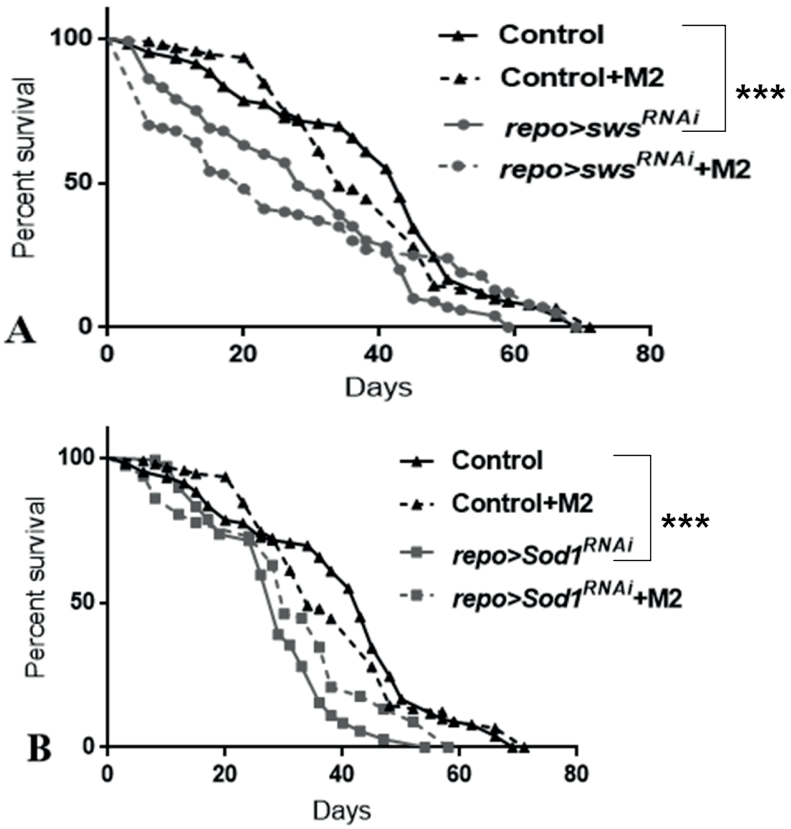

Fig. 2. Survival curves for control, knockdown of *sws* (A) and *Sod-1* (B) genes in glia on standard conditions and after larva treatment by M-2. ***P < 0.0002

nificant influence of the M-2 on the longevity of either control (P = 0.9) or experimental individuals (P = 0.2) (Fig. 2).

Locomotor activity

We observed a decrease in motor activity of 21-old day males flies with knockdown of *sws* and *Sod-1* genes in glia compare to control flies (P = 0.02 and P = 0.03, respectively). However, we did not observe a statistically significant influence of M-2 on locomotor activity of any flies (Fig. 3).

Oxidative stress markers

The levels of main oxidative stress markers the TBARS and CD were calculated on 21-day old flies under standard conditions and under the action of M-2. In the studied mutant Sod^{X-39} flies we observed a significant increase in the content of both CD and TBARS compared with control flies (P = 0.002 and P = 0.025, respectively). After the use of M-2, levels of CD and TBARS in control individuals did not change, while levels of both oxidative stress markers in mutants significantly decreased (Fig. 4). In particular, using of M-2 leads to the reduction of CD level by 47.6% (P = 0.003), and TBARS by 27.5% (P = 0.04).

DISCUSSION

To determine the effectiveness of a new neuroprotective drug, it is necessary to estimate several phenotypic manifestations which are common characteristics of neurodegeneration in *Drosophila* and human – lifespan, behavior changes, and cells degeneration extent. Any improvement of these indicators, even if it does not fully meet all the control levels, may be considered a positive effect.

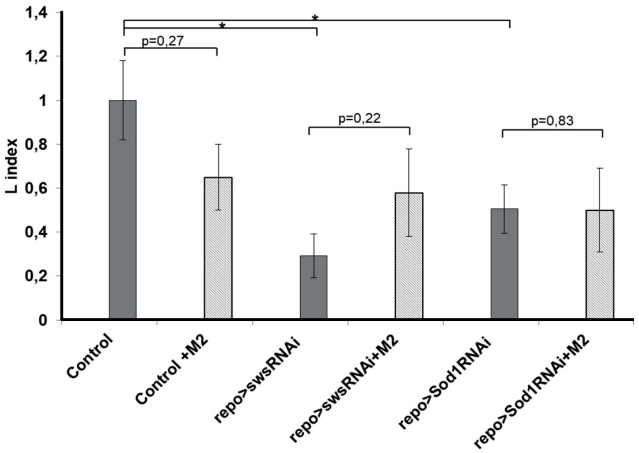

Fig. 3. Locomotor activity of 21-day old males flies of control group with knockdown of sws and Sod1 genes in glia on standard conditions and after larva treatment by M-2. *P < 0.05

The presence of a genetic marker for gliocytes in the brain of the *Drosophila* adult fly, such as the expression of *genes reversed polarity (repo)*, makes it possible to distinguish them clearly from neurons [13]. UAS/GAL4 system gives us a possibility to induce a functional inhibition of genes translation in certain cells or tissues. It was previously discovered that flies with a knockdown of the *sws* gene in the glyocytes are characterized by a specific type of pathology in the glial region, in particular, a significant vacuolization of the brain tissue was observed in the region corresponding to the transition of lamina to retina and medulla, where the highest concentration of gliocytes was observed [6]. Also, it is definitely known, that this type of pathology is not related to oxidative stress. The flies with knockdown of *Sod-1* gene in glial tissue also have specific neurodegenerative phenotype, in particular, the brain tissue degeneration zones appeared in the form of oval areas localized in the lobule and medulla [14]. Thus, we observed decrease in neurodegenerative zones after M-2 treatment

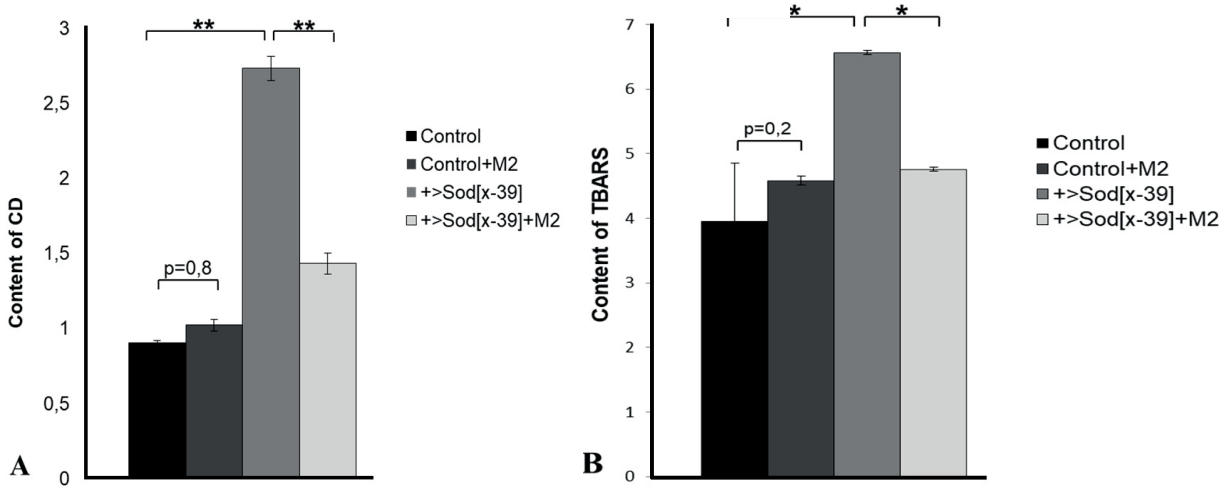

Fig. 4. Levels of oxidative stress markers CD (A) and TBARS (B) in flies under standard conditions and after M-2 treatment. *P < 0.05; **P < 0.004

of *repo>sws*^{RNAi} and *repo>Sod1*^{RNAi} flies, which may indicate a non-specific neuroprotective effect of the tested neuropeptide.

Neurodegenerations, in addition to changes in the structure of the brain, also can affect lifespan and behavioral reactions. Motor activity is a universal behavioral reaction, as well as the component of almost all more complex behavioral reactions such as sexual activity, photo and geotaxis, olfactory behavior and others. Changes in brain cells caused by mutations in the *sws* gene can determine behavioral abnormalities, in particular, the olfactory and motor behavior [15, 16]. In this study, we did not observe any influence of M-2 on longevity or locomotor activity in spite of brain degeneration decrease. Moreover, in our previous study of M-2 on *sws* mutants model [17], we observed life span increase after M-2 treatment. We can assume that in the current model of *sws* and *Sod-1* glial knockdown, neuroprotector M-2 delays degeneration of glial cells. However, contrary to expectations, this did not improve the functions of the neurons.

Also, oxidative stress, either as a primary cause or as an additional factor in expanding neurodegeneration when it develops, can be involved in the mechanism of neurodegenerative disorders progression. It is known [18] that the negative effect of the oxidative stress is the peroxide oxidation of lipids, the main markers of which are CD and TBARS. We studied these markers in *Sod-1* mutants and found that their level reduced after M-2 using. It could be evidence of the antioxidant effect of M-2.

It has to be noted that M-2 did not make influence any phenotype control flies, so we can assume that M-2 has an impact on specific paths that are associated with neurodegeneration. However, this mechanism, in case of its existence, needs further research.

CONCLUTION

1. Larvae feeding of experimental nootropic drug M-2 reduces degeneration zones in the brain tissue of *Drosophila* individuals with *Sod-1* gene knockdown in glia (*repo>Sod1*^{RNAi}).

2. M-2 did not effect the on life expectancy or motor behavior of flies in both *sws*- and *Sod-1*-dependent neurodegeneration models (*repo>sws*^{RNAi} and *repo>Sod1*^{RNAi}).

3. M-2 use reduced the level of oxidative stress markers (CD and TBARS) in old *Sod-1* mutants.

Experiments on D. melanogaster were approved by the Ethics Committee of the Ivan Franko Lviv National University, (Lviv, Ukraine) where this study was carried out.

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НЕЙРОПРОТЕКТОРНІ ЕФЕКТИ НОВОГО НООТРОПНОГО ЗАСОБУ МІТОХОНДРИН-2

Досліджували вплив нейропептиду мітохондрин-2 (М-2) на нейродегенеративних модельних системах Drosophila з патологією мозку, викликаною функціональним нокдауном генів *swiss cheese (sws)* і *Superoxide dismutase 1 (Sod-1)* у гліоцитах. Слід відмітити, що після використання М-2 зона дегенерації мозку зменшується і маркери оксидативного стресу (дієнові кон'югати і ТБК-позитивні продукти) сягали рівня контролю. Однак ми не спостерігали жодного покращення у тривалості життя чи руховій активності. Це свідчить про певну нейротропну і антиоксидантну дію М-2, особливо на гліальні клітини, але його вплив на підтримку функції нейронів залишається незрозумілим. Потрібні подальші дослідження для виявлення механізму дії пептидних нейропротекторів.

Ключові слова: *Drosophila melanogaster*; нейродегенерація; гени swiss cheese і Sod-1; нейропептиди.

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НЕЙРОПРОТЕКТОРНОЕ ДЕЙСТВИЕ НОВОГО НООТРОПНОГО СРЕДСТВА МИТОХОНДРИН-2

В данной работе мы исследовали влияние нейропептида митохондрин-2 (М-2) на нейродегенеративных модельных системах Drosophila с патологией мозга, вызванной функциональным нокдауном генов *swiss cheese (sws)* и *Superoxide dismutase 1 (Sod-1)* в глиоцитах. Следует отметить, что после использования М-2, зона дегенерации мозга уменьшается и маркеры оксидативного стресса (диеновые конъюгаты и ТБК-положительные продукты) достигали уровня контроля. Однако мы не наблюдали никакого улучшения продолжительности жизни или двигательной активности. Это свидетельствует об определенном нейротрофическом и антиоксидантном воздействии М-2, особенно на глиальные клетки, но его влияние на поддержание функции нейронов остается неясным. Требуются дальнейшие исследования для выявления механизма действия пептидных нейропротекторов.
Ключевые слова: *Drosophila melanogaster*; нейродегенерация; гены swiss cheese и Sod-1; нейропептиды.

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