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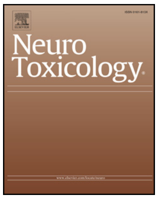

Full Length Article

Neuroprotective action of Eicosapentaenoic (EPA) and Docosahexaenoic (DHA) acids on Paraquat intoxication in *Drosophila melanogaster*

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ABSTRACT

Several studies have shown the protective effects of dietary enrichment of omega-3 (ω-3) long-chain fatty acids in several animal models of neurodegenerative diseases. Here we investigate if eicosapentaenoic (EPA) and Docosahexaenoic (DHA) acids (ω-3) protect against neurodegeneration mediated by the exposure to a widely used herbicide Paraquat (PQ) (1,1′-dimethyl-4-4′-bipyridinium dichloride), focusing on mitochondrial metabolism using *Drosophila melanogaster* as a model. Dietary ingestion of PQ for 3 days resulted in the loss of citrate synthase content, respiratory capacity impairment and exacerbated H_2O_2 production per mitochondrial unit related to complex I dysfunction, and high lactate accumulation in fly heads. PQ intoxication lead to 1) the loss of ELAV (embryonic lethal abnormal vision) and α-spectrin, essential proteins of neuronal viability and synaptic stability; 2) increased gamma-secretase activity, an enzyme related to APP release; and 3) increased the amyloid fibrils contents. All these toxic effects induced by PQ were prevented by concomitant dietary ingestion of EPA/DHA, suggesting that a neuroprotective effect of ω-3 also involves mitochondrial protection. In conclusion, concomitant EPA and DHA ingestion protects against PQ-induced neuronal and mitochondrial dysfunctions frequently found in neurodegenerative processes reinforcing its protective role against environmental neurodegenerative diseases.

1. Introduction

Exposure to some pesticides is considered as one of the environmental risk factors in the etiology of neurodegenerative diseases (for review Jett, 2011), particularly Alzheimer Disease (AD, Yan et al., 2016). Accounting for ~80% of dementia cases, AD is the most common neurodegenerative illness (Reitz and Mayeux, 2014), predicted to affect 81 million people worldwide in 2040 (World Health Organization, WHO, World Health Organization, 2012).

Characterized by progressive loss of memory and cognitive abilities (Simoncini et al., 2015; Devassy et al., 2016), the AD is frequently associated with high levels of β-amyloid peptide (Aβ) (40–43 amino acids) derived from the amyloid precursor protein (APP), severe lost of cholinergic neurons (Ferreira-Vieira et al., 2016; Brunkan and Goate, 2005) and mitochondrial dysfunction (Breuer et al., 2012; Simoncini et al., 2015). While epidemiological data has pointed a widely used herbicide Paraquat (PQ, N,N′-dimethyl-4,4′-bipyridinium dichloride) as a risk factor of AD (Baldi et al., 2003; Hayden et al., 2010), scientific experiments have demonstrated that PQ exposure raises Aβ levels, induces mitochondrial dysfunction, and oxidative damage specifically in mitochondria, being such events directly correlated with impaired associative learning and memory (Chen et al., 2012). Impairment of mitochondrial ATP synthesis is a typical condition of aging and neurodegenerative processes (Johri and Beal, 2012). Furthermore, due to its ability to induce degeneration of cholinergic and dopaminergic neurons, PQ has been used extensively as a model to replicates some specific features of Parkinson Disease (Baltazar et al., 2014; Franco et al., 2010; Berry et al., 2010).

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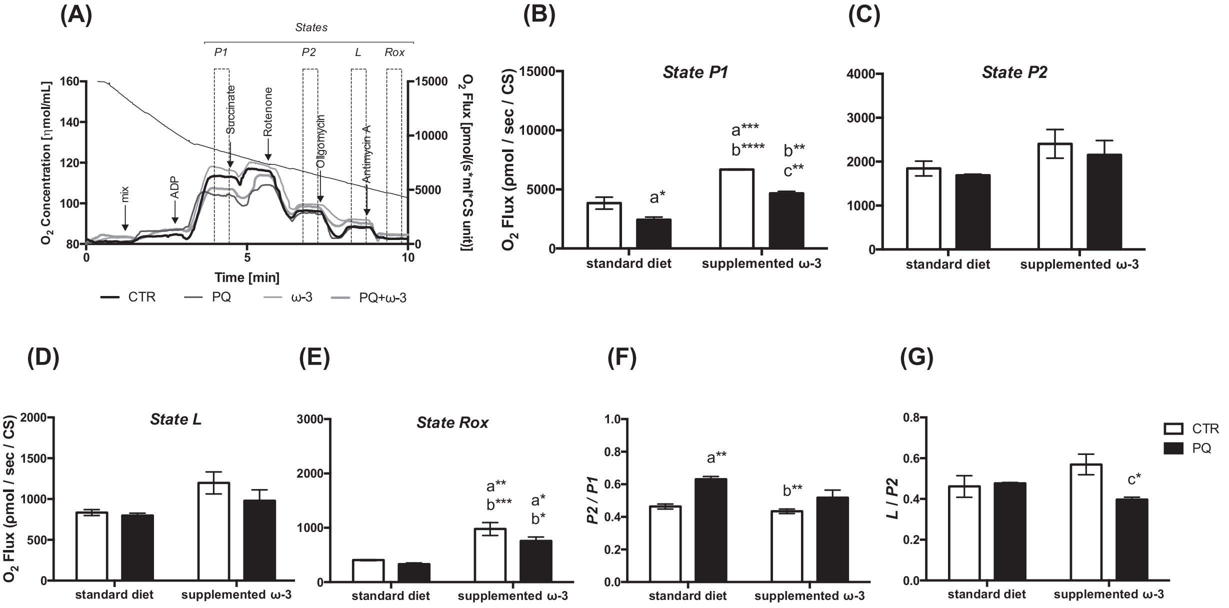

Fig. 1. Mitochondrial respiratory parameters of heads of *D. melanogaster* fed on a diet supplemented with PQ and/or ω-3. (A) Representative Oxygraph trace of heads of *D. melanogaster* (20 per chamber) indicating that was added NADH-linked substrates (mix), ADP, succinate, rotenone, oligomycin and antimycin A (Ant. A), as described in Materials and Methods, to determine the States of OXPHOS (*P1*) and OXPHOS (*P2*), LEAK (*L*), and RESIDUAL (*Rox*). Respiratory rates per CS units in *States* of **(B)** *P1*, **(C)** *P2,* **(D)** *L* and **(E)** *Rox*. Flux control ratio of *States* **(F)** *P2/P1* and **(G)** *L/P2*. The values represent the mean ± S.E.M of three independent experiments. Two-way ANOVA, interactions: **(B)** $F_{(1,8)} = 1.049$, $p = 0.3358$, **(C)** $F_{(1,8)} = 0.037$, $p = 0.8516$ **(D)** $F_{(1,8)} = 0.8619$, $p = 0.3804$, **(E)** $F_{(1,8)} = 1.074$, $p = 0.3303$, **(F)** $F_{(1,8)} = 2.465$, $p = 0.1550$, **(G)** $F_{(1,8)} = 6.447$, $p = 0.0348$. The results were considered statistically significant when $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$: ^{a} *vs* CTR, ^{b} *vs* PQ and ^{c} *vs* ω-3.

Docosahexaenoic acid (22:6 n-3, DHA) and eicosapentaenoic acid (20:5 n-3, EPA) are dietary omega-3 (ω-3) long-chain fatty acids, which promote several benefits to brain functions (Swanson et al., 2012) related to changes in neuronal membranes, facilitating neurotransmitter release, and acting as a neurothophic factors (Zhang et al., 2013, 2016). In *Drosophila melanogaster*, both fatty acids are essential since this fly lacks the capability to synthesize the C20 and C22 polyunsaturated fatty acids (PUFA). Dietary DHA and EPA are absorbed but the majority of DHA is shortened into EPA and incorporated into body tissues (Shen et al., 2010). Although the biochemical mechanism and physiological significance to brain functions remain unclear, PUFA deficiency strongly affects synaptic transmission in the *D. melanogaster* visual system, defects that were rescued by diets containing omega-3 or omega-6 PUFA alone or in combination (Ziegler et al., 2015). Recently we have shown that EPA/DHA ingestion protected *Drosophila melanogaster* against neurodegeneration and neuromuscular dysfunction induced by PQ intoxication at low concentration. In the muscle these ω-3 acted restoring mitochondrial biogenesis, dynamics and respiratory capacity (De Oliveira Souza et al., 2017). However, the protective role of EPA/DHA on mitochondrial function in brain remains uncharacterized. Therefore, the aim of this study was to describe protective effects of ω-3 (EPA/DHA) against neurodegeneration mediated by PQ exposure at low concentration focusing on mitochondrial metabolism in brain using D. melanogaster as a model.

2. Materials and methods

2.1. Chemicals

Reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO), except antibodies (described below), Bradford protein assay (cat. no. #500-0006) from Bio-Rad, lactate assay (Labtest, Brazil, cat. no. #138-1/50), Amplex Red (cat. no. A22177) from Invitrogen Molecular Probes, and peptides NMA-GGVVIATVK-(DNP)-DRDRDR-NH_2 from by AminoTech Research and Development Ltda (Brazil).

2.2. Drosophila stock

Female Drosophila *melanogaster* wild-type strain Canton Special flies were reared under 25 ± 1 °C and 12:12 h light/dark cycle (He and Jasper, 2014; Bosco et al., 2015), and separated into four groups, which received: 1) standard diet containing water, 0.5% agar, 1.5% yeast, 2.5% sucrose, 8.5% corn extract, 5% dextrose, 0.5% Nipagin, and 0.5% acid mix (8.2% propionic acid and 45.8% phosphoric acid); 2) standard diet supplemented with omega-3 (Centrum lot 4406HQ, Pfizer, 39% EPA and 24% DHA) at a final concentration of 1 mg/mL (2 mM); 3) standard diet containing PQ at a final concentration of 200 μg/mL (0.8 mM); 4) standard diet containing EPA/DHA and PQ at a final concentrations mentioned above. Female *D. melanogaster* were synchronized young (1 day old) and submitted to experimental settings for 3 days. After 3 days, flies were transferred into vials with fresh food for maintaining the best physiological conditions, and avoid insect death due to other causes (sticking to the humid food, mold, or bacterial growth) (Bosco et al., 2015; Oka et al., 2015). Flies were anesthetized with CO_2, transferred to 15 mL centrifuge tubes, flash frozen with liquid N_2 and the tubes were shaken vigorously for five time (10 s each time) to separate the heads from the body (Berglund et al., 2013). Heads were collected on dry ice and stored at −80 °C until use.

2.3. Oxygen consumption

Twenty heads were mechanically macerated in 0.02 mL buffer for *ex vivo* tissue maintenance (BIOPS, 2.77 mM Ca^{2+}-EGTA, 7.23 mM EGTA, 6.56 mM $MgCl_2.6H_2O$, 20 mM taurine, 15 mM phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol (DTT), 50 mM 2-[N-morpholine]-ethanesulfonic acid (KMES) and 5.77 mM ATP). The homogenate was added in 2 mL MiR05 respiration buffer (20 mM HEPES, 10 mM KH_2PO_4, 110 mM sucrose, 20 mM taurine, 60 mM K-lactobionate, 0.5 mM EGTA, 3 mM $MgCl_2$, 1 g/L fatty acid-free BSA, pH 7.1) at 25 °C and 600 rpm, and oxygen consumption was monitored using a High-Resolution Respirometer (OROBOROS Oxygraph) equipped with DatLab 5.0 (OROBOROS, Innsbruck, Austria) for data acquisition (2-

sec-time intervals) and analysis. Respiration rates at phosphorylating states were determined in the presence of exogenous NAD^+-linked substrates (1.5 mM pyruvate, 0.28 mM malate, 1.3 mM glutamate) and 240 μM ADP (*State P1*, phosphorylation supported by complex I activity), following 10 mM succinate and 0.5 μM rotenone (*State P2*, phosphorylation supported by succinate). Then, non-phosphorylating state (*State L*, leak) and *State Rox* (oxygen consumption not related to the mitochondrial electron transport) were determinate after addition of 0.5 μg/mL oligomycin and 0.6 μg/mL antimycin A, respectively (Fig. 1A). Upon terminating the assay session, the medium containing the tissue was collected to determine protein and the citrate synthase (CS) contents, values used to calculate the rates of oxygen consumption. The rate of oxygen consumption at Rox State was subtracted from the rates in others respiratory states.

2.4. Citrate synthase (CS) activity

Fifteen heads were submitted to 2 cycles of homogenization followed by centrifugation in 0.02 mL triethanolamine-HCl buffer (0.1 M) pH 8, to disrupt mitochondrial membranes. Then, the homogenates were centrifuged at 12000xg for 10 min at 4 °C, the supernatant collected (Peleg et al., 2016) and protein concentration determined. The reaction (10 min) was started by adding 10 μg protein to ~170 μL triethanolamine-HCl buffer containing 0.1 mM Acetyl-CoA, 0.1 mM 5′,5′-Dithiobis-2-nitrobenzoic acid (DTNB), 0.2 mM oxaloacetate and 0.1% Triton X-100. The reduced CoA (CoA-SH) formed by CS activity converts the DTNB into 2-nitro-5-benzoic acid (TNB). CS activities were evaluated by the rate of TNB formation, measured spectrophotometrically at 412 ηm according to Srere (1969) using a Model Cary 50MPR Varian Spectrophotometer (Varian Ltd., Melbourne, Australia). The increase in absorbance was linear for up to 2 min, at rates less than 0.06 A/min. The CS activity was calculated by the following equation: Units (μmol/mL/min) = ((ΔA_{412}) / min x V (mL) / ε^{mM} x L (cm), where A_{412} is the absorbance of TNB in 412 ηm, V (mL) is the reaction volume (mL), ε^{mM} is the extinction coefficient of TNB (13.6 $mM^{-1}.\ cm^{-1}$) and L (cm) is the pathlength for absorbance measurement. Each sample was run in quintuplicate and the absorbance values were expressed per total amount of protein in the sample.

2.5. H_2O_2 production

Fifteen heads were incubated in 2 mL MiR05 respiration buffer containing 2 μM N-acetyl-3,7-dihydroxylphenoxazine (Amplex Red) and 2 U/mL horseradish peroxidase (HRP) (Rhee et al., 2010) in the presence of NAD^+-linked substrates (1.5 mM pyruvate, 0.28 mM malate, 1.3 mM glutamate) or 10.5 mM succinate, at room temperature for 10 min. In the presence of HRP, the Amplex Red reacts with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent oxidation product, resorufin, measured at 563/587 ηm ex/em in a fluorescence spectrophotometer (F-4500 Hitachi Ltd., Tokyo, Japan) with continuous stirring. Upon terminating the assay session, the medium containing the tissue was collected to determine protein and the citrate synthase (CS) contents, values used to calculate the rates of H_2O_2 production.

2.6. Lactate content

Fifteen heads were macerated and homogenized three times in 0.02 mL 0.1 M sodium phosphate buffer pH 7.4. The homogenate was centrifuged at 10000xg for 10 min at 4 °C and supernatant collected. Lactate content was measured by lactate oxidase method. The reaction (5 min) was started by adding 10 μL sample to 1 mL of assay buffer, according manufacturer instructions (Labtest, Brazil, cat. no. #138-1/50). Absorbance was monitored spectrophotometrically at 550 ηm using a Model Cary 50MPR Varian Spectrophotometer (Varian Ltd., Melbourne, Australia). Each sample was run in quintuplicate and the absorbance values were expressed per total amount of protein in the sample.

2.7. Western blot analyses

Twenty heads were submitted to 2 cycles of homogenization followed by centrifugation in 0.02 mL PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing protease inhibitor cocktail. Soluble proteins (30 μg) were homogenized in buffer (40 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.05% Triton X-100) and separated by 10% of SDS-PAGE and transferred to nitrocellulose membrane. Western blot was performed using primary antibodies against α-spectrin (1:3000, cat. no. 3A9, Developmental Studies Hybridoma Bank - DSHB, University of Iowa, Iowa), ELAV (1:2000, cat. no. 7E8A10, DSHB) and α -Tubulin (1:2000, cat. no. C4: 12G10, DSHB). Secondary antibodies (1:10,000) against rabbit (cat. no. 12-348, EMB Millipore), rat (cat. no. 14-16-12, KLP SeraCare) or mouse (cat. no. 12-349, EMB Millipore) conjugated with horseradish peroxidase (HRP) and HRP substrate were used for chemiluminescent detection (Millipore, #WBKLS0050, Merck Millipore, USA).

2.8. Gamma-secretase activity

Fifteen heads were lysed in 0.02 mL hypotonic buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA; and 1 mM EGTA) containing 0.2% CHAPS plus protease inhibitor cocktail. The reaction (12 h) was started by adding 10 μg protein to 0.1 M sodium phosphate buffer, pH 7.4, containing 10 μM fluorescent conjugated peptide substrate [NMA-GGVVIATVK(DNP)-DRDRDR-NH2] a APP sequence for gamma-secretase cleavage (Hayashi et al., 2012). The rate of substrate cleavage was determined fluorometrically (at 355/440 ηm ex/em) in a fluorescence spectrophotometer (Synergy 2, BioTek Instruments, Inc., Winooski, USA). Each sample was run in quintuplicate and the fluorescence values (A.U.F.) were expressed per total amount of protein in the sample.

2.9. Amyloid fibrils concentration

Fifteen heads were submitted to 2 cycles of homogenization followed by centrifugation in 0.02 mL PBS buffer containing protease inhibitor cocktail. The samples were centrifuged at 2000xg for 2 min at 4 °C. Supernatant (5 μL) was incubated for 5 min in 195 μL PBS containing 200 μM thioflavin T (ThT). A standard curve of β42 was made (0–11 μM) and incubated in PBS during 24 h for fibril formation (Xue et al., 2017). The binding of amyloid fibrils and ThT was determined fluorometrically (at 450/482 ηm ex/em) (Naiki et al., 1989) in a fluorescence spectrophotometer (Synergy 2, BioTek Instruments, Inc., Winooski, USA). Each sample was run in triplicate and the fluorescence values were expressed per total amount of protein in the sample.

2.10. Protein assay

The protein concentration was determined by Bradford assay using BSA as the standard (Bradford, 1976).

2.11. Statistical analyses

The data are presented as mean ± S.E.M. N means the number of flies per group used in each experiment. Statistical analysis was performed using two-way ANOVA for multiple comparisons using the GraphPad ©Prism 6 software (San Diego, CA, USA). Results were considered significant when $P < 0.05$ ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$) and ns for $P > 0.05$.

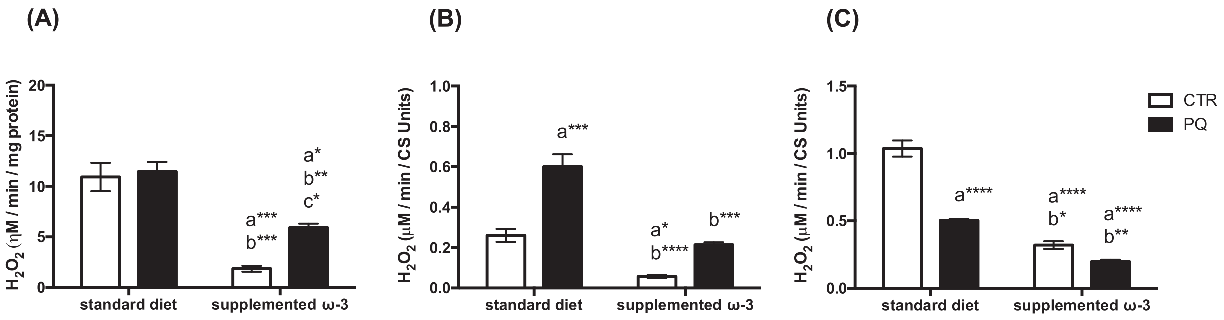

Fig. 2. H_2O_2 production in heads of *Drosophila melanogaster* fed on a diet supplemented with PQ and/or ω-3. (A) Rates of H_2O_2 production per mg of tissue protein in the presence of NAD^+-linked substrates (pyruvate, malate and glutamate) and **(B)** Rates of H_2O_2 production per CS units in the presence of NAD + -linked substrates and **(C)** succinate, as described in Materials and Methods. The values represent the mean ± S.E.M of three independent experiments. Two-way ANOVA, interactions: **(A)** $F_{(1,8)} = 4.056$, $p = 0.0788$, **(B)** $F_{(1,8)} = 6.845$, $p = 0.0308$ and **(C)** $F_{(1,8)} = 35.39$, $p = 0.0003$. The results were considered statistically significant when $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$: ^{a} *vs* CTR, ^{b} *vs* PQ and ^{c} *vs* ω-3.

3. Results

3.1. Mitochondrial parameters

PQ supplementation induced a reduction of citrate synthase (CS) activity, an enzyme of Krebs Cycle which can be an indicative of mitochondrial content (Figs. 2 and Fig. 3). PQ supplementation also decreased the respiration rates in States of OXPHOS (*P*, oxidative phosphorylation) supported by NAD^+-linked substrates (*State P1*) (Fig. 1B), but not in OXPHOS supported by succinate in the presence of rotenone (*State P2)* (Fig. 1C), indicating Complex I impairment. The analyses of *P2/P1* ratio reinforce this result, demonstrating high complex II relative to complex I in heads of flies exposed to PQ (Fig. 1F). No any marked changes were observed in LEAK State (*L*, independent of oxidative phosphorylation) (Fig. 1D) and *L/P2* ratio (Fig. 1G), indicating similar levels of mitochondrial coupling, or *Rox* (oxygen consumption not related to the respiratory chain activity) (Fig. 1E). The administration of EPA/DHA (ω-3) alone increased *States P1*, *L* and *Rox* (Fig. 1E), suggesting an increased phosphorylation capacity, H^+ leak and cellular oxygen consuming reactions (enzymes/autooxidation, including the activity of peroxidases and oxidases), respectively. Therefore, the administration of ω-3 in combination with PQ prevented the changes in *State P1 and P2/P1* (Figs. 1**B and 1 G**), and increased State *Rox* as found in ω-3 alone, and in particular decreased *L/P2*, an indicative of elevated coupling degree of mitochondria.

3.2. H_2O_2 production

After 3 days of PQ exposure, heads of *D. melanogaster* presented similar the rates of hydrogen peroxide (H_2O_2) generation per mg of tissue protein when energized with NAD^+-linked substrates (entry of electrons from complex I, Fig. 2A). However, when these rates were normalized by CS units, an elevated H_2O_2 production per mitochondria is observed in these heads (Fig. 2B). It was reversed when mitochondria were energized with succinate (Fig. 2**C**) (entry of electrons from complex II), indicating that in heads of PQ supplemented animals H_2O_2 generation is related to complex I dysfunction. Under this condition, heads of control animals showed increments in H_2O_2 generation as a result of electron back flow from complex II to complex I. The exposure to ω-3 alone or in combination with PQ tightly reduced H_2O_2 production per tissue or per mitochondria energized with any of the substrates.

3.3. Lactate concentration

PQ ingestion by *D. melanogaster* significantly increased lactate concentration in heads, an effect prevented by EPA/DHA ingestion in combination (Fig. 4).

3.4. Neuronal viability and synaptic stability

PQ exposure markedly affected the contents of ELAV (embryonic lethal abnormal vision) (Fig. 5A), a protein localized in the nucleus of neurons of Drosophila, expressed at all stages of neural development and involved in the maintenance of the nervous system in adults (Colombrita et al., 2013), and also negatively affected the contents of α-spectrin (Fig. 5B), a protein involved in the stability and structure of cell membrane that controls the shape of a cell (for review, Zhang et al., 2013). In neurons, α-spectrin works stabilizing the synapses, the axons, and in the formation of axon initial segments and nodes of Ranvier (Bennett and Baines, 2001; Baines, 2010; Rasband, 2010) (Fig. 5B). EPA/DHA exposure increased ELAV and α-spectrin levels and, when in combination with PQ was effective to prevent the fall in ELAV and α-

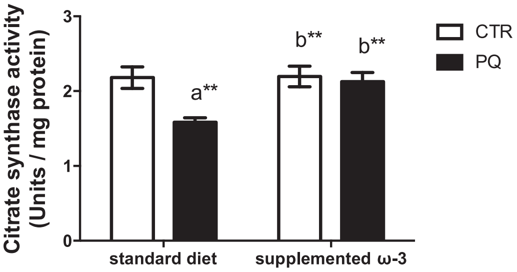

Fig. 3. Citrate synthase (CS) activity in heads of Drosophila melanogaster fed on a diet supplemented with PQ and/or ω-3. The values represent the mean ± S.E.M of five experiments. Two-way ANOVA, interaction ($F_{(1,62)} = 4.708$, $p = 0.0339$). The results were considered statistically significant when $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$: ^{a} *vs* CTR, ^{b} *vs* PQ and ^{c} *vs* ω-3.

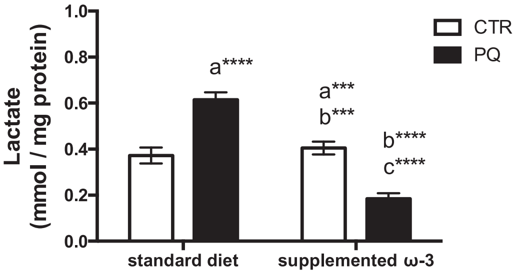

Fig. 4. Lactate concentration in heads of D. melanogaster fed on a diet supplemented with PQ and/or ω-3. The values represent the mean ± S.E.M of five independent experiments. Two-way ANOVA, interaction ($F_{(1,36)} = 58.91$, $p < 0.0001$). The results were considered statistically significant when $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$: ^{a} *vs* CTR, ^{b} *vs* PQ and ^{c} *vs* ω-3.

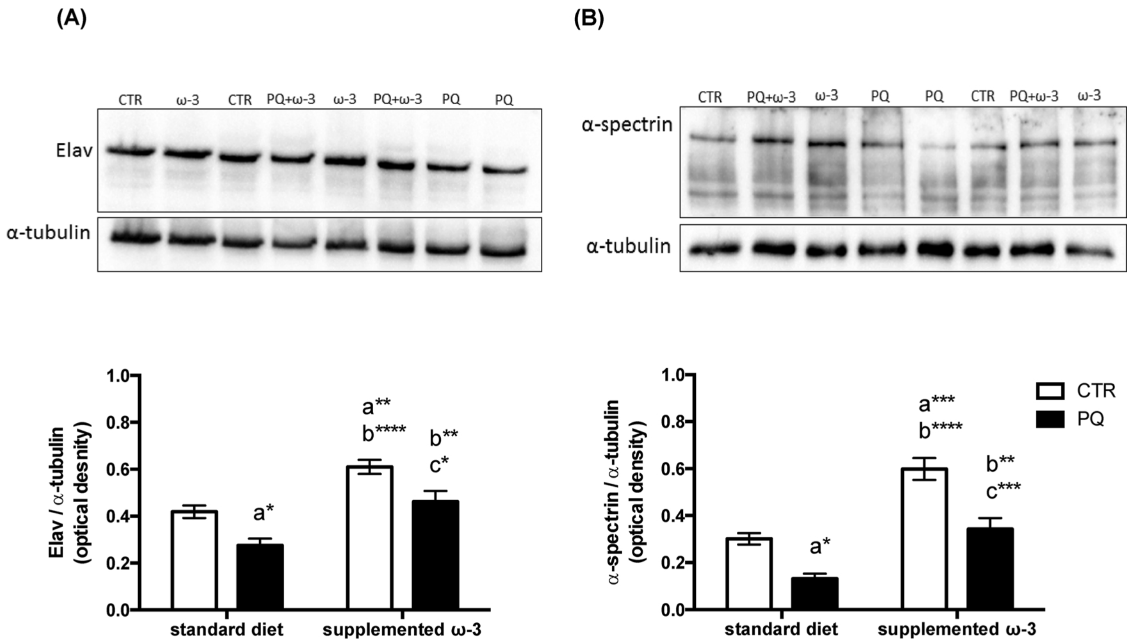

Fig. 5. Western blot analysis and quantitative densitometry of proteins in heads of *D. melanogaster* fed on a diet supplemented with PQ and/or ω-3. Western blot analysis of **(A)** ELAV and **(B)** α-spectrin were performed as described in Material and Methods. Individual blottings are in supplementary material. ELAV and α-spectrin protein expressions were normalized by total α-tubulin protein expression. The values represent the mean ± S.E.M of three experiments. Two-way ANOVA, interactions: **(A)** $F_{(1,21)} = 0.00477$, $p = 0.9455$ and **(B)** $F_{(1,18)} = 1.348$, $p = 0.2608$. The results were considered statistically significant when $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$: ^{a} *vs* CTR, ^{b} *vs* PQ and ^{c} *vs* ω-3.

spectrin contents, suggesting that these ω-3 protects the head against of loss of mature neurons and/or reduction of neuronal differentiation, and synaptic dysfunction promoted by herbicide intoxication.

3.5. *Gamma-secretase activity and production of amyloid fibrils*

PQ supplementation significantly increased gamma-secretase activity in the heads (Fig. 6A), suggesting increased release of APP (a precursor for amyloid beta peptide 42 (Aβ42) formation), and the content of amyloid fibrils (Fig. 6B). ω-3 in combination with PQ significantly reduced enzyme activity and amyloid fibril content, reaching control levels. Increased gamma-secretase activity was found in human neuroblastoma SH-SY5Y wildtype cells overexpressing APP (Grimm et al., 2015), a peculiar condition for Alzheimer's disease.

4. Discussion

In addition to $\Delta\psi m$-dependent accumulation into the mitochondrial matrix (Cochemé and Murphy, 2008), PQ may interact with electron transport chain (ETC) complexes, especially with complex I and III promoting a redox cycling: divalent PQ cation (PQ^{2+}) is reduced to highly reactive mono cation radical (PQ^{+}), which in turn reacts with the molecular oxygen to generate superoxide radical ($O_2^{\cdot -}$) and subsequently other oxygen reactive species (Hosamani and Muralidhara, 2013; Blanco-Ayala et al., 2014; Medina-Leendertz et al., 2014). This property of PQ has been implicated in the oxidative stress-mediated neuronal damage (Baltazar et al., 2014). In rat lung, PQ-induced $O_2^{\cdot -}$ formation and lipid peroxidation inhibit complex I activity (Fukushima et al., 1994). In addition, other ETC complexes (I, II and III), as well as CS activity, were found less active after acute (24 h) exposure to high PQ concentrations (10–40 mM) in whole *D. melanogaster* (Hosamani

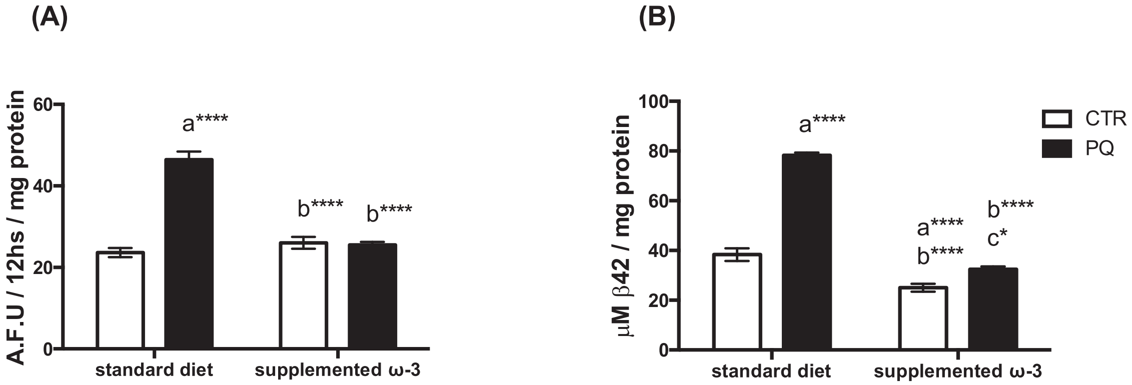

Fig. 6. Gamma-secretase activity and β42 oligomer concentration in heads of *D. melanogaster* fed on a diet supplemented with PQ and/or ω-3. (A) Gamma-secretase activity and **(B)** β42 oligomer concentration assays were performed as described in Material and Methods. Gamma-secretase activity was expressed as arbitrary fluorescence units (A.U.F) of substrate cleaved by 12 hs per mg protein. The values represent the mean ± S.E.M from three experiments. Two-way ANOVA, interactions: **(A)** $F_{(1,56)} = 67.16$, $p < 0.0001$ and **(B)** $F_{(1,32)} = 94.96$, $p < 0.0001$. The results were considered statistically significant when $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$: ^{a} *vs* CTR, ^{b} *vs* PQ and ^{c} *vs* ω-3.

and Muralidhara, 2013). Here, in heads of this model, we carefully showed that at low concentration (diet containing 200 μg/mL or 0.77 mM PQ, for 72 h), PQ exposure not only diminishes CS activity as well as specifically impairs complex I activity, reducing mitochondrial respiratory/phosphorylation capacity. The total mass of PQ ingested/fly (~0.9 μg) was *in vitro* added to the head homogenates and no effect in respiration and H_2O_2 production were found (data not show) showing that the effects found here are related to *in vivo* PQ ingestion. As neurons are the main O_2 consumers for oxidative phosphorylation, these results suggest a diminished ATP availability in neurons, which is essential to the activity and viability of these cells (for review, Bélanger et al., 2011). Consistent with us, PQ-induced mitochondrial dysfunction at acute low (0.1 mM) doses was also demonstrated in embryos of zebrafish (vertebrate), in which maximal respiration was 70% reduced (Wang et al., 2018).

As a vicious cycle, mitochondrial complex I inhibition can, in turn, stimulate more electron leakage for ROS formation (Takeshige and Minakami, 1979; Turrens and Boviers, 1980; Turrens et al., 1982). Elevated mitochondrial superoxide generation and oxidative stress biomarkers are present in whole *D. melanogaster* after acute exposure to high concentrations of PQ (Hosamani and Muralidhara, 2013). Here, in heads of flies exposed to low PQ concentrations we accurately found that complex I is the main site of ROS formation, which can be associated with impaired activity of this complex. Nevertheless, the total rates of H_2O_2 production in the whole head tissue of PQ exposed flies were not changed, probably as a consequence of mitochondrial content reduction.

The diminished mitochondrial metabolism in heads of PQ-supplemented flies observed here can also be associated with high lactate concentration found. As the final product of glycolysis, lactate is produced mainly by astrocytes and also by neurons at high energy demand (for review, Bélanger et al., 2011). Lactate from astrocytes is shuttled to neurons through proton-linked monocarboxylate transporters (MCTs) being converted to pyruvate to entry into mitochondria (Proia et al., 2016) following the oxidative metabolism. Raised lactate levels are a useful biomarker of neuronal degeneration related to mitochondrial dysfunction detected at cerebrospinal fluid (Albanese et al., 2016) and serum (Amorini et al., 2014) of humans with multiple sclerosis, and brains of mammals in aging (Ross et al., 2010), with Parkinson (Bowen et al., 1995) or Alzheimer (Harris et al., 2016) diseases.

Here we showed for the first time that PQ intoxication decreases α-spectrin contents, a protein that stabilizes the axons and synapses, and plays an important role in synaptic functions including presynaptic vesicle tethering and postsynaptic receptor aggregation (Pielage et al., 2006; Featherstone et al., 2001). α-spectrin deletion in *D. melanogaster* impairs neurotransmissions and disrupts the subcellular localization of several synaptic proteins (Featherstone et al., 2001). In fact, we and others have demonstrated dysfunctions in cholinergic (De Oliveira Souza et al., 2017), dopaminergic (Cassar et al., 2015) and glutamatergic (Baltazar et al., 2014) synaptic transmissions in *D. melanogaster* induced by PQ.

Approaching the aspects of AD, PQ ingestion induced abnormal amyloid fibrils formation in heads of *D. melanogaster*, which can trigger the pathological cascade of AD (Chen et al., 2012). Amyloid fibril formation initiates with accumulation of Aβ42 oligomer, a product of proteolytic cleavage of APP in the transmembrane domain by PS1, the catalytic component of γ-secretase complex (Haass and Selkoe, 1993; De Strooper, 2003), which was also found folded active after PQ exposure. PQ also reduced the contents of ELAV, a neuronal RNA-binding protein that works in post-transcriptional regulation and is involved in the maintenance of the adult nervous system (Robinow and White, 1991; Ogawa et al., 2018). In mammalian brain, the protein kinase C (PKC)α-dependent ELAV protein recruitment and activation has a positive role in the differentiation, regeneration and learning programs (Pascale et al., 2005), and their derangement can contribute to AD pathophysiology (Talman et al., 2016).

As described by us in the muscle (De Oliveira Souza et al., 2017), here the dietary ingestion of EPA and DHA also protected the heads of *D. melanogaster* against the loss of mitochondrial content and respiratory capacity, as well as against the modulations in α-spectrin, ELAV, γ-secretase and Aβ42 induced by PQ. In addition, ω-3 prevented the rise in lactate levels suggesting that ω-3 neuroprotective action in the brain really involves the mitochondrion. These beneficial ω-3 effects in mitochondria have been related to the control of oxidative stress and mitochondrial membrane permeability (Kumar et al., 2014; Zhang et al., 2016). Related to neuronal proteins, previous studies have shown that tissue lost/deficiency of ω-3 is associated to elevated spectrin breakdown after traumatic brain injury (Desai et al., 2014), whilst lower levels of Aβ and amyloid plaques are found in the brain of AD transgenic mouse model, expressing the double-mutant form of human APP, fed a DHA enriched diet (Amtul et al., 2011). To our knowledge, we are the first to show that ω-3 supplementation elevates ELAV content in the brain.

Finally, ω-3 increased the O_2 flux to non-mitochondrial oxygen consuming reactions in the head, suggesting stimulated EPA/DHA oxidation. This reaction can be catalyzed by ciclooxigenase (COX), lipoxygenase and cytochrome P450 for synthesis of lipid-derived mediators called oxylipins (Mosblech et al., 2009; Martínez and Campos-Gómez, 2016). Specific oxylipins derived from DHA and EPA can reduce neuroinflammation, an important player in the advancement of the AD, and prevent cognitive decline (for review, Devassy et al., 2016).

5. Conclusion

PQ ingestion by *D. melanogaster* resulted in neuronal changes frequently found in neurodegenerative processes, including mitochondrial dysfunction, lactate accumulation, negatively affected key proteins related to synapse organization and stability, and events characteristics of Alzheimer's disease. Concomitant EPA and DHA ingestion protected *D. melanogaster* following PQ-induced neuronal changes and mitochondrial dysfunction, reinforcing its protective role against environmental neurodegenerative diseases.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2018.11.013.

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