Short Communication

Neurodegeneration-related beta-amyloid as autocatabolism-attenuator in a micro-in vivo system

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ARTICLE INFO

Keywords:
Invertebrate
Beta-amyloid
Autocatabolism
Organ shrinkage
Metabolism
Bdelloid rotifer

Chemical compounds studied in this article:
Acridine orange (PubChem CID: 62344)
BisANS (PubChem CID: 16213473)
Concanamycin A (PubChem CID: 6438151)
NaOH (PubChem CID: 14798)
Neutral red (PubChem CID: 11105)
Propidium-iodide (PubChem CID: 104981)

ABSTRACT

Investigation of human neurodegeneration-related aggregates of beta-amyloid 1–42 (Aβ42) on bdelloid rotifers is a novel interdisciplinary approach in life sciences. We re-applicated an organ size-based in vivo monitoring system, exploring the autocatabolism-related alterations evoked by Aβ42, in a glucose-supplemented starvation model. The experientially easy-to-follow size reduction of the bilateral reproductive organ (germovitellaria) in fasted rotifers was rescued by Aβ42, serving as a nutrient source- and peptide sequence-specific attenuator of the organ shrinkage phase and enhancer of the regenerative one including egg reproduction. Recovery of the germovitellaria was significant in comparison with the greatly shrunken form. In contrast to the well-known neurotoxic Aβ42 (except the bdelloids) with specific regulatory roles, the artificially designed scrambled version (random order of amino acids) was inefficient in autocatabolism attenuation, behaving as negative control. This native Aβ42-related modulation of the ‘functionally reversible organ shrinkage’ can be a potential experiential and supramolecular marker of autocatabolism in vivo.

1. Introduction

Rotifers are widely accepted animal models of aging-, metabolism-, starvation-, pharmacology- and micro-in vivo OMICS research and methodologies (Macsai et al., 2015; Snell, 2014). Bdelloids, such as Philodina or Adineta species, have high tolerance to normal environmental changes due to their capability of adaptive phenotypic plasticity (van Cleave, 1932; Azevedo and Leroi, 2001). Marotta et al. (2012) found that the organs of these animals appeared to be compressed during starvation. The germovitellaria (the combined site of the germarium and vitellarium glands) showed significant reduction in size and in fine structure under caloric restriction. These organ tissues could also function as nutrient storage; therefore, their content is used by rotifers via autophagy (Mizushima and Komatsu, 2011). In acidic vesicular organelles (AVOs) detection, acidotropic dyes are applied such as acridine orange (AO) or neutral red (NR). These indicators show good correlation with each other (Morishita et al., 2017) and in some cases these fluorescent probes, without cross-linking, are more promising quantitative approaches than immunofluorescence to evaluate the late phase of autocatabolism. The vacuolar-type H+–ATPase inhibitors (e.g. Concanamycin A) can hinder the catabolic processes of metabolic autophagy (Goto-Yamada et al., 2019). The direct connection between anatomical changes (e.g. organ shrinkage) and autophagy has been proved in rotifers (Marotta et al., 2012; Cervellione et al., 2017). Treatments with various exogenous Aβ isoforms are well-known models of Alzheimer’s disease and numerous studies applying in vitro and in vivo systems to discover their exact impacts. Studies are available using human neuroblastoma cells (Datki et al., 2003; Poeggeler et al., 2005), invertebrates, rodents, and primates (Harkany et al., 2000; Kong et al., 2016; Sharma et al., 2017). Despite these facts, only one publication

Abbreviations: Aβ, beta-amyloid; AO, acridine orange; AVOs, acidic vesicular organelles; BisANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt; ConA, Concanamycin A; D0, Day 0; D20, Day 20; D25, Day 25; FROS, functionally reversible organ shrinkage; FROS, FROS index; NFI%, percentage of normalized fluorescence intensity; PI, propidium-iodide; S-Aβ42, scrambled isoform of Aβ; SEM, standard error of the mean.

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https://doi.org/10.1016/j.ibror.2020.10.002
Received 25 June 2020; Accepted 2 October 2020
Available online 6 October 2020
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deals with the effects of Aβ on bdelloid rotifers, e.g. Philodina species by Poeggerler et al. (2005). This research group administered beta-amylol to test the potential protective effects of novel drug candidates (e.g.: antioxidant LPBNAH). These phylogenetically ‘simple’ animals are inappropriate to model higher, species-specific (e.g. human) physiological processes (e.g. neurodegeneration); nevertheless, they are suitable for demonstrating various interdisciplinary concepts (metabolic connection between non-pathogenic invertebrates and natural aggregates; Ricci and Boschetti, 2003; Ramulu et al., 2012). An adequate example for this is the phenomenon that bdelloids are able to exceed-ingly catabolize the highly resistant peptide- or protein aggregates (well-known neurotoxins), such as beta-amylol (Aβ), alpha-synuclein and scrapie prions under extreme conditions (e.g. starvation). Investigation of neurodegeneration-related Aβ42 on rotifers is the central research topic of our team. In an exceptional way, the human-type aggregates are potential nutrient sources to bdelloid rotifers (Datki et al., 2018); however, the general foods of these animals are aggregated organic masses in their natural habitat (Fontaneto et al., 2011). Bdelloid content (mg/L) were: Ca\(^{2+}\) 31.05; Mg\(^{2+}\) 17.6; Na\(^+\) 0.9; K\(^{+}\) 0.25; Fe\(^{2+}\) 0.001; HCO\(^{3-}\) 153.097; SO\(^{4-}\) 3; Cl\(^{-}\) 0.8; F\(^{−}\) 0.02; H\(_{2}\)SO\(_{4}\) 3.3 (pH = 7.5) (Olah et al., 2017). For standard food of cultures, we used homogenized baker’s yeast (EU-standard granulated instant form, 2-0 1-42 0674/001-Z12180/HU) which was heat-inactivated and filtered (Whatman filter with 10 μm pore; 6728-5100).

2. Materials and methods

2.1. The invertebrate models
The experiments were performed on Philodina acuticornis and Adineta vaga species; therefore, no specific ethical permission was needed according to the current international regulations. They were obtained from a Hungarian aquavarist, originating from an agricultural farm in Szarvas, Hungary. The species have been maintained in standard laboratory conditions for 6 years. The rotifers were cultured based on the following methods of our previous publications. The standard medium content (mg/L) were: Ca\(^{2+}\) 31.05; Mg\(^{2+}\) 17.6; Na\(^{+}\) 0.9; K\(^{+}\) 0.25; Fe\(^{2+}\) 0.001; HCO\(^{3-}\) 153.097; SO\(^{4-}\) 3; Cl\(^{-}\) 0.8; F\(^{−}\) 0.02; H\(_{2}\)SO\(_{4}\) 3.3 (pH = 7.5) (Olah et al., 2017). For standard food of cultures, we used homogenized baker’s yeast (EU-standard granulated instant form, 2-0 1-42 0674/001-Z12180/HU) which was heat-inactivated and filtered (Whatman filter with 10 μm pore; 6728-5100).

2.2. Treatment and monitoring
The Aβ42 and its scrambled isofrom (S-Aβ42: LKADFIDGVEYNNV- GEGFAISGHVAHVD/SVMGFEI GV/RDV/HQA) were prepared at the Department of Medical Chemistry, University of Szeged, Hungary. The concentrations of the stock solutions were 1 mg/mL in distilled water; the aggregation time was 3 h (25 °C, pH 3.5); the neutralization (to pH 7.5) was performed with NaOH (1 N) (Bozso et al., 2010; Kalweit et al., 2015). The final concentrations of Aβ were 100 μg/mL. For in vivo investigations we applied Concanamycin A (ConA; 27689, 50 nM), 4, 4′-dianilino-1,1′-binaphthyl-5,5′-disulonic acid dipotassium salt (BisANS, D4162; 50 μM), propidium-iodide (PI, 81845; 5 μM), AO (13000; 15 μM) and NR (N-4638; 50 mM) dyes obtained from Sigma-Aldrich, USA.

Monitoring the size of the germovitellaria, in the presence of glucose (1 mM), started on Day 0 (D0) providing a reference control. Following twenty days (D20) of starvation and five days (D25) later, the organ regeneration was presented. On D20, one-time feeding (600 μg/mL yeast homogenate) was applied and followed-by five days of recovery. The treatment protocol was performed on a ‘one-housed rotifer’ (one animal/well) setup in a 96-well plate (Costar Corning Inc., CLS3595). There were 150 ± 30 rotifer/well in all fluorescence-related experiments. Investigated rotifer populations have adequate number of individuals to provide similar and equable size distribution.

The entities were photographed (Nikon DS100 camera, Japan) under inverted microscope (Leitz Labovert FS). The two separated germovitellarium were digitally colored in blue. The process of shrinkage with functional (egg production) regeneration capability was named ‘functionally reversible organ shrinkage’ (FROS).

To investigate (n = 5, well) the amount of protein in the animals, BisANS was applied (Datki et al., 2019) parallelly with detecting the total amount of nucleic acid, where PI was used (Mozes et al., 2011) after 10 min incubation and washing. There was no extraction, the labelling and measurements were directly performed on the animals. The extinction/emission were 405/520 nm for BisANS and 530/620 nm for PI, measured by a microplate reader (NOVOSstar, BMG, Germany).

In AVs-detection methods, we used a slightly modified version of Kang et al. (2018). The AO and the NR labellings were performed under the same conditions as the BisANS and PI applications. These two dyes were used in different wells, but the conditions and the number of animals were the same. The extinction/emission of AO was 480/620 nm in red and 480/520 nm in green. The wavelengths of NR were 540/630 nm in red and 450/590 nm in yellow. In order to inhibit the autoysis-related processes, ConA was added to the wells on D0. The percentage of normalized fluorescence intensity (NFIs%) was calculated from the ratio of fluorescence data divided by the number of rotifers in each well.

In the experiential monitoring of FROS, each data (n = 36; individual one-housed rotifer/well) sums the whole (bilateral) size of the germovitellaria in one individual. The organs were circled by using a freehand tool (allowing to create irregularly shaped selections) in ImageJ program (64-bit for Windows, (Collins, 2007). The scale bar was 20 μm.

To demonstrate the functional recovery of the reproductive organs, the number of laid eggs was counted after the regeneration phase (from D20 to D25). As a reference control, the treatment-free species-specific laid egg production was determined after 5 days in normal culturing conditions.

2.3. Statistics
Statistical analysis was performed with SPSS 23.0 (SPSS Inc, USA) using one-way ANOVA with Bonferroni post hoc test. The FROS index (FROS) was calculated by the following formula: FROS = A/B/A/C/D/E (germovitellaria size on A; D0; B; D20; C, D25; D, species-specific number of laid eggs under standard feeding; E, number of laid eggs on D25). The error bars represent the standard error of the mean (SEM). The different levels of significance are indicated as follows: p< \(\leq \) 0.05, p< \(\leq \) 0.01, p< \(\leq \) 0.001, p< \(\leq \) 0.0001 (*, significant difference from the untreated controls; #, significant difference from the same S-Aβ42-treated groups of the given rotifer species; n, significant difference from the D0 and D25 groups of the given rotifer species).

3. Results and discussion
The Aβ42 is a well-known neurotoxin, which is prone to form highly-resistant aggregates in an aquatic environment (Lin et al., 2019). The bdelloid rotifers are able to catabolize these aggregates, with no physiological damage (Datki et al., 2018). Our aims were to reveal the special role of Aβ42 in autocraticanism-related processes during a 25-day period (Fig. 1). To explore the possible sequence specificity of this molecule, we applied its scrambled version as control. The molecular content and weight of S-Aβ42 were the same as that in wild-type human form, with different order of amino acids (Datki et al., 2018; Bartus et al., 2019).

On D0, the reference germovitellaria of P. acuticornis (Fig. 2A) or A. vaga (Fig. 2D) can be seen. Their size and fine structure reduced after a 20-day long glucose-supplemented (ATP-source for autolytic processes) starvation (Fig. 2B and E). On D20, the animals were fed once, providing standard nutrient for the regeneration phase. The organs were
The ConA treatment hindered the observed autolysis-related vesicular acidification in both species on D20 compared to the untreated controls. Significant increase in autocatabolism-related vesicular acidification (Fig. 2G). In line with the above mentioned investigations, we detected shrinkage period with one-time drug treatment in glucose supplemented environment; D25 = Day 25, organ regeneration period with one-time standard feeding).

Fig. 1. Schematic protocol of the functionally reversible organ shrinkage in a micro-invertebrate system (D0 = Day 0, starting day; D20 = Day 20, organ shrinkage period with one-time drug treatment in glucose supplemented environment; D25 = Day 25, organ regeneration period with one-time standard feeding).

Fig. 2. Functionally reversible organ shrinkage (FROS) in P. acuticornis and A. vaga rotifers. The germovitellaria (digitally pseudo-colored in blue; scale bar: 20 μm) of P. acuticornis (PA; A–C) and A. vaga (AV; i–I) was monitored on D0 (A and D), D20 (B and E) and D25 (C and F). The FROS-related alterations are presented (G) with the protein- (green), nucleic acid- (red) and AVOs amount in PA (full columns) and AV (striped columns) rotifers. The D0 means 100 %. ConA was applied parallelly with all investigations. The error bars represent SEM. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance are p*** ≤ 0.001 and p**** ≤ 0.0001 (*, significant difference from all the groups) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

then rebuilt, and showed similar characteristics (Fig. 2C and F) to the reference ones. These results suggest that the organic shrinkage is reversible depending on the availability of food. To connect the autocatabolism-related processes with starvation-induced organ shrinkage (Puente et al., 2016), we applied ConA (Johnson et al., 2010) during the experiments. The amounts of protein and nucleic acid were measured parallelly with AVOs. Consequently, we found that the total protein decreased on D20, indicating that the animals catabolized it for survival. The ConA-administration inhibited these processes. The amounts of nucleic acid did not show any changes in either species (Fig. 2G). In line with the above mentioned investigations, we detected significant increase in autocatabolism-related vesicular acidification (Tan et al., 2018) in both species on D20 compared to the untreated reference values of D0. The ConA treatment hindered the observed alterations. On D25, there were no significant changes either in AO or in NR. The decrease of protein amount (associated with stable nucleic acid content) and the occurrence of AVOs under starvation show good conceptual correlation with organ shrinkage. These phenomena are adequate physiologic and experiential markers of autolytic metabolism in the aforementioned bdelloid rotifers.

The AJs are stigmatized as negative multifunctional agents (e.g. in the human brain) in academic literature. Based on this concept, the core question is how Aβ42 may influence the autocatabolism-related processes in our microinvertebrate species? In FROS-related investigations the Aβ42, S-Aβ42 or ConA were added to the treatment medium on D0 (Fig. 3A); therefore, these data served as references to the upcoming ones. In both rotifer species the organ shrinkage was less pronounced on D20 compared to the given untreated controls. Significantly higher regeneration was detected on D25 in Aβ42-treated groups compared to the other ones on D25. These results indicated that the native aggregate has a potential specific modulatory effect. Decrease of organ-size was lower in Aβ42-treated groups compared to the ones influenced by S-Aβ42. The same phenomenon was observed at the end of regeneration, where the animals showed significantly higher rate of recovery in the presence of Aβ42. These data showed that the attenuation of shrinkage via modulation is likely sequence-specific, since the order of amino acid is the only difference between the two types of AJs (Vadukul et al., 2017). On D20, ConA significantly inhibited the FROS in both species in a glucose-containing, but food-free environment. We have no knowledge about the treated animals eating the ConA itself, but the individuals remained alive in a good shape. On D25, the ConA had no effects on the monitored phenomenon.

In the FROS acronym, besides reversibility (‘R’) of organ shrinkage, ‘F’ stands for functionality, which refers to the remaining reproduction ability of rotifers. The measured parameter on D25 was the number of laid eggs, which was compared to the reference values (Fig. 3B). In all groups, the amount of eggs was low on D20 due to the minimal calorie intake. In the presence of AJs, the number of eggs significantly elevated in both species compared to the control and ConA peers; moreover, the Aβ42-modulated animals laid significantly higher number of eggs than the S-Aβ42-treated ones. Integration of egg-number-data into the time-dependent organ size alterations resulted a special formula, named...
Fig. 3. Modulation of the functionally reversible organ shrinkage (FROS) by aggregated Aβ42. The FROS was assessed in both P. acuticornis (PA) and A. vaga (AV) with applying Aβ42, S-Aβ42 or ConA. The germovitellaria were monitored on D0, D20 and D25. (A) The organ-size during FROS (A) and the amount of eggs on D20 and D25 (B) are presented. The D0 means 100 %. The error bars represent SEM. One-way ANOVA with Bonferroni post hoc test was used for statistical analysis, the levels of significance are p* 0.01 and p** 0.001 (*, significant difference from the same untreated control groups of the given rotifer species; †, significant difference from the same S-Aβ42-treated groups of the given rotifer species; ‡, significant difference from the D0 and D25 groups of the given rotifer species). FROS index (FROSᵢ; relative unit) is presented (C) in untreated control, Aβ42, S-Aβ42 and ConA groups.

FROS index, which is a representative unit for the current treatment agents. This index shows a linear correlation with the level of autocalabolism. The FROS, was positively lower in all agent-influenced experiments compared to the controls in both species (Fig. 3C). The FROSᵢ of native aggregates, in contrast to their scrambled version, demonstrated that the Aβ42 is not only a food source for rotifers, but it is also a potential regulator of their systemic metabolism. Both Philodina and Adineta species showed alterations with the same tendencies; therefore, these effects of Aβ42 are not limited to only one species.

Rotifers are extremely resistant to environmental alterations and they successfully adapt to different types and amounts of nutrients present in their natural habitat. The natural decomposition of organic materials is a process that results in the formation of precipitates and aggregates, which represent potential nutrients for rotifers (Wallace and Snell, 2010). The metabolic utilization of all these available organic material resources is their special property (Castro et al., 2005). Nobody has investigated before the in vivo catabolism of the Aβ as food sources or as potential autocalabolism-regulator for multicellular entities. The starvation-induced shrinkage of germovitellaria, with their regeneration and reproduction capability in these animals, is an adequate physiologic and experiential marker of autocalabolism, summarized by FROSᵢ. By applying these microinvertebrates, the hitherto unknown roles of Aβ42 were demonstrated, providing additional tools for exploring relations between neurotoxic aggregates and metabolism.

Conflicts of interest

No competing interests declared.

CRediT authorship contribution statement

Evelin Balazs: Validation, Investigation, Writing - review & editing, Project administration. Zita Galik-Olah: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration. Bence Galik: Formal analysis, Investigation, Resources, Writing - review & editing, Funding acquisition. Zsolt Bozso: Investigation, Writing - review & editing, Project administration. Janos Kalman: Resources, Writing - review & editing, Funding acquisition. Zsolt Datki: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Acknowledgements

The authors wish to thank to Anna Szentgyorgyi MA, a professional in English Foreign Language Teaching for proofreading the manuscript. This research was conducted within the project which has received funding from the European Union’s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement, Nr. 754432 and the Polish Ministry of Science and Higher Education, and from financial resources for science in 2018-2023 granted for the implementation of an international co-financed project and Developing scientific workshops of medical-, health sciences and pharmaceutical training (grant number: EFOP 3.6.3-VEKOP-16-2017-00009; Hungary).

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