A Novel Way of Amelioration of Amyloid Beta Induced Toxicity in *Caenorhabditis elegans*

Kopal Saharia^{a, c} Ranjeet Kumar^a Kuldeep Gupta^a Shrilekha Mishra^c
Jamuna R. Subramaniam^{a, b}

^aDepartment of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, ^bCenter for Preclinical and Translational Medicine Research, Central Research Facility, Sri Ramachandra University, Chennai, and

^cDepartment of Bioscience and Biotechnology, Banasthali University, Banasthali Vidyapith, India

Key Words

Alzheimer disease · Amyloid beta · Neuropeptide · FLP-11 · Acetylcholine

Abstract

Background: With an incidence of 1 in 85 persons above the age of 60 years succumbing to the disease, Alzheimer's disease (AD), has been predicted to create havoc globally. In spite of enormous efforts and exhaustive research, no cure is in sight. Hence, it is critical to unravel the mechanism of AD development/protection and identification of a cure soon. **Purpose:** This study is aimed at investigating the mechanism of reserpine action, which alleviates the toxicity of amyloid beta (A β) (AD-causing peptide) in *Caenorhabditis elegans* [1, 2]. **Methods:** Determination of alleviation of A β toxicity with reserpine manifested as reduction in progressive paralysis, in the background of GFP reporter driven by the promoter of the FMRFamide neuropeptide, FLP-11 (AD; P_{FLP-11}::GFP) and acetylcholine contribution through aldicarb (which inhibits acetylcholine esterase) treatment. **Results:** The most significant protection against A β toxicity was obtained in the back-

ground of P_{FLP-11}::GFP. This protection had 2 components. The promoter of FLP-11 with the reporter GFP, P_{FLP-11}::GFP, per se gave significant protection. Further reserpine treatment provided additional alleviation. Together they could almost eliminate A β toxicity. These 2 components of A β toxicity alleviation are dependent on acetylcholine levels, as an increase in acetylcholine by aldicarb treatment reduces the protective effect. **Conclusion:** A unique way to alleviate A β toxicity is reserpine treatment in combination with P_{FLP-11}::GFP. Reserpine should be evaluated as a potential drug in a pilot study in AD patients. Furthermore, identification of the mechanism of P_{FLP-11}::GFP-mediated reduction in A β toxicity is a potential pathway to develop therapeutics for AD.

© 2016 S. Karger AG, Basel

Introduction

Alzheimer's disease (AD) is a predominant neurodegenerative disease of epidemic proportion affecting people 50–60 years of age, leading to progressive cogni-

tive deficits, primarily dementia due to the formation of amyloid beta (A β) plaques and neurofibrillary tangles [3–5]. In addition, there are added complications because of the dysfunction of various neurotransmitter systems, making the management of AD patients a challenging task, personally, financially, and socially. Despite rigorous research, no potential drug is in sight for AD.

Models of AD (AD causing A β toxicity) in tractable organisms such as *Caenorhabditis elegans* [6] and mice [7, 8] are widely used. They aid in understanding the disease mechanism and in the development of therapeutics. The major advantages of *C. elegans* system [9] are: manifestation of in vivo A β aggregation/toxicity effect, amenable, preclinical drug screening, understanding the mechanism through forward and reverse genetics, presence of nervous system with 302 neurons of 118 classes [10], and neurotransmitter system similar to the mammalian system [11], such as acetylcholine, glutamate, GABA, etc., short lifespan in days, being a hermaphrodite can produce a large number of worms, short generation time of 3 days to develop from embryo to adult, high throughput screening of drugs, and less expensive [9]. The identified lead compounds can be evaluated in the transgenic (Tg) mouse models of AD. Though time consuming, the prominent deficit, dementia or loss of memory, reversal in the AD models in mice by identified lead compounds will take us closer to the potential drug candidates.

One of the commonly used well-established A β toxicity model in *C. elegans* (CL2006) is the unc-54 promoter-driven toxic A β 42-peptide expression in muscles [6]. The A β toxicity is manifested as progressive paralysis [6].

Various pharmaceutical, natural, supplementary therapies are being tried for AD [12, 13]. Some of the natural compounds—alkaloid-reserpine [2], components of red seaweed [14], lycopene [15], 18-alpha glycyrrhetic acid proteasome activator [16], traditional Chinese medicines such as Shengmai [17] and Liuwei Dihuang [18]—were reported to alleviate A β toxicity in the *C. elegans* model.

Of these, earlier studies have reported that reserpine, an FDA approved anti-hypertensive and anti-psychotic drug, which also happens to be an Ayurvedic drug called ‘sarpagandha’ from the roots of *Rauwolfia serpentina*, alleviates amyloid toxicity in the above described model in *C. elegans* [1, 2].

Reserpine reduces the plaque forming A β 42 significantly in the Tg2576 Tg mouse model of AD [19] and improves the working memory [20] in the 5XFAD AD Tg

mouse model [8]. Moreover, earlier studies have shown that reserpine modulates acetylcholine release in *C. elegans* [2]. However, the mechanism, genes, and pathways are not known.

Neuropeptides are a large family of short (approximately 5–20 amino acids), bioactive peptides acting on the nervous system. *C. elegans* possesses more than 100 different neuropeptides [21]. These are encoded as Pre-Pro- with several neuropeptides generated from a single protein by stepwise specific protease cleavage. Furthermore, a single type of receptor can bind diverse neuropeptides, making their study a great challenge. One class of neuropeptides is the short FMRFamide peptide. One among them is the neuropeptide, FLP-11 (K02G10.4, X chromosome) whose exact function is not known [21, 22]. FLP-11 expression is driven by its own ~2.8 kb promoter region [22].

In this study, we evaluate whether the ~2.8 kb promoter region of FLP-11 driving the expression of GFP can be a critical mediator of the alleviation of A β toxicity through acetylcholine and reserpine in *C. elegans*.

Methods

Strains

Bristol N2: wild-type, RB918: *acr-16* (*ok789*) V, NY2040: *ynls40V ynls40* (*P_{flp-11}::GFP*) were obtained from the *Caenorhabditis* Genetics Center, University of Minnesota. The Tg *C. elegans* line that expresses A β _{3–42} in the body wall muscles (CL2006) is a kind gift from C.D. Link [6] (University of Colorado, Boulder). All the *C. elegans* strains including CL2006 were maintained following standard protocols [23] at 20°C using NGM plates with *Escherichia coli* OP50 as food.

Reserpine Treatment

Worm strains were treated with reserpine in acetic acid as reported earlier [1, 24, 25] at a concentration of 60 μ M [1] at 20°C. Reserpine was spread on NGM plates with *E. coli* OP50 bacterial lawn.

Paralysis Assay

For the paralysis assay, the L4 stage worms were transferred to plates with or without reserpine and monitored for 12 days [26]. The worms were scored as paralyzed when they did not move even after repeated prodding. The assay was repeated at least thrice.

Aldicarb Assay

Aldicarb assays were carried out as reported, with minor modifications [2]. Aldicarb plates were prepared by adding aldicarb to the NGM media (final concentration 0.1 mM) before pouring to the plates. L4 stage worms were placed on the aldicarb plates and followed for paralysis. All assays were carried out minimum 3 to several times.

Fig. 1. Neuropeptide, FLP-11 promoter region, can ameliorate A β toxicity in AD model worms. **a** GFP expression pattern of P_{flp-11} in the Tg worm. **b** In Tg P_{flp-11}::GFP promoter reporter, the paralysis due to A β toxicity is delayed and further reduced by treatment with reserpine. CL2006 control – black solid (black circles); CL2006 treated – black dash (black triangle) AD;

P_{flp-11}::GFP control – red dash (open circle); AD; P_{flp-11}::GFP treated – red dot-dash (open triangle). (**c, d**) Percentage protection against A β toxicity in AD; P_{flp-11}::GFP worms. On treatment with reserpine, a drastic reduction in paralysis is observed in AD; P_{flp-11}::GFP. Number and p values are given in table 1.

Statistical Analysis

Mean $\pm$ SD and p values were calculated using the software SigmaPlot 10.0. Progressive paralysis was plotted using SigmaPlot 10.0.

Results

Abolition of A β Toxicity

Initial studies revealed that modulation of acetylcholine release contributes to reserpine-mediated alleviation of A β toxicity [2] in *C. elegans*. But the upstream or downstream mechanism is not known. Hence, the strain NY2040, which expresses the GFP reporter under the FLP-11 (P_{flp-11}) promoter [22] was screened. It was ob-

served that A β toxicity-induced paralysis was reduced to ~40% in the P_{flp-11}::GFP background (fig. 1b, c; table 1). Further reserpine treatment, nearly reduced it to 10% (fig. 1b, c; table 1). Overall, a dramatic protection was observed with a 60% reduction (fig. 1d) in A β toxicity. But, this did not reduce the A β aggregates (data not shown) determined by immunohistochemistry as reported earlier [1].

To identify the potential peptide molecule that could be transcribed from the FLP-11 promoter, an ORF analysis was carried out in the FLP-11 promoter region (~2.8 kb), which gave multiple ORFs. The authors attempted to amplify the ORF with specific primers by RT-PCR. But no PCR product was obtained, suggesting the absence of existence of any of these ORFs.

Fig. 2. a, b Reserpine could alleviate the aldicarb-induced paralysis in AD and AD; P_{flp-11}::GFP. CL2006 aldicarb – black solid (black circle); CL2006 AldRes – black dash (black triangle); AD; P_{flp-11}::GFP Ald – red solid (open circle); AD; flp-11 AldRes – red dash (open triangle). Number and p values are given in table 1.

Table 1. Reserpine-mediated alleviation of Aβ toxicity in P_{flp-11}::GFP

Strain	% paralysis, n		% protection	p value
	acetic acid	reserpine		
AD	72.07 (111)	40.9 (110)	31.2	<0.001
AD + aldicarb	93.84 (130)	73.6 (125)	20.2	
AD; P _{flp-11} ::GFP	39.64 (285)	10.56 (246)	29	
AD; P _{flp-11} ::GFP + aldicarb	76.10 (226)	41.49 (241)	34.6	

Acetylcholine Participates in the P_{flp-11}::GFP Mediated Aβ Toxicity Alleviation

Subsequently, it was determined whether P_{flp-11}::GFP effect is dependent on acetylcholine since the authors had previously reported that reserpine modulates acetylcholine to alleviate Aβ toxicity [2]. Chronic aldicarb treatment increases the acetylcholine concentration leading to the paralysis of the Aβ toxicity model worms (fig. 2; table 1). In contrast, in the AD; P_{flp-11}::GFP worms, there was a significant reduction (20%) of the paralysis of the worms with aldicarb (table 1; fig. 2). Though reserpine reduced paralysis by 34% in the presence of aldicarb (table 1; fig. 2), the overall reduction is only upto 30% and not to the near abolition observed with reserpine alone. This suggests that the endogenous and below endogenous (by reserpine) acetylcholine concentration in the neuromuscular junction strongly influences the alleviation of Aβ toxicity.

Discussion

Reserpine, the FDA approved anti-psychotic and anti-hypertensive drug, extends the lifespan of *C. elegans* [25] and alleviates Aβ toxicity [1, 2]. To understand the mechanism through which Aβ toxicity is alleviated, a novel approach was considered. Small molecule neurotransmitters and neuropeptides act in tandem. Unlike neurotransmitters, a huge number of neuropeptides (~100) exist in *C. elegans* [21]. Identifying and understanding their function is a huge challenge [21].

To determine the role of the FMRF amide neuropeptide in the alleviation of Aβ toxicity, the *C. elegans* strain, NY2040 [22], in which GFP reporter was driven by FLP-11 promoter, was screened with reserpine. The FLP-11 promoter drives the GFP expression mostly in different neurons of the head and the ventral cord (fig. 1a). More-

over, the GFP seems to be transported/secreted out as the ventral nerve cord or dorsal nerve cord, and the long axonal processes in the head region show GFP fluorescence. A β toxicity is reduced per se and drastically alleviated upon reserpine treatment (fig. 1; table 1). The in silico analysis could generate several ORFs from the promoter. But RT-PCR did not yield any PCR product. Therefore, it is unclear how the ~2.8 kb FLP-11 promoter specifically resulted in (i) GFP to be transported out of the neuronal cell bodies into the processes and (ii) the alleviation of A β toxicity.

Furthermore, the modulation of acetylcholine level (2) is necessary (fig. 2; table 1) to bring about the protective effect in the P_{flp-11}::GFP background, suggesting acetylcholine level modulation as the primary step in this context. Furthermore, mapping and identification of the specific region in the promoter could shed light on how the protective effect is brought about.

Thus, the FLP-11 promoter encoded information is able to bring about alleviation of A β toxicity. Near abolition of A β toxicity could be achieved in the P_{flp-11}::GFP background, proving that reserpine utilizes novel ways to alleviate A β -induced toxicity. Reserpine should be evaluated in a pilot study in AD patients as: (i) a drug to treat

AD is the need of the hour; (ii) reserpine being used as an anti-hypertensive drug; and (iii) more importantly, reserpine alleviates A β toxicity.

Acknowledgement

The authors gratefully acknowledge the financial support from the Department of Biotechnology, India, Dr. C.D. Link, University of Colorado, Boulder, USA for CL2006 strain and *C. elegans* Genetics Center, Minnesota, USA supported by National Institutes of Health for the NY2040 strain.

Authorship Contribution

K.S., R.K. and K.G. (few) carried out the experiments. J.R.S. designed, supervised, analyzed the data and wrote the manuscript. S.M. (minor) contributed to the design and writing of the manuscript.

Disclosure Statement

The authors declare no conflict of interest. Ethical approval is not required. This article complies with International Committee of Medical Journal Editor's standard requirements for manuscript.

References

- Arya U, Dwivedi H, Subramaniam JR: Reserpine ameliorates abeta toxicity in the Alzheimer's disease model in *Caenorhabditis elegans*. *Exp Gerontol* 2009;44:462–466.
- Saharia K, Arya U, Kumar R, Sahu R, Das CK, Gupta K, Dwivedi H, Subramaniam JR: Reserpine modulates neurotransmitter release to extend lifespan and alleviate age-dependent A β proteotoxicity in *Caenorhabditis elegans*. *Exp Gerontol* 2012;47:188–197.
- Glenner GG, Wong CW: Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun* 1984;120:885–890.
- Selkoe DJ: Alzheimer's disease: genes, proteins, and therapy. *Physiol Rev* 2001;81:741–766.
- López OL, DeKosky ST: [Neuropathology of Alzheimer's disease and mild cognitive impairment]. *Rev Neurol* 2003;37:155–163.
- Link CD: Expression of human beta-amyloid peptide in transgenic *Caenorhabditis elegans*. *Proc Natl Acad Sci U S A* 1995;92:9368–9372.
- Borchelt DR, Ratovitski T, van Lare J, Lee MK, Gonzales V, Jenkins NA, Copeland NG, Price DL, Sisodia SS: Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* 1997;19:939–945.
- Oakley H, Cole SL, Logan S, Maus E, Shao P, Craft J, Guillozet-Bongaarts A, Ohno M, Distenhof J, Van Eldik L, Berry R, Vassar R: Intraneuronal beta-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *J Neurosci* 2006;26:10129–10140.
- Arya U, Das CK, Subramaniam JR: *Caenorhabditis elegans* for preclinical drug discovery. *Curr Sci* 2010;99:1669–1680.
- Hobert O: Neurogenesis in the Nematode *Caenorhabditis elegans*, ed. The *C. elegans* Research Community, WormBook, 2010.
- Jorgensen EM: Ed. *Neurobiology of Behavior*, ed. The *C. elegans* Research Community, WormBook, 2010.
- Wollen KA: Alzheimer's disease: the pros and cons of pharmaceutical, nutritional, botanical, and stimulatory therapies, with a discussion of treatment strategies from the perspective of patients and practitioners. *Altern Med Rev* 2010;15:223–244.
- Savonenko AV, Melnikova T, Hiatt A, Li T, Worley PF, Troncoso JC, Wong PC, Price DL: Alzheimer's therapeutics: translation of preclinical science to clinical drug development. *Neuropsychopharmacology* 2012;37:261–277.
- Sangha JS, Wally O, Banskota AH, Stefanova R, Hafting JT, Critchley AT, Prithiviraj B: A cultivated form of a red seaweed (*Chondrus crispus*), suppresses β -amyloid-induced paralysis in *Caenorhabditis elegans*. *Mar Drugs* 2015;13:6407–6424.
- Chen W, Mao L, Xing H, Xu L, Fu X, Huang L, Huang D, Pu Z, Li Q: Lycopene attenuates A β 1–42 secretion and its toxicity in human cell and *Caenorhabditis elegans* models of Alzheimer disease. *Neurosci Lett* 2015;608:28–33.
- Papaevgeniou N, Sakellari M, Jha S, Tavernarakis N, Holmberg CI, Gonos ES, Chondrogianni N: 18 α -Glycyrrhetic acid proteasome activator decelerates aging and Alzheimer's disease progression in *Caenorhabditis elegans* and neuronal cultures. *Antioxid Redox Signal* 2016, Epub ahead of print.
- Zhang W, Zhi D, Ren H, Wang D, Wang X, Zhang Z, Fei D, Zhu H, Li H: Shengmai formula ameliorates pathological characteristics in AD *C. elegans*. *Cell Mol Neurobiol* 2016, Epub ahead of print.
- Sangha JS, Sun X, Wally OS, Zhang K, Ji X, Wang Z, Wang Y, Zidichouski J, Prithiviraj B, Zhang J: Liuwei Dihuang (LWDH), a traditional Chinese medicinal formula, protects against β -amyloid toxicity in transgenic *Caenorhabditis elegans*. *PLoS One* 2012;7:e43990.

- 19 Go J, Cho SI, Kim JI, Lee YJ, Kwak MH, Koh EK, Song SH, Sung JE: Effect of reserpine on the behavioral defects, A β -42 deposition and NGF metabolism in Tg2576 transgenic mouse model for Alzheimer's disease. *J Life Sci* 2013; 23:812–824.
- 20 Vasantharaja R, Kumar A, Kumar A, Subramaniam JR: Reserpine improves working memory. *J Behav Brain Sci* 2016;6:107–112.
- 21 Li C, Kim K: Neuropeptides, ed. The C. Elegans Research Community, WormBook, 2008. <http://www.wormbook.org>.
- 22 Kim K, Li C: Expression and regulation of an FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *J Comp Neurol* 2004;475:540–550.
- 23 Brenner S: The genetics of *Caenorhabditis elegans*. *Genetics* 1974;77:71–94.
- 24 Duerr JS, Frisby DL, Gaskin J, Duke A, Asermely K, Huddleston D, Eiden LE, Rand JB: The cat-1 gene of *Caenorhabditis elegans* encodes a vesicular monoamine transporter required for specific monoamine-dependent behaviors. *J Neurosci* 1999;19:72–84.
- 25 Srivastava D, Arya U, SoundaraRajan T, Dwivedi H, Kumar S, Subramaniam JR: Reserpine can confer stress tolerance and lifespan extension in the nematode *C. elegans*. *Biogerontology* 2008;9:309–316.
- 26 Cohen E, Bieschke J, Perciavalle RM, Kelly JW, Dillin A: Opposing activities protect against age-onset proteotoxicity. *Science* 2006;313:1604–1610.