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Reserpine modulates neurotransmitter release to extend lifespan and alleviate age-dependent Aβ proteotoxicity in *Caenorhabditis elegans*

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ABSTRACT

Aging is a debilitating process often associated with chronic diseases such as diabetes, cardiovascular and neurodegenerative diseases like Alzheimer's disease (AD). AD occurs at a very high incidence posing a huge burden to the society. Model organisms such as *C. elegans* become essential to understand aging or lifespan extension – the etiology, molecular mechanism and identification of new drugs against age associated diseases. The AD model, manifesting Aβ proteotoxicity, in *C. elegans* is well established and has provided valuable insights. Earlier, we have reported that Reserpine, an FDA-approved antihypertensive drug, increases *C. elegans* lifespan with a high quality of life and ameliorates Aβ toxicity in *C. elegans*. But reserpine does not seem to act through the known lifespan extension pathways or inhibition of its known target, vesicular monoamine transporter, VMAT. Reserpine's mode of action and the pathways it activates are not known. Here, we have evaluated the presynaptic neurotransmitter(s) release pathway and identified acetylcholine (ACh) as the crucial player for reserpine's action. The corroborating evidences are: i) lack of lifespan extension in the ACh loss of function (hypomorphic) – synthesis (*cha-1*) and transport (*unc-17*) mutants; ii) mitigation of chronic aldicarb effect; iii) lifespan extension in dopamine (*cat-2*) and dopamine and serotonin (*bas-1*) biosynthetic mutants; iv) no rescue from exogenous serotonin induced paralysis in the AD model worms; upon reserpine treatment. Thus, modulation of acetylcholine is essential for reserpine's action.

1. Introduction

The world's aging population is increasing at an alarming rate and are being burdened with debilitating chronic-diseases such as diabetes, cardiovascular and neurodegenerative diseases like Alzheimer's disease (AD). Understanding their mechanisms and finding therapeutics is crucial for prolonged healthy living. However, the aging and longevity mechanism of an organism is poorly understood. The lifespan ranges from few days to several decades in metazoans. Since the discovery of single gene mutations in the insulin/IGF-1 signaling (IIS) (Friedman and Johnson, 1988; Kenyon et al., 1993; Kimura et al., 1997) that alters lifespan in *C. elegans*, more than 100 divergent genes have been shown to modulate longevity. These include genes that are involved in (Friedman and Johnson, 1988) insulin signalling (Kenyon et al., 1993; Kimura et al., 1997), calorie restriction (Lakowski and Hekimi, 1998; Wood et al., 2004), response to stress, chemosensation and reproductive signaling (Kenyon, 2010). Of these, calorie restriction and IIS pathways are shown to be conserved across species. Japanese and Jewish Centenarians show variants of IGF-1 receptor (Kenyon, 2010) validating the importance of insulin/IGF-1 signaling in lifespan extension. Similarly, calorie restriction works from yeast to mice. Overall, this makes the *C. elegans* paradigm a worthwhile and amenable approach.

In the age associated neurodegenerative or protein misfolding diseases (NDs) such as Alzheimer disease (AD), and Huntington disease (HD) multiple pathways become perturbed including signaling involved in oxidative stress (Trushina and McMurray, 2007), mitochondrial dysfunction (Trushina and McMurray, 2007), axonal transport defects (Gunawardena et al., 2003) and microglial activation (Minghetti et al., 2005; Bruijn et al., 2004). In addition to the presence of protein aggregates (Selkoe, 2003; Kopito and Ron, 2000), impaired or excessive neurotransmission is implicated as a major contributor to NDs (Garcia et al., 2007). Importantly, in AD, manifested as loss of cognitive behaviour, reduced cholinergic neurotransmission due to degeneration of cholinergic neurons in the brain is a major dysfunction (Liu et al., 2007; Tabet, 2006).

To understand the etiology, molecular mechanism and identification of new therapeutics for NDs like HD and AD, studies have been conducted in the well established models including *C. elegans*. The toxic protein is expressed in the muscles of *C. elegans* (Link, 1995). The proteotoxicity builds up and manifests as progressive paralysis. In the AD model, intracellular Aβ accumulation (Zhang et al., 2004) in the muscle represents the more toxic early event preceding extracellular Aβ plaque formation. In addition, muscle is the postsynaptic

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target for several neurotransmitters, hence, a relevant system to assess the proteotoxicity as a manifested phenotype. Moreover, the neuromuscular junction is the equivalent of synapse and increased neurotransmission is reported to accelerate progressive paralysis in the HD model worms (Garcia et al., 2007).

The major cause of Alzheimer's disease is Amyloid beta (Aβ) toxicity due to abnormal processing of the amyloid precursor protein, APP, leading to the formation of toxic Beta Amyloid (1-42) peptide, which forms plagues in the human brain and initiate cascade of events leading to AD pathology in humans. In *C. elegans*, there is a well established transgenic Aβ toxicity model, CL2006 (Link, 1995). In this model, the toxic human Aβ (1–42) peptide is constitutively expressed in the body wall muscles under the control of *unc-54* promoter, which leads to manifestation of Aβ protein deposits (Arya et al., 2009) and $A\beta_{1-42}$ toxicity induced progressive paralysis (Link, 1995). A positive correlation between lifespan extension and protection against Aβ (Cohen et al., 2006) or polyQ toxicity (Hsu et al., 2003) in *C. elegans* has been reported.

The FDA approved antihypertensive drug, reserpine, extends lifespan (Srivastava et al., 2008) and ameliorates the proteotoxic effect of Aβ (Arya et al., 2009) in *C. elegans*. The *r*eserpine *m*ediated *l*ifespan *e*xtension (RMLE) is independent of *daf-16* dependent reduced IIS pathway or *eat-2* dependent calorie restriction (Srivastava et al., 2008). The alleviation of Aβ toxicity is not because of reduction in the expression of Aβ mRNA levels or the gross Aβ aggregates (Arya et al., 2009). The mechanism by which reserpine brings about lifespan extension or decrease the proteotoxic effect of Aβ remains elusive. In this study, we identify that modulation of acetylcholine release is crucial for reserpine's action.

2. Materials and methods

2.1. Strains

Bristol N2: wild-type, CD1111: *cat-1(e1111)X*, CB1112: *cat-2(e1112) II*, MT7988: *bas-1(ad446)III*, NM440: *unc-104(e1265) II-jsls1*(SNB::GFP), CB246: *unc-64(e246)III*, CF1038: *daf-16(mu86) I*, NM1968: *slo-1(js379)V*, PR1152: *cha-1*(*e1152*) , CB933: *unc-17*(*e245*), DH404: *unc-63* (*b404*)*I* and RB918: *acr-16* (*ok789*) were obtained from the *Caenorhabditis* Genetics Center (CGC), University of Minnesota. The transgenic *C. elegans* line constitutively expressing $A\beta_{1-42}$ in the body wall muscles (CL2006) is a kind gift from C.D. Link (Link, 1995) (University of Colorado, Boulder). All the *C. elegans* strains including CL2006 were grown on NGM plates with OP50 *E. coli* lawn as food at 20 °C following standard protocols (Brenner, 1974).

2.2. Reserpine treatment

Reserpine treatment was carried out as reported earlier (Srivastava et al., 2008; Arya et al., 2009; Duerr et al., 1999) at 25 °C. Briefly, reserpine in acetic acid at a final concentration of 60 μM (Arya et al., 2009) was spread on the *E. coli* OP50 bacterial lawn containing NGM agar plates and used.

2.3. Lifespan, locomotion and pharyngeal pumping assays

All these assays were carried out as described in Srivastava et al., 2008). Around 30–40 embryos or L4 stage worms were transferred to 35 mm plate with or without reserpine and maintained at 25 °C. The lifespan assay was based on the Libina et al. (2003) procedure with minor modifications. In the AD model, synchronous worms (CL2006) at L4 stage, grown on OP50 *E. coli* lawn on NGM agar were shifted to the control and reserpine plates, maintained at 20 °C and followed for all the assays. After the worms reached the young adult stage, they were shifted everyday to fresh plates till the end of reproduction and then on shifted once in every two or three days. The lifespan was counted from L4 onwards. The L4 stage day was counted as day 0. The assay had been repeated several times with 30–40 worms per plate. The worms which crawled off the plates were excluded. The death was determined as when the worms did not move at all when prodded with the platinum wire. Locomotion and pharyngeal pumping assays were carried out at various ages as described (Srivastava et al., 2008; Huang et al., 2004). The locomotion and pharyngeal pumping assays were carried out as 20 worms per set and determined thrice independently. In all assays, control is vehicle (acetic acid) instead of reserpine.

2.4. Paralysis assay

For the paralysis assay, the worms were monitored everyday and were considered paralysed when they did not move even after repeated prodding. The paralysis was followed till day 12 of adulthood as described (Cohen et al., 2006).

2.5. Aldicarb/levamisole assay

The standard aldicarb (Rand and Russell, 1985; Opperman and Chang, 1991; Mahoney et al., 2006; Nguyen et al., 1995)/levamisole (Lewis et al., 1980) assay was carried out with slight modifications. Aldicarb (1 mM)/levamisole (200 μM) plates were prepared ½ h before the assay, by adding to M9 buffer and spreading on the OP50 containing NGM agar plate. For these assays, first the L4 worms were placed on control/reserpine plates and used when they were young adults. The young adult worms were placed on the aldicarb/levamisole plates and followed for paralysis every 30 min. for 5 h. The worms were considered paralysed when they did not move in response to repeated prodding. For chronic aldicarb treatment the plates were prepared by adding aldicarb (final concentration 0.1 mM) (Nguyen et al., 1995) to the agar before pouring. Chronic aldicarb/reserpine treatment was from embryo stage till death except in the AD model worms where the assay was started at L4 stage. All assays were carried out minimum three to several times.

2.6. M^{-}NGM medium for aldicarb/reserpine assays

The medium was identical to NGM except that $MgSO_4$, Calcium Chloride and Potassium Phosphate buffer was replaced with 1 M Tris pH 7.0 alone. Otherwise the composition and protocol was the same as described (Brenner, 1974). The assay was repeated at least three to four times.

2.7. Exogenous serotonin sensitivity assay

4 days old, Aβ expressing CL2006 worms with and without reserpine treatment (from L4 onwards) were placed in 5 mg/ml serotonin in Dempsey et al. (2005) and scored for paralysis every 5 min till all the worms got paralyzed approximately within 30 min. The assay was repeated minimum three times.

2.8. Protein extraction and immunoblotting

The *C. elegans* extract for immunoblotting was prepared as described in Dr. Richard Morimoto (Northwestern University, Chicago, IL, USA) lab protocol. The SDS-PAGE gel running and western was performed according to standard methods (Sambrook, 2001). The Aβ species were detected using 6E10 antibody (anti mouse, Covance) as described (Wu and Luo, 2005). Briefly, CL2006 control and reserpine treated worms were washed with 1X PBS buffer and spun at 2000 rpm for 10 min. The worm pellet was then suspended in 1X PBS containing 1X protease inhibitor cocktail. It was then homogenized, sonicated and dissolved in 2% SDS containing sample buffer, heated with 5% β-mercaptoethanol and loaded on tricine gel. The