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Ameliorative influence of atorvastatin in transgenic Drosophila Melanogaster model of neurodegenerative diseases

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ABSTRACT

Background: The common features in the pathogenesis of Alzheimer's disease (AD) and Parkinson disease (PD) (two most common neurodegenerative diseases) are chronic and progressive aggregation and accumulation of misfolded proteins (amyloid-beta and tau proteins in AD as well as α-synuclein in PD) leading to the destruction of vulnerable neurons and synaptic connections and ultimately neuronal cell death brain mass loss. Despite our knowledge of the molecular mechanisms implicated in AD and PD pathogenesis and primary target of therapeutic intervention being the misfolded protein aggregates, no efficient treatments are available. The fruit fly, *Drosophila* melanogaster (*Drosophila)*, is a valuable model organism for neurodegenerative disease owing to its short lifespan and plethora of genetic tools for exquisite targeted manipulation of the genome. Thus, in this study the protective action of atorvastatin on genetic model of AD and PD in mice.

Methods: To model PD and AD in *Drosophila*, the bipartite system of GAL4 transcriptional activator was placed under a cell-type specific promoter; embryonic lethal abnormal visual system-GAL4 (ELAV-GAL4) or dopa decarboxylase (Ddc-GAL4) for expression of amyloid-beta (Aβ42) or α-synuclein, respectively, under the control of the upstream activating sequence (UAS) in *Drosophila*. The flies were was either maintained on media supplemented with vehicle or atorvastatin (85, 170 or 340μM; HMG-CoA reductase inhibitor – antihyperlipidemic drug). The effect of treatments on larva motility, climbing activity, fecundity and lifespan were recorded.

Results: Supplementation of fly media with different concentration of atorvastatin ameliorated the deficits in larva motility and climbing activity. Moreover, supplementation of fly media with atorvastatin prolonged the survival of drosophila but atorvastatin (384μM) reduced fecundity.

Conclusion: Findings from this study showed that atorvastatin improved spontaneous motor activity and prolonged lifespan in *Drosophila* possibly through reduction of misfolded protein aggregates.

1. Introduction

The whole world is witnessing a significant increase in lifespan and aging population, but neurodegenerative diseases have emerged as a critical health concern to both caregivers and poses a huge burden on the society[1]. The brain and spinal cord are extraordinarily complex, consisting of a highly organized network of neuronal and supporting cells that communicate in a highly specialized manner. One approach to tackling problems of such complexity is to address the scientific questions in simpler, yet analogous systems. The fruit fly, *Drosophila melanogaster* (*Drosophila*) is a valuable model organism for human diseases with over half of its genes have counterparts in rodents or human associated with neurodegeneration, enabling many major drug discovery and molecular markers. The plethora of genetic tools available in *Drosophila* allows for exquisite targeted manipulation of the genome as a results of its relatively short lifespan. Hence, complex questions of brain function can be addressed more rapidly than in other model organisms, such as the mouse and rats[1]. In this study, we evaluated the potential of atorvastatin in *Drosophila* models of Alzheimer's disease (AD) and Parkinson's disease (PD using

the bipartite GAL4-Upstream activating sequence (UAS) system. The GAL4 system allow expression of exogenous genes in distinct, small subsets of the adult nervous system. GAL4 is a yeast transcription protein that regulates genes induced by galactose to control both spatial and temporal expressions of target genes at a specific cell type (neurons) or time (adult)[1]. It binds to 17 base pair sites known as the upstream activating sequences (UAS) to activate the GAL10 and GAL1 target genes[2]. In this study, the GAL4 transcriptional activator was placed under embryonic lethal abnormal vision (*ELAV*) or dopa decarboxylase (*Ddc*) for expression pan-neuronally and dopaminergic neurons, respectively, to enable spatial or temporal control of transgene (Aβ42 or α-syn) up-regulation by UAS[2]. The ability to make targeted gene manipulations is crucial to the investigation of biological phenomena and is the hallmark of modern genetics[3]. Various inducible-gene expression systems are available in *Drosophila,* the majority of which require the use of two distinct transgenic constructs containing, respectively; an effector gene (EG) and a transactivator (TA) gene[4]. The expression of the effector gene is controlled by a promoter, which is regulated by the transactivator. Thus, the expression pattern of the EG is indirectly controlled by the nature of the promoter directing TA expression. The majority of the GAL4 drivers available are solely useable during the pre-adult stages because the expression pattern of GAL4 across the life span of the adult is generally ignored, despite the fact that both the magnitude and localization of expression can change with age[5,6].

Atorvastatin is an HMG-CoA reductase inhibitor used to treat hypercholesterolemic conditions associated with hypertension[7]. Atorvastatin has been shown to reduce early brain edema and neuronal death after subarachnoid hemorrhage[8]. Moreover, atorvastatin decreased striatal neurodegeneration induced by 3-nitropropionic acid (3NP) via attenuation of inducible nitric oxide synthase and c-Jun levels as well as activation of extracellular signal-regulated kinase and Akt[9]. Thus, in this study, we evaluated the protective action of atorvastatin on genetic model of AD and PD in mice.

2. Materials and method

2.1 Reagents and Drug: Methyl-p-hydroxyl-benzoate and propionic acid obtained from LOBA Chemical Laboratory, Mumbai, India; Agar from Himedia Laboratories Pvt. Ltd., Mumbai, India; Orthophosphoric acid from Thermo Fischer Scientific India Pvt. Ltd., Mumbai, India; atorvastatin (Codix Pharma Limited, Lagos, Nigeria), yeast obtained from STK Industry Ltd, China, malt from Sigma Aldrich (St. Loiuis MO, USA), corn flour obtained from Latyf Food and Beverages Ventures limited, Ogun State, Nigeria.

2.2 Fly Stocks and Culture

Wild type W118, *ELAV-gal4/FM*, UAS-Aβ42/TM3, *Ddc-Gal4/TM3, UAS-α-synuclein/CyO,* and Caxton S (Cs) flies were obtained from Dr. Rakesh Mishra *Drosophila* Laboratory, Centre for Cellular and Molecular Biology, Hydrabad. India. Flies strains were maintained on standard corn flour, malt, yeast, sugar, orthophosphoric acid, propionic acid and agar at 26±2°C with 60-75% relative humidity and a 12h light/dark cycle.

2.3 Construction of Aβ42 and *α-synuclein* transgenic *Drosophila* models

To construct the *UAS-Aβ42/TM3* or *UAS-α-synuclein/CyO* transgenic *Drosophila,* respectively, with Elav-GAL4/FM or *Ddc-Gal4/TM3* genetic background. Virgin females of Elav-GAL4/FM or *Ddc-Gal4/TM3* were mated with UAS-Aβ42 or *UAS-α-synuclein* transgenic *Drosophila*, respectively. *Elav-Aβ42* and *Ddc-α-synuclein Drosophila* were collected within 48 hours for lifespan or behavioural assays[10].

2.4 Fecundity assay

Fecundity, also known as *reproductive rate* is a measure of the number of offspring produced by an organism over time. Assessment of fecundity allows monitoring of the effect of atorvastatin on reproductive activity. Parameters recorded include, the number of eggs laid, number of dark pupa and the number of flies that emerged from the dark pupa through the course of the study.

Cs fly strain was used; male and female mated at a ratio of 1:2 (5 males to 10 females) n=3 vials per group

2.5 Larval Motility

Third instar larvae were removed from their individual medium, after a quick wash in distilled water, twenty larvae from each group, comprising media only, media supplemented with atorvastatin (85, 170 or 340μM) (n=3 vials) were gently placed on 2%$^{w}/_{v}$ agar slabs in 245mm×245mm square petri-dishes using a small paint brush; all on the surface of a 2B sheet with partitioned square boxes measuring 0.5cm by 0.5cm. The larvae was allowed to acclimatize for one minute and the number on line crosses in one minute was recorded.

2.6 Longevity assay

Individual aging manifests at the population level as an increase in age - dependent mortality and this assay allows monitoring of the effect of atorvastatin on *Drosophila* lifespan. Briefly, Lifespan was measured in once mated female or male flies kept at 20/vial (3 vials per group) on standard food or atorvastatin supplemented; transferred to new food three times a week. To determine whether the life length of *Drosophila* was shortened, the survival rate curve was done according to the average number of survival flies[11].

2.7 Climbing assay

Newly enclosed *Elav-Aβ42* and *Ddc- α-synuclein* or Cs *Drosophila* were randomly divided into 4 groups (20 flies per vial, n=3 vials per group) comprising media only, media supplemented with atorvastatin (85, 170 or 340μM). *Drosophila* creeping tubes were transparent plastic pipes with 15 cm in height, 2 cm in diameter, and 0.1 cm in thickness. The test was taken on days 7, 14, and 21 28 post-eclosion. Each strain was divided into 4 groups and each group of 20 *Drosophila*. When the test was made, gently shake the tube body so that the *Drosophila* would drop into the bottom of the tubes. Due to the characteristics of negative geotaxis, the fruit flies will leave the bottom of the tube to crawl. Record the numbers of *Drosophila* at different positions under 5 cm in 5 seconds. Each group was tested 5 times, and the test interval was 5 minutes[10].

2.8 Statistical Analysis

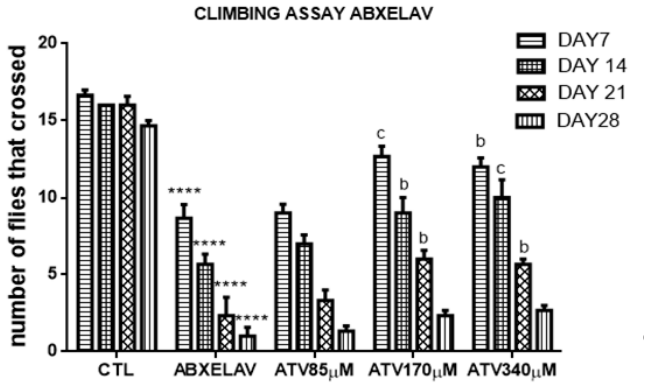
Statistical analyses were performed using GraphPad™ Prism 7 software (GraphPad Software, Inc.) Results were expressed as the means±SEM.

3. RESULTS

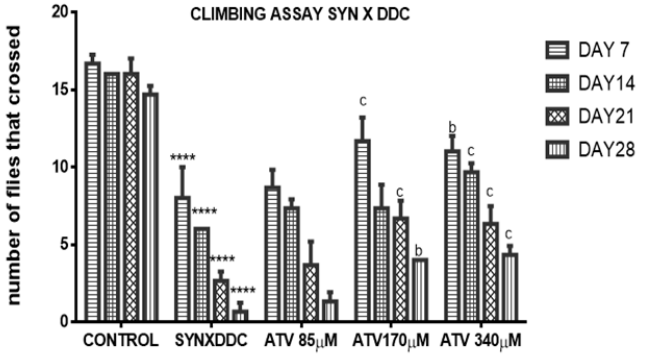
3.1 Fly climbing assay

Climbing assay was conducted periodically for 28 days to assess neuroprotective effect of atorvastatin in elav>Aβ flies. One-way ANOVA (P<0.05) showed a significant decrease in climbing ability of elav>Aβ flies cultured on normal feed media compared to normal Cs control; indicative of AD pathology. Atorvastatin 170 and 340μM showed significant increase in climbing ability on Day 7, 14 and 21 when compared elav>Aβ flies *Drosophila* on normal media. However post hoc analysis (P<0.05) showed no significant change in climbing ability at atorvastatin 85μM treatment (Figure 1).

In another experiment, post hoc analysis (P<0.05) showed significant decrease in climbing ability of Ddc-*α-synuclein* flies compared to the normal Cs control suggestive of motor deficit. However, maintenance of Ddc- *α-synuclein* transgenic *Drosophila* on atorvastatin 170 and 340μM improves climbing activity on days 7, 21 and 28 compared with Ddc-*α-synuclein* transgenic flies maintained on unsupplemented media. Conversely, atorvastatin at 85μM produced no significant change in climbing activity (Fig. 2).



Values are expressed as means ± SEM. $P < 0.0001$ versus control; $^{b}P < 0.01$, $^{c}P < 0.001$ versus elav>$A\beta_{42}$. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison test. Note: CTL = Cs fly

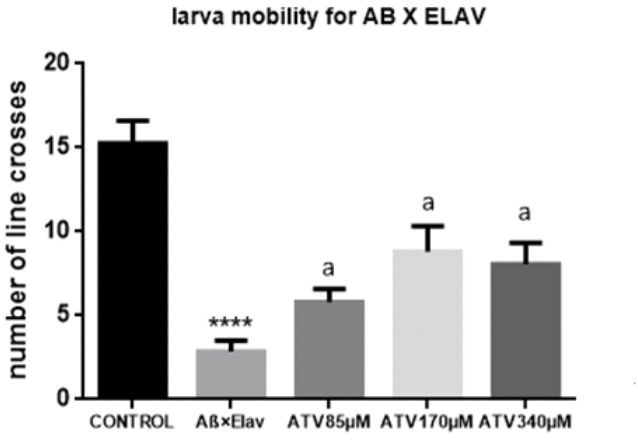


expressed as means ± SEM. ****$P < 0.0001$ versus control, $P < 0.001$, $^{b}P<0.01$ versus alpha synuclein flies. Data were analyzed by two-way ANOVA followed by Tukey's multiple comparison test. Note: CTL= Cs fly

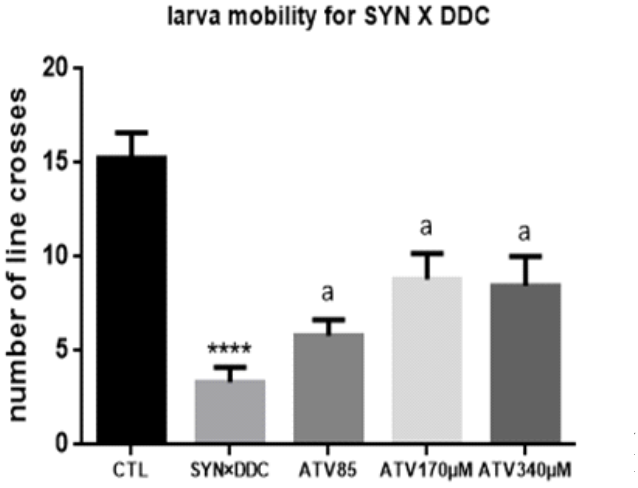
3.2 Larva Mobility

Larva assay was carried in third instar to assess the effect of atorvastatin on locomotor activity or chemotaxis. Post hoc analysis showed significant (P<0.05) decrease in number of line crosses (indicative of locomotive dysfunction) in the Elav>Aβ42 expressing flies when compared to control Cs flies. However, Elav>Aβ42 maintained on media supplemented with atorvastatin (85,170 and 340μM) produced significant increase in larva motility compared to Elav>Aβ42 transgenic larva on media only (Figure 3).

In another study, *post hoc* analysis showed that Ddc- α-syn overexpression caused significant decrease in number of line crosses in larva compared to that of Cs control. However, the supplementation of fly media with atorvastatin (85, 170 and 340μM) caused significant increase in larva mobility when compared with Ddc- *α-synuclein* (PD model) cultured in normal feed media (Fig. 4).



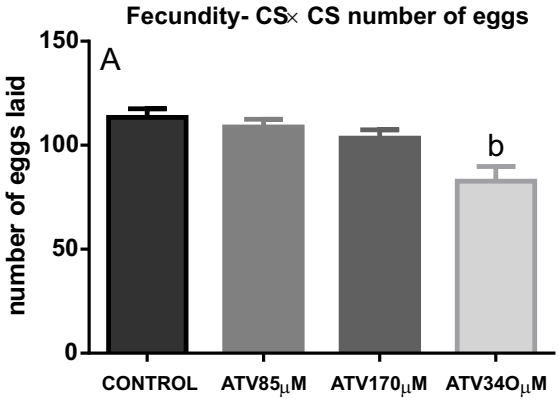
control, [a]P < 0.0001 versus
Aβ × elav control.

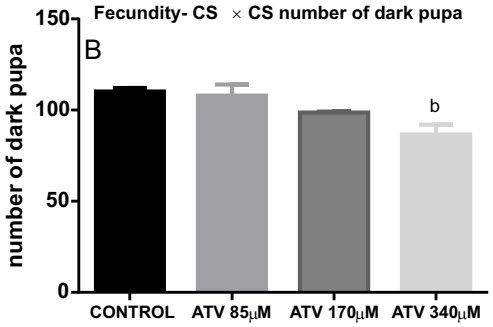


control.

3.3 Fecundity

Supplementation of fly media with atorvastatin 340µM but not 85 and 170 µM significantly reduced number of egg laid (Fig. 5a), dark pupa (Fig. 5b) and number of adult flies that eclosed (Fig. 5c) when compared to control normal media.





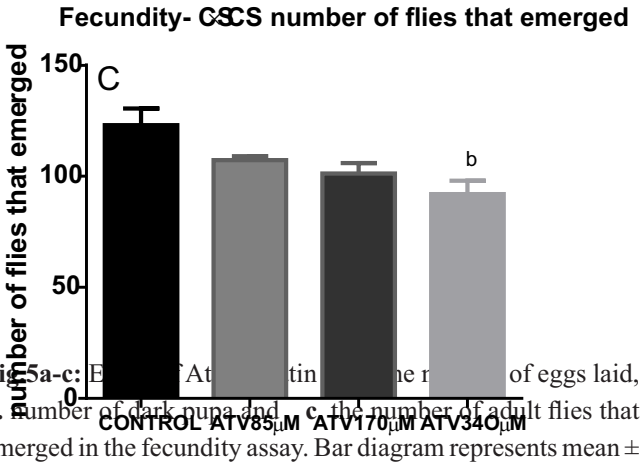
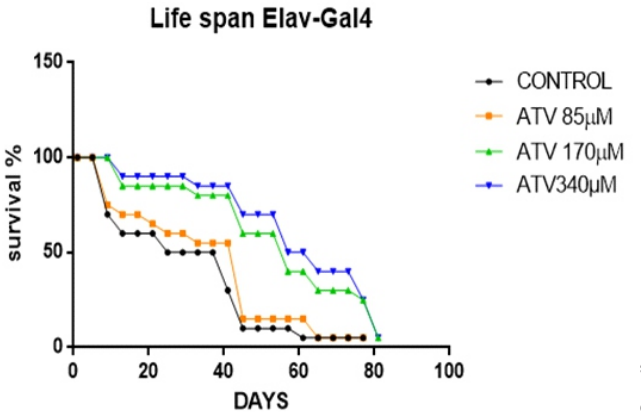


Fig. 5a-c: E[illegible] At[illegible]tin [illegible]e r[illegible] of eggs laid, **b.** number of dark pupa and **c.** the number of adult flies that emerged in the fecundity assay. Bar diagram represents mean ± SD; [b] P < 0.05 versus control. One-way ANOVA was used to analyze followed by Tukey's *post hoc* multiple comparison test.

3.4 Lifespan Assay

Figure 6 shows the effect of atorvastatin on survival of *Elav-GAL4* fly strain. Elav-Aβ42 transgenic group showed significant decrease in lifespan when compared to normal control. However, the supplementation of the fly media with atorvastatin caused dose-dependent increase in lifespan when compared with Elav-Aβ42 transgenic *Drosophila* with peak effect at 340µM.



was plotted against lifespan duration in days and analysis was done using two-way ANOVA followed by Dunnett's *post-hoc* multiple comparison test.

4. Discussion

Findings from this study showed that *Elav-Aβ* and *DDC-α-synuclein* transgenic *Drosophila* displayed motor deficits (in larva and adults) and shorten lifespan. However, supplementation of *Drosophila* media with atorvastatin reversed the deficits in spontaneous motor activity (both geotaxis and chemotaxis) and prolonged the lifespan of both the PD and AD transgenic *Drosophila.* Conversely, caution should be taken when administering atorvastatin, as the highest concentration used in this study, though beneficial but reduced fecundity in wild type Cs flies, including decrease in egg laid, number of third instar larva and eclosed adult flies.

Drosophila has been a widely used model in evolutionary biology because of its ease-of-use and short generation time. Of central interest has been the characterization of life history traits (e.g., life span, fecundity (number of egg laid; widely used proxy for fitness), mating competitiveness) under laboratory conditions are closely linked to fitness[12,13]. In this study, exposure of Cs Drosophila to atorvastatin 340μM reduced fecundity including decrease in egg laid, third instar larva and eclosed flies indicative of reduction in their reproductive fitness. Thus, caution should be taking when administering atorvastatin to pregnant mothers and ladies of child bearing age.

Sensory navigation results from coordinated transitions between distinct behavioral programs. During chemotaxis in the *Drosophila* larva, the detection of positive odor gradients extends runs while negative gradients promote stops and turns[14]. Although their movement is often characterized as a series of runs and directed turns, it can also be modeled as a continuous modulation of turning extent by the detected change in stimulus intensity as the animal moves through the gradient[15]. In this study, the exposure of Drosophila larva to atorvastatin (170 and 340μM) prevented motor deficit induced by amyloid beta and alpha-synuclein aggregation in neurons evidenced in significant increase in larva chemotaxis.

Neurodegenerative diseases are characterized by age-dependent deterioration in memory functions and movement coordination. Here we use behavioral assays, including the negative geotaxis assay to show that some of the behavior characteristics associated with human neurodegeneration can be recapitulated in flies. In the negative geotaxis assay, the natural tendency of flies to move against gravity when agitated is utilized to study genes or conditions that may hinder locomotor capacities[16]. In this study, ELAV-Aβ42 and Ddc- α-synuclein transgenic construct flies showed time course decrease in climbing activity suggestive of ageing and motor deficit as seen in AD and PD. However, the supplementation of fly feed with atorvastatin improves the flies motor function which was consistent up to day 28. Interestingly, previous studies have shown neuroprotective effect of atorvastatin in different neurological conditions including PD[8,9],[17,18]. It is plausible that treatment with atorvastatin might prevent neurodegeneration possibly through attenuation of oxidative stress or inhibition of oligomeric protein seeding activity[19].

Statins have been reported to significantly increased the mean and maximum lifespan of Drosophila and enhanced cardiac function in aging flies by significantly reducing heart arrhythmias and increasing the contraction proportion of the contraction/relaxation cycle through decreased protein prenylation[20]. In this study, atorvastatin produced dose related increase in lifespan of *ELAV-Aβ42* and *ddc- α-synuclein* transgenic *Drosophila* indicative of potential to promote longevity.

5. CONCLUSION.

Findings from this study showed that atorvastatin promotes motor activity and lifespan in transgenic models of Parkinson disease and Alzheimer's disease in *Drosophila melanogaster*. Thus, atorvastatin could be a promising option in the treatment of PD and AD.

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Conflict of Interest

We do not have any conflict of interest to declare.

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