2023

Pharmacologic Proteasome Activators Ameliorate Alzheimer's-Like Pathology in Ad Fly Models

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PHARMACOLOGIC PROTEASOME ACTIVATORS AMELIORATE ALZHEIMER'S-LIKE PATHOLOGY IN AD FLY MODELS

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A THESIS

Submitted to graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2023

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MULTIDISCIPLINARY BIOMEDICAL SCIENCE

THEME NEUROSCIENCE

ABSTRACT

The proteasome is a large multi-subunit protease responsible for the degradation and removal of oxidized, misfolded, and polyubiquitinated proteins. The proteasome plays a critical role in nervous system processes. This includes the maintenance of cellular homeostasis in neurons (1). It also includes synaptic efficacy and plasticity as well as protein turnover, presynaptic vesicle transport, and neuronal protesostasis. Proteasome function is impaired as a consequence of aging, which is aggravated by conditions like Alzheimer's Disease and Related-Dementias (AD, ADRD) (2). According to earlier work from our lab the proteasome is critical to how quickly AD progresses. In Drosophila, human cells, and model of Alzheimer's disease, proteasome depletion has been shown to accelerate AD-like pathology and cognitive deficits, whereas proteasome augmentation has been shown to attenuate these features (2). We also demonstrated that proteasome activators enhanced turnover of the amyloid precursor protein and prevention of overall proteostatic dysfunction and the protective effects of proteasome overexpression, at least

in part, from increased degradation of APP, resulting in reduced Aβ. However, it is not clear if the protective effects are fully dependent on the ability of proteasome to turnover amyloid precursor proteins. In this study we investigate if proteasome augmentation may protect against AD progression independent of modulation of Aβ machinery. Here we used Elav-Gs-GAL4>UAS-Aβ42 flies, which overexpress pre-formed Aβ1-42 we demonstrate using treatment with proteosome activators (TAT1-8,9TOD, TAT1-DEN). That proteasome augmentation can protect against cognitive and survival deficits from Aβ42 but not in motor deficits.

DEDICATION

To my late grandfather, Mr. Gulam Rasool Buzdar, who lost his battle with Alzheimer's Disease. Baba your journey will always be an inspiration to me.

ACKNOWLEDGEMENTS

I want to thank my mentor, Dr. Andrew M Pickering, who has been a guiding light in my life. His unwavering support, extensive knowledge, boundless generosity and freedom of work have helped me achieve my professional and personal goals. You inspire me to be a better scientist and a better person every day. I want to thank every member of the Dr. Pickering Lab-Danitra Parker, Morgan Barkley, Kanisa Davidson, Nisi Jiang, Harper S Kim, Andy Delgado, Nayana Nagaraj and Parnesh. I would like to thank my Co-Mentor Danitra Parker for helping me survive through the process by being extremely supportive al kind and maintaining the best lab environment. I would like to thank my committee members- Dr. Goldberg, Matthew and Dr. Arrant Andrew for their valuable time and constructive feedback. I would like to thank Dr. Eric Roberson for attending my first committee meeting as my committee member. I would also like to thank Dr. John Shacka for his valuable guidance and motivation throughout the course.

I would like to thank US Department of State and Fulbright Association for awarding me Fulbright scholarship and sponsoring my master's program.

Any number of words would never suffice how deeply grateful I am to my Mother, Saeeda Parveen for being most supportive, motivating and inspiring and my Baba, Haq Nawaz Buzdar for giving me wings to fly freely and confidently. They have made me the person I am today, and I hope I can always make them proud. I would like to thank my brothers, Changez Khan and Agha Shah Jahan and my sister Gran Naz for being so progressive and supportive and making this world a better place. I would like to thank my niece Mahekan

for patiently waiting for her gifts. I would like to thank my maternal Grandmother for her unconditional love and always believing in me. I would like to thank my uncle Ameer Alam for being so humble, caring and always checking on me.

I want to thank my cousins, Barseena Seemak, Mehreen , Nosheen , Abdullah ,Shahmeer and Mustafa for sending memes and making me smile every time I was having a bad day.

I would also like to thank my support system, my friends-Shamza Manzoor, Ramsha Kamran, Ahsan, Asbha, Bagelz, Rumaisa, Ranveer, Rejoice, Sejal, and Ching for believing in me and picking me up every time I failed to believe in myself.

Lastly, I would like to thank myself for consistently trying to get better with each passing day, one step at a time and not giving up when times get tough.

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CHAPTER 1

INTRODUCTION

Alzheimer's Disease Overview

According to the Alzheimer's Association (2019), Alzheimer's disease (AD) dementia describes a specific age-related start and course of cognitive and functional deterioration that ultimately leads to death. Alois Alzheimer first described the disease in 1906 when he discussed the case of Auguste Deter, a 51-year-old lady with cognitive impairment, disorientation, delusions, and other behavioral problems whom he first encountered in 1901 (3).

Despite being qualitatively characterized by Alzheimer's in 1906, the two distinguishing pathologies of the disease—β-amyloid peptide identified in plaques and hyperphosphorylated-tau protein found in neurofibrillary tangles (NFTs)—were not discovered until the mid-1980s. (4,5,6,7,8). Following these initial findings, neuropathologic evaluation of AD has advanced to include recognition of other concomitant neuropathologies that further contribute to clinical dementia (,9,10,11,12,13).

Today, Alzheimer's disease is the most prevalent type of neurodegenerative dementia, impacting millions of people worldwide. The disease's early stages are marked by deficits in the capacity to encode and store new memories. The later stages are accompanied by further gradual changes in cognition and behavior. Synaptic loss, a decrease in synaptic strength, and neurodegeneration are all caused by changes in amyloid

precursor protein (APP) cleavage and synthesis of the APP fragment beta-amyloid (Aβ) along with hyperphosphorylated tau protein aggregation 2019 (14).

Proteasome Dysfunction as a Modulator of Alzheimer's Disease

Alzheimer's disease (AD) is also characterized by impaired proteostasis, including reduced proteasome function in brain tissues (15,16). Reduced proteasome function may contribute to the toxicity of intracellular Aβ, which is a cleavage product of the Amyloid Precursor Protein (17,18,19), and a key factor in AD (20,21). That leads to neurotoxic effects and the formation of insoluble plaques and soluble oligomers (22,23) which then contributes to neurodegeneration, formation of neuronal aggregate, and cognitive impairments (24,25,26). Additionally, hyperphosphorylated microtubule-associated protein tau can also form non-fibrillary aggregates in synapses, which is linked to disturbed proteostasis (27).

Proteosome assembly

The proteasome system is crucial in maintaining proteostasis in eukaryotic cells. The 26S proteasome is composed of one or two 19S regulatory caps bound to the top or bottom alpha rings of the core 20S proteasome (1). The 26S degrades polyubiquitin-tagged proteins, and the 19S regulatory cap will selectively bind to proteins carrying a polyubiquitin tag, unfolding the protein and feeding it into the 20S core. while the core 20S proteasome destroys disordered, misfolded, and oxidatively damaged proteins (1). In neurons, the proteasome system plays a critical role in synapse formation, establishing long-term potentiation for learning and memory and promoting dendritic spine growth (1,2). Therefore, protease failure in the nervous system is a major risk factor that may contribute to aging and neurodegenerative disorders, including Alzheimer's disease.

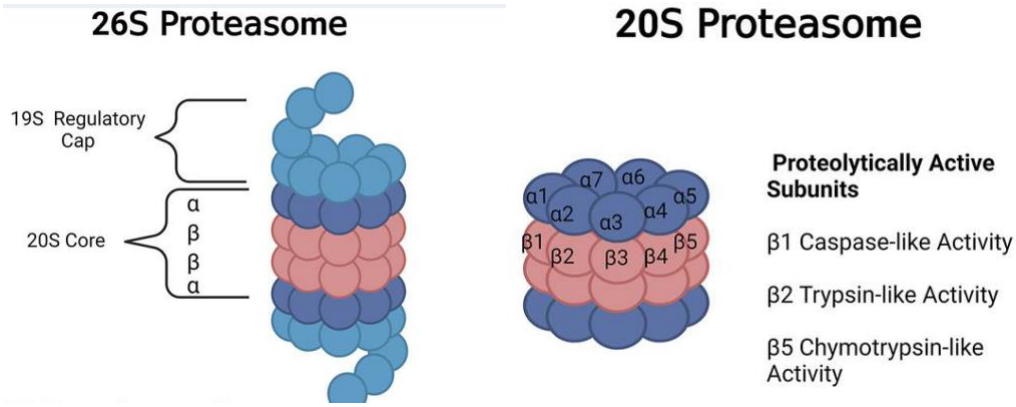

Fig 1. Forms of the proteasome. The 20S core of the 26S proteasome is bound by a 19S regulatory cap at either the top or bottom. There are 3 proteolytically active subunits in the core–β1, β2, and β5–associated with caspase-like, trypsin-like, and chymotrypsin-like activities, respectively (1).

Proteasome Dysfunction in AD study models

Previously, to investigate the biological significance of proteasome dysfunction in Alzheimer's disease (AD), Our lab used a Drosophila AD fly model with pan-neuronal expression of hAPP and hBACE1. The significance of this fly model is that it produces a peptide that resembles human Aβ40/42 (28). It has been also demonstrated that these flies produce Aβ deposits (29) and form plaques (28). Also, seen Aβ accumulation in the mushroom body which is responsible for learning and memory (30). We also demonstrated accelerated age-related cognitive deficiencies based on an odor aversion training assay and survival deficits, mimicking aspects of AD pathology (31,32).

We examined if augmentation of proteasome function could reduce or protect again AD-like pathology in this model. This was examined first through overexpression of a rate-

limiting proteasome subunit Prosβ5 (33). We showed augmentation of proteasome levels to reduce deficits similar to Alzheimer's disease in associative learning and memory, spontaneous activity and survival (1). We subsequently developed a set of proteasome activators which we showed to also protect against AD-like deficits. **Our work demonstrates proteasome augmentation to reduce protein levels of the Amyloid Precursor Protein and this to produce a subsequent decline in β-amyloid. However, it is unclear if reduced β-amyloid content represents the sole route by which protective effects are produced.**

Hypothesis

Coinciding with the previous findings, that age-related proteasome dysfunction is a key driver of the exponential increase in AD susceptibility during aging. We propose that age-related impaired proteasome will trigger, or at least accelerate AD progression. Previously, our lab team has developed novel genetic and pharmacologic interventions to augment proteasome function. It has been found that these interventions slow AD progression in fly and cell culture models of AD. Currently, we are testing the same set of proteosome activators to investigate if AD impairs proteasome function leading to aberrant neuronal function, independent of the roles of proteasome in the synthesis and processing of abundance of Aβ or Aβ machinery. With this aim we will Use novel pharmacologic agents created and characterized by our group, to determine if genetic or pharmacologic proteasome augmentation delays AD-like progression in fly models; and Determine if AD-protective effects of proteasome augmentation occur via mechanisms that are independent of degradation of amyloid-β (Aβ) machinery or APP.

CHAPTER 2

MATERIAL AND METHODS

Fly lines and strain maintenance

Elav-GS-GAL4>UAS-Aβ42, and Elav-GS-GAL4;UAS-hAPP;UAS-hBACE1 (56756) stocks were obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537). All lines were maintained on agar-cornmeal-dextrose-yeast growth medium (36) in a humidified 24°C incubator with 12-hour light/dark cycles. All crosses were set up with female virgins of the respective GAL4 driver line and male UAS- Aβ42 and W1118 flies. Progeny were collected within 48 hours of enclosion and allowed to mate on 10% sugar/yeast (SY10) medium (37) for another 48 hours. Females were then separated and sorted into sets of 25 flies per vial containing SY10 medium supplemented with either 400 μM mifepristone (RU-486) or ethanol vehicle, mixed directly into the food. Blue dye #1 (8 μM) was added to food containing RU-486 for the purpose of identification. Carbon dioxide was used to briefly anesthetize flies for sorting. Flies were moved to vials of fresh medium every 2 to 3 days.

Experimental Design:

A cohort of 200 flies per sex / genotype will be treated with ± 200 μM RU486 (mixed directly into stock food) to induce post-development transgene expression along with either vehicle, 1 μM, or 10 μM TAT1-8,9TOD (to into the stock food. Flies will be aged to 30 and 70 days then evaluated for cognitive and motor deficits including

spontaneous activity and learning and memory. We will measure lifespan in separate independent cohorts.

We used an RU486-inducible driver to induce a range of levels of overexpression and ensure that controls and experimental flies are genetically identical siblings. To test for off-target effects of RU486, we will run parallel experiments in flies that are genetically identical except for the absence of the transgene.

Drosophila life span assays

Flies were transferred to fresh medium, and survival was scored every 2 to 3 days. dLife software (38) was used to record survival and to compare median and maximum life span via log-rank analysis. Vials were randomized in terms of tray position and semi-blinded to reduce the impacts of environment or investigator bias.

Olfactory aversion training

Animals were exposed (via an air pump) in alternation to two neutral odors (3-octanol and 4-methylcyclohexanol, prepared as a 1:10 dilution in mineral oil) for 5 min under low red light, and a 100-V 60-Hz shock was applied during exposure to one of the two odors. The odor associated with the electric shock was alternated between vials. After three training rounds per odor, animals were given 1 hour to recover and then placed in a T maze (CelExplorer Labs) with opposing odors from either side. Flies were allowed 2 min to explore the maze, after which the maze sections were sealed, and the number of flies in each chamber was scored.

Spontaneous activity and circadian rhythm

Spontaneous activity was monitored using a TriKinetic activity monitor, in which vials containing 20 to 25 flies were secured, and activity was recorded in a humidified 24°C

incubator with 12-hour light/dark cycles as described above. Flies were allowed to acclimate for 8 hours before data collection. The activity was averaged for each 12-hour cycle and normalized per fly.

Statistics

Experimental cohorts will each contain 250 flies per condition. Measures of learning, memory, and cognitive function will be evaluated using Fisher's exact tests (21) based on 2*2 contingency tables where the rows correspond to the outcome: the first column controls and the second column is the RU486 treatment. The null hypothesis is that the outcome has the same hypergeometric distribution in both columns. The two sexes will be evaluated separately due to distinct behavioral differences. Survival will be evaluated using a log-rank Mantel–Cox test (39). The number of flies chosen was based on a formal power analysis in which we have a 90% ability to detect at least a 10% change.

CHAPTER 3

RESULTS

Previous reports have shown that APP, BACE1, and γ-secretase–activating protein (GSAP) are all degraded by the proteasome and that treatment of cells with proteasome inhibitors increases levels of BACE1 and GSAP (1). Therefore, the protective effects of proteasome augmentation may stem from the degradation of the Aβ precursor machinery and substrate, leading to decreased Aβ production. However, a substantial part of AD-induced proteasome dysfunction is independent of ligase activity and is thought to occur via direct inhibition of the proteasome (40, 41). Thus, it is plausible that part of the pathology in AD stems from the inhibition of the proteasome by Aβ. To test this concept, we will introduce proteosome activators to Aβ overexpressing flies to test if restoration of proteasome function reduces the Aβ that can lessen or ablate cognitive deficits and neurodegeneration in models of AD where the proteasome cannot influence Aβ synthesis.

To determine if proteasome activators alter pathology and disease symptoms, we used Elav-GS-GAL4>UAS-Aβ42. This fly model expresses the pre-formed 42-aa (Aβ) fragment of APP under the control of the pan-neuronal RU486 inducible Elav-GS-GAL4 driver (42). The GeneSwitch-GAL4 RU486 inducible drivers were used to cause the synthesis of Aβ42 to produce deficits in lifespan and cognitive measures. A cohort of 200 flies per sex / genotype / drug treatment, were treated with ± 200 μM RU486 along with either vehicle, 1 μM, TAT1-8,9TOD, or TAT1-Dendrite was mixed directly into the stock

food. Flies were aged to 25 days and then evaluated for cognitive and motor deficits including spontaneous activity, circadian rhythmicity, and learning and memory. We used GeneSwitch-GAL4 RU486 inducible drivers to cause synthesis of Aβ42 to produce deficits in lifespan and cognitive measures.

TAT1-8,9TOD and TAT1-DEN reduce, cognitive deficits, and proteostatic dysfunction in a fly model of AD.

Data from olfactory aversion showed overexpression of Aβ42 to produce cognitive deficits which were lessened under treatment with proteasome agonists. We showed under training that controlled flies learned to avoid the odor associated with an electric shock (**Figure 2A**). In contrast, our fly AD model did not displace training inducted odor avoidance **(Figure 2A, B**). However, treatment with proteasome agonists restored training-induced odor avoidance (**Figure 2B**). This suggests a protective effect against cognitive deficits produced in this model from proteasome augmentation.

Experiments were performed by exposing flies (via an air pump) in alternation to two neutral odors (3-octanol and 4-methylcyclohexanol, prepared as a 1:10 dilution in mineral oil) for 5 min under low red light, and a 100-V 60-Hz shock was applied during exposure to one of the two odors. The odor associated with the electric shock was alternated between vials. After three training rounds per odor, animals were given 1 hour to recover then placed in a T maze (CelExplorer Labs) with opposing odors from either side. Flies were allowed 2 min to explore the maze, after which the maze sections were sealed, and the number of flies in each chamber was scored.

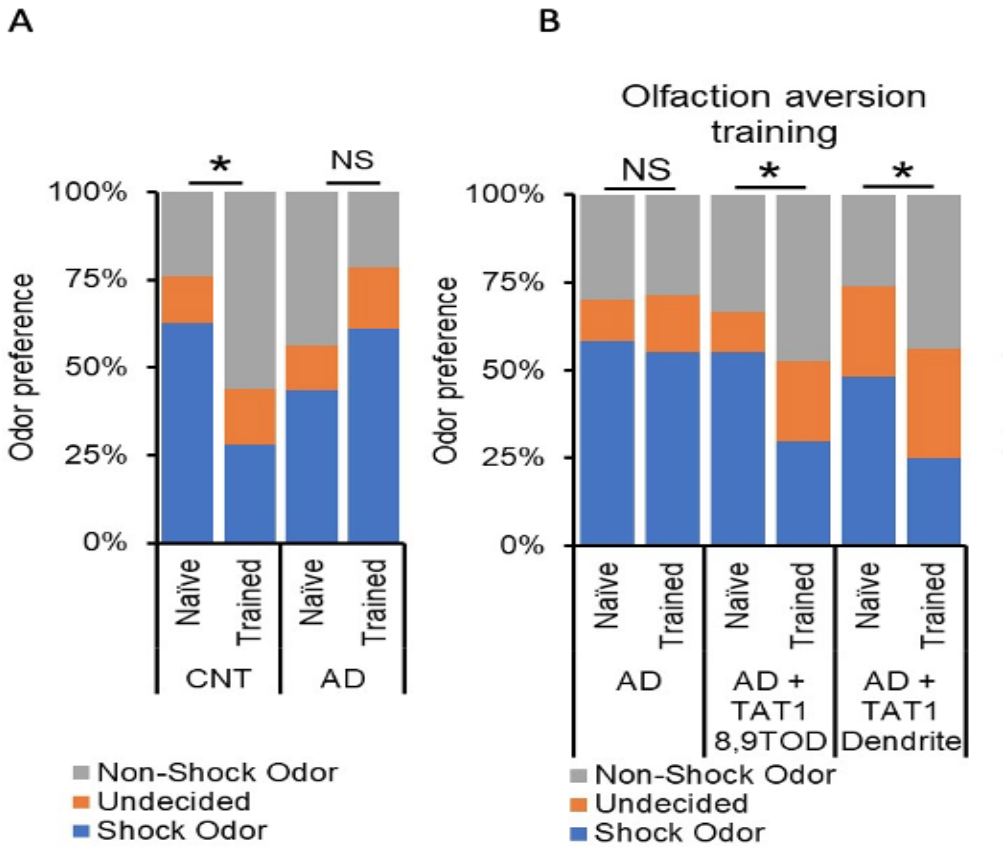

Fig.2. TAT1-8,9TOD and TAT1-DEN reduce, cognitive deficits, and proteostatic dysfunction in a fly model of AD.

*Olfaction aversion training in Elav-GS-GAL4 >UAS-Aβ40-42 flies treated for 25 days with 10 μM TAT1-8,9TOD, 10 μM TAT1-DEN, or vehicle mixed directly into food. (A). Control flies exhibited considerable increase in avoidance of the "shock" odor in contrast to RU486 treated AD flies. (**B**) Flies fed with peptidomimetics (TAT1-8,9TOD and TAT1-DEN) restored impairments similar to those seen in AD. AD+TOD ***P = 0.007975 and AD+DEN ***P = 0.007348. Significance was based on chi-square test. Values shown as percentage of flies in each chamber at the end of the assay.*

Proteasome agonists TAT1-8,9TOD and TAT1-DEN did not restore motor deficits in an AD fly model.

We additionally evaluated the impacts of peptidomimetics on spontaneous motor activity. This was monitored using a TriKinetic activity monitor in which vials containing 20 to 25 flies were secured, and activity was recorded in a humidified 24°C incubator with 12-hour light/dark cycles as described above. Flies were allowed to acclimate for 8 hours before data collection. Activity was averaged for each 12-hour cycle and normalized per fly.

We observed that our AD model displayed deficits in spontaneous activity however treatment with proteasome agonists did not correct these deficits (**Figure. 3).** This suggests that proteasome augmentation may protect from cognitive but not motor deficits produced by Aβ42.

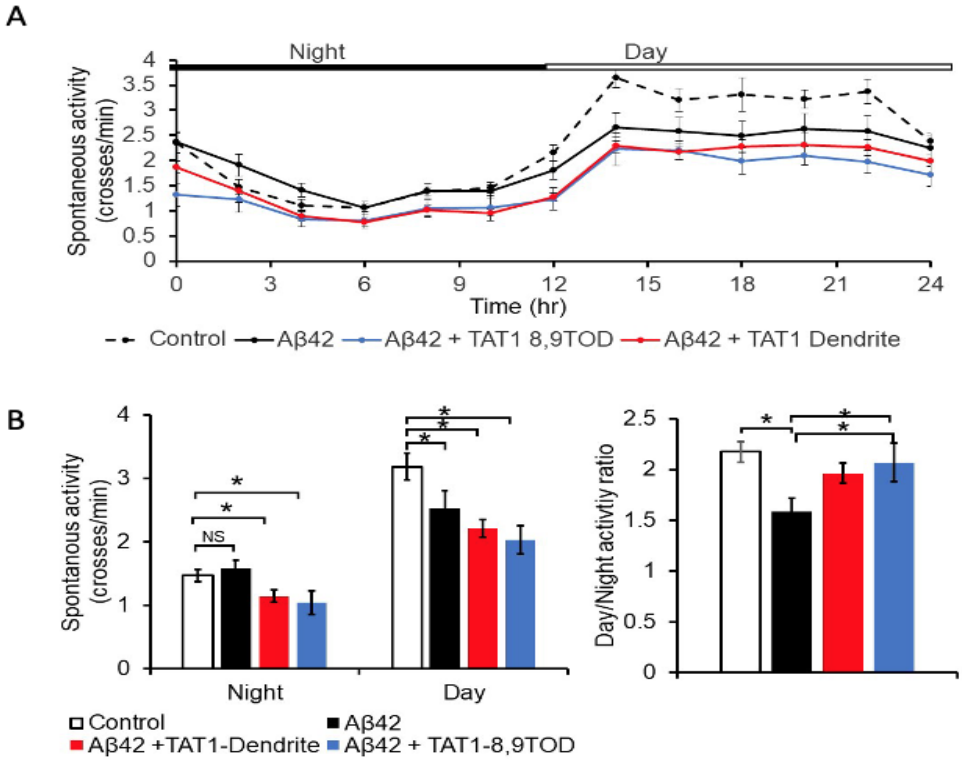

Fig.3. TAT1-8,9TOD and TAT1-DEN failed to reduce motor deficits in a fly model of AD. *Elav-GS-GAL4>UAS-Aβ3-42 flies maintained on SY10 medium with 10 μM TAT1-8,9TOD, 10 μM TAT1-DEN and ±200 μM RU-486 until day 25. Proteasome activators did not restore motor deficits in 25-day-old flies, N = 250-300 per group.*

Proteosome activators partially restored the median life span.

Subsequently, A lifespan assay was performed in four separate groups. Flies were divided randomly among ±200 μM RU-486, ± 1μM TAT1-8,9TOD, and ±1μM TAT1-DEN and Control group. As per our anticipated results, the Aβ42 expression did reduce the average lifespan. Whereas flies exposed to proteasome activators partially restored the median life span to a comparable life span of non-disease controls **(Figure.4).**

For this purpose, flies were transferred to fresh medium, and survival was scored every 2 to 3 days. dLife software (38) was used to record survival and to compare median and maximum life span via log-rank analysis. Vials were randomized in terms of tray position and semi blinded to reduce the impacts of environment or investigator bias.

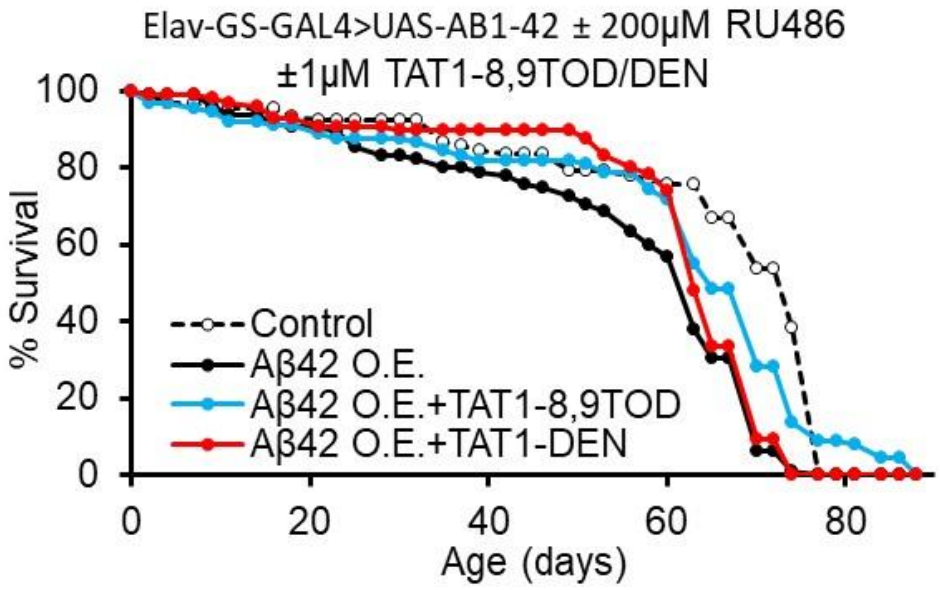

Fig.4. life span assay of Elav-GS-GAL4 >UAS-Aβ40-42 flies. *Flies treated with ± 1μM TAT1-8,9TOD, ±1μM TAT1-DEN, and ±200 μM RU-486 the vehicle mixed directly into food. AD flies treated with ±200 μM RU-486 displayed a reduced life span while proteosome activators partially restored the median life span by reducing early deaths compared to the life span of non-disease controls. N = 150-200 per group.*

Off-target effects of proteosome activators in wild type Elav-GS-GAL4>W^{1118} flies.

To test for off-target effects of proteosome activators, we performed parallel experiments in wild-type Elav-GS-GAL4>W^{1118} flies that are genetically identical except for the absence of the transgene to ensure that the drugs employed do not alter our experimental outcomes in a non-AD context.

Here we showed no difference olfaction aversion training following treatment with our proteasome agonists in wild-type flies (**Figure 5.A)**. We also observed no deficits in spontaneous activity under treatment with our proteasome agonists (**Figure 5.B)**. Collectively, these data suggest that proteasome activators reduced the AD-like deficits from Aβ toxicity but were only partially protective against non-neuronal deficits.

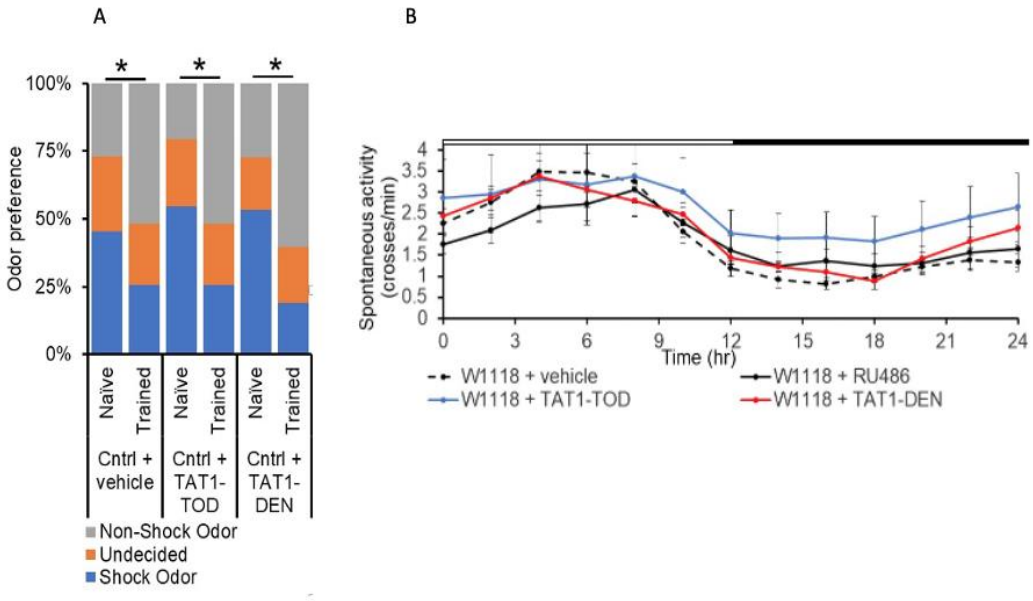

Fig.5. Off-target effects of Proteosome activators in wild type Elav GS-GAL4>W^{1118} flies.

(A)*treated with ± 1μM TAT1-8,9TOD and ±1μM TAT1-DEN does not exhibit any change in odor preference followed olfactory aversion training.*

(B) *Flies treated with ± 1μM TAT1-8,9TOD partially reduced the circadian rhythmicity in spontaneous assay activity when compared to other cohorts.*

*CNT ***P = 0.0003, TAD+TOD ***P = 0.0004 and AD+DEN ***P = 0.0001. N=200-250 per group.*

CHAPTER 4

DISCUSSION

Alzheimer's disease (AD) affects millions worldwide, producing cognitive deficits and increased mortality. Many hallmarks of the disease point to disrupted proteostasis. The proteasome is the major, multifunctional, and multi-subunit protease of the ubiquitin-proteasome pathway (1,2).

The proteasome is essential for numerous cellular processes, such as the transportation of presynaptic vesicles and synaptic plasticity. (2). Both Aβ, either internal or internalized, and aggregated tau may inhibit the proteasome's activity (17,20,21). According to studies in cell culture and mouse models of AD, impaired proteasome function has a significant impact on neuronal proteostasis and is a distinctive characteristic of the disease (1). Our lab has previously explained how the proteasome function relates to the turnover of APPs and downstream Aβ, as well as preventing proteasome malfunction decreased the quantity of APP and, in turn, prevented the accumulation of Aβ in our study models. These results seem solid and repeatable across many APP overexpression AD models (1)

To investigate more thoroughly whether proteasome overexpression may protect against impairments similar to AD. Dr. Pickering and the members of our lab improved proteasome function in flies that express hAPP and hBACE1 which led to noticeably lower amounts of hAPP protein. That was accomplished by genetically augmenting proteasome

levels or by amplifying catalytic activity with new pharmacologic agents. The 20S proteasome core subunit PSMB5/Pros5, which houses the peptidase activity's catalytic center, was overexpressed for the former. (2, 43,44).

Following the initial positive findings, they created a set of novels, as of yet unreported proteosome activators that can improve proteasome performance. They also discovered that these substances shield against AD-like symptoms in invertebrates and cell culture (1).

However, prior study has not tested if manipulation of proteasome impacts AD-like outcomes arising from high Aβ, through mechanisms independent of its synthesis from APP processing to critically dissect the mechanisms by which proteasome augmentation can protect against AD progression. Therefore, this time we tested the hypothesis that the protective effects of proteosome have an independent role in the synthesis and processing of Aβ machinery. By using the AD fly model *Elav-gs- GAL4>UAS-Aβ42* flies where Aβ get generated independent from APP processing and subjected them to pharmacologic proteasome activators to evaluate them for cognitive deficits, synaptic loss, and survival. Subsequently a set of novel peptidomimetics that was used in this study was previously developed by our lab that reported to allosterically activate the proteasome in vitro (37). These compounds activate the core proteasome (37) and the 26*S*holoenzyme. Also, shown that these activators were nontoxic, and could penetrate the blood-brain barrier (28).

Additionally, we have demonstrated that our proteosome activators improved disease traits similar to AD in Drosophila models of AD through learning and memory assays. However, the motor deficiencies showed no appreciable improvement. The information we provided also supports the notion that the Aβ machinery plays a partially

independent role in the proteasome-protective process. Additionally, it is both conceivable and feasible that some protective effects of proteasome overexpression result from processes unrelated to the turnover of APP/tau. A parallel experiment was also performed in Elav-GS-GAL4>W^{1118} to test for TAT1-8,9TOD and TAT1-DEN off-target effects.

Limitation:

We have investigated and produced evidence that proteasome dysfunction is a risk factor for developing AD. However, one of the major drawbacks of employing fly models was that they did not provide us with the framework to explore the increased proteasome dysfunction in various stages of AD in comparison to age-matched controls. Likewise, this study did not observe covariance between proteasome dysfunction and the Braak stage or prevalence of Aβ / Aβ machinery dysfunction. Therefore, this study cannot fully replicate the course of AD like mild cognitive impairment (MCI) and early clinical stages of AD compared to age-matched controls. Additionally, while performing the spontaneous assay, we see a non-significant improvement in motor impairments, which requires additional examination.

Future Direction:

Whether enhancing proteasome function can have a similar effect in tau models of AD will be a crucial subject in future studies. Similar to Aβ, tau oligomers directly interact with and impair proteasome activity, and Aβ -induced proteasome inhibition appears to be what causes tau accumulation (28). Last but not least, a group of new proteasome agonists that our lab produced hold therapeutic potential for AD and were used in this investigation. Also, this suggests that reduced proteasome function is a factor in the development of the

disease and justifies further pre-clinical studies using the proteasome agonist as a treatment for AD.

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