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Article

Effects on learning and memory of a mutation in Dα7: A *D. melanogaster* homolog of Alzheimer's related gene for nAChR α7

Anushka Sanyal[1], Sonia Cuellar-Ortiz[2]
[1] Homestead High School, Cupertino, California
[2] Schmahl Science Workshop, San Jose, California

SUMMARY

The nicotinic acetylcholine receptor α7 (nAChR α7) is a ligand-gated channel that releases neurotransmitters in excitatory neurons. Alzheimer's disease (AD) involves the reduction of cholinergic activity due to a decrease in neuronal levels of nAChR α7. This significant reduction makes nAChR α7 an intriguing target to study in the context of AD, specifically with regards to learning, memory, and locomotion, which are key affected abilities of the disease. Dα7 is the *Drosophila melanogaster* homolog of the nAChR, subunit α7. In this work, we explore the role of the nAChR α7 in learning and memory retention, using *Drosophila melanogaster* as a model organism. The performance of silenced Dα7-allele flies (PΔEY6) was analyzed in locomotive and olfactory-memory retention tests in comparison to wild type (WT) flies and an Alzheimer's disease model Arc-42 (Aβ-42). WT flies performed with average success rates of 80% in both the climbing assay and olfactory shock learning tests. In contrast, for the climbing assay, PΔEY6 flies performed with average success rates of 61% and the Aβ-42 flies performed with an average success rate of 60%. For the olfactory shock learning, the PΔEY6 flies performed with an average success rate of 45% and the Aβ-42 flies showed an average success rate of 43%. These results suggest that the lack of the *D. melanogaster*-nAChR causes learning, memory, and locomotion impairments, similar to those observed in Alzheimer's models Arc-42.

INTRODUCTION

Alzheimer's disease (AD) is an incurable condition that eventually causes substantial memory loss and death. Up to 5.6 million Americans currently live with AD. According to the Society for Neuroscience, AD is the seventh leading cause of death in the United States and the fifth leading cause in people over the age of 65. In 2018, the estimated cost of caring for Americans with AD and other dementias was $277 billion, not including unpaid caregiving (1).

Nicotinic acetylcholine receptors (nAChRs) are a family of ligand-gated channels widely expressed in the mammalian brain. These receptors are assembled from five identical (homomeric) or different (heteromeric) subunits. The receptor α7 nAChR is a homopentamer of the α7 subunit (2).

nAChR α7 plays an essential role during the process of releasing neurotransmitters in excitatory neurons, enhancing human cognitive function (2). Studies have indicated a loss of nAChR α7 ranging from 17% to 50% in Alzheimer's patients (1). Amyloid-β is a long, sticky protein that originates from mutations of amyloid precursor protein, which has an unknown function. When released in cells, they aggregate in the brain and clump around neurons, eventually causing neuronal death by blocking off synapses (3). As AD progresses and amyloid-β protein accumulates, decaying neuronal cells, cholinergic activity and neuronal levels of nAChR α7 decrease (3). Other studies have suggested that nAChR α7 interaction with G-proteins is also related to Alzheimer's progression (3-4).

Other subunits of nAChR receptors, such as β2 and α4β2, have been studied for the purposes of AD therapy. A reduction in nAChR α7 binding sites and consequent neuronal loss are among the earliest events detected in Alzheimer's. In addition, amyloid-β, the primary protein aggregate which drives AD progression, binds to nAChR α7 very tightly (1). However, the receptor has not been studied extensively in the context of AD. The fact that nAChR α7 displays various changes during the progression of Alzheimer's makes it a potential AD therapeutic target. *Drosophila melanogaster* is a model organism widely used to study mammalian genes, particularly genes related to human diseases. Nearly 75% of human disease-causing genes are believed to have a functional homolog in the fruit fly. Also, these flies are able to form memories using genes that are identical or closely related to genes found in the human body, making them ideal for this work (5). In particular, fruit flies are reliable models for neurodegenerative diseases such as Alzheimer's and Parkinson's due to their similarity to the human nervous system. Since flies have had a long history as a research model, a wide variety of genetic manipulation tools are available (5).

Dα7 is the homolog in fruit fly of the human gene expressing the α7 nAChR receptor (6). The *D. melanogaster* strain Dα7PΔEY6 (referred here as PΔEY6) has a P-element (a *Drosophila*-specific transposable element that interrupts the sequence and produces a labeled mutation) inserted in the regulatory regions of Dα7 that has been shown to stop Dα7 gene expression (6). No work had been done regarding

this allele in the context of AD. The *D. melanogaster* strain Arc-42 (also known as Aβ-42) overexpresses the human gene for the Amyloid-β protein (7). Arc-42 is known to have effects on a fly's cognitive processes in a way that mimics AD in humans and is widely regarded as an AD model (8).

We analyzed the behavior of PΔEY6 flies in comparison to wild type flies (WT) and unaltered Aβ-42 flies to study the role of α7 nAChR in AD. In this work, the PΔEY6 mutants that carried a defective allele of Dα7 performed similarly to the AD model Aβ-42 in locomotive and olfactory-memory retention tests, suggesting that α7 nAChR receptor may play a role in AD.

RESULTS

Olfactory Perception Test

To confirm odor perception in the mutants is not affected, an olfactory perception test was used. Success was standardized to odorant identification of apple cider vinegar over mineral oil. The WT flies in this test performed with a success rate of 67.23%, while the Aβ-42 flies had a 66.25% success rate, and the PΔEY6 flies had a 66.71% rate of success (**Figure 1**). The difference between the two mutants was not significant (p = 0.995). No statistically significant differences were detected among the fly mutants and the WT (PΔEY6 & WT: p = 0.995; Aβ-42 & WT: p = 0.995), suggesting that both mutants can detect the odor at the same level as the WT and act upon their perception.

Climbing Assay

As locomotive deterioration is a key symptom in AD, a climbing assay was used to examine the PΔEY6 fly's abilities in this regard. Success was standardized to being able to climb to the benchmark distance of 5 cm in under 18 seconds. The success rates of the flies in the climbing assay were 61.2%, 60.2%, and 79.5% for the PΔEY6, Aβ-42, and WT populations respectively (**Figure 2**). The PΔEY6 showed an average decline of 23% decline in locomotive abilities relative to the WT. This difference was statistically significant (p = 10^{-7}). Additionally, PΔEY6 and Aβ-42 showed no significant differences (p = 0.98) in locomotion abilities in the climbing assay, indicating the Dα7 defective mutants are as impaired as the AD model flies.

Olfactory Shock Learning

To examine impact in learning and memory of the absence of the receptor Dα7, an olfactory shock learning test was performed comparing PΔEY6 to WT flies. This test associated electrical shocks to one odor stimuli (CS-) and no shocks to the other (CS+). Memory retention is measured as the percental fraction of the flies successfully choosing the CS+ odor in both the short- and long-term memory tests. AD model flies have been previously shown to display a significant reduction in their performance in this test, when compared to the WT (8). To verify that our experimental setting worked as reported, the AD model Aβ-42 was included as a control in this experiment for one-week old flies.

Results are presented for one-week-old flies PΔEY6 and Aβ-42 flies in comparison to the WT (**Figure 3a-b**). PΔEY6 flies had about a 40% decline in short- and long-term memory in comparison to WT, which was statistically significant (p < 10^{-25} for both time points). Additionally, the PΔEY6 mutant strain exhibited no significant differences in short- and long-term memory when compared to the AD model Aβ-42 (short-term: p = 0.93; long-term: p = 0.93), suggesting that PΔEY6 mutants are as impaired as AD flies in the learning and remembering processes. Two-week old PΔEY6 flies showed a significant reduction in short- and long-term memory, compared to WT flies (p < 10^{-43} and p < 10^{-6} respectively) (**Figure 3c-d**). The differences between the results of the same fly strain in the short- and long-term memory tests were

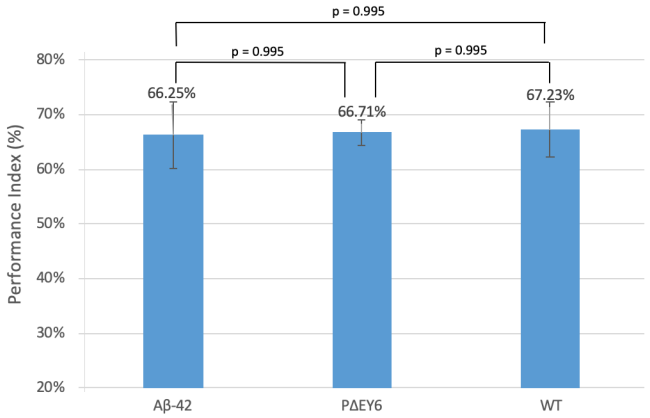

Figure 1. All tested fly strains are able to perceive odors. The average percentage of successful odorant identification of apple cider vinegar over mineral oil for WT, PΔEY6, and Aβ-42 flies. Error bars represent standard deviation (n = 3 trials of 20 flies for each strain).

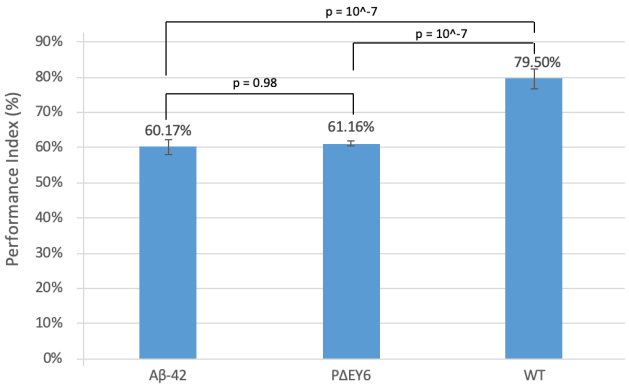

Figure 2. Mutant fly strains climb less successfully than the wild type. The average percentage of successful climbing to benchmark distance of 5 cm in under 18 seconds for WT, PΔEY6, and Aβ-42 flies. Error bars represent standard deviation (n = 3 trials of 50 flies for each strain). Statistical significance determined with Chi-square test.

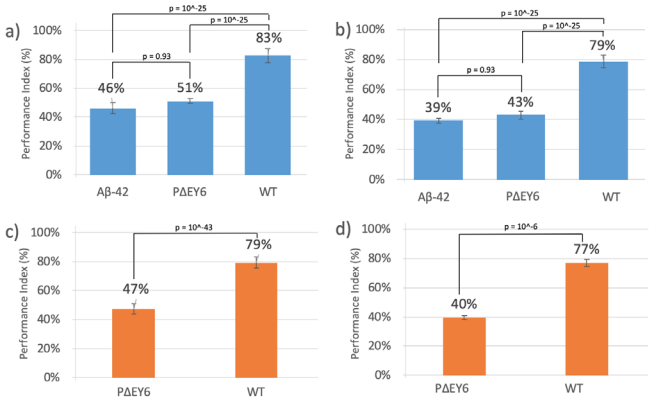

Figure 3. Mutant fly strains learn & retain memory less successfully than the wild type. The average percentage of flies that successfully chose the CS+ odor in the olfactory shock learning test. a) One-week-old trained WT, PΔEY6, and Aβ-42 flies were tested after one hour to assess short-term memory. b) One-week-old trained WT, PΔEY6, and Aβ-42 flies were tested after one week to assess long-term memory. c) Two-week-old train WT and PΔEY6 were tested after one hour to assess short-term memory. d) Two-week-old train WT and PΔEY6 were tested after one week to assess long-term memory. Error bars represent standard deviation (n = 5, 8, or 11 populations/trials of 50 flies for WT, PΔEY6, and Aβ-42 flies, respectively). Statistical significance determined with Chi-square test.

not statistically significant. There was no performance impact when switching the odor (data not shown). Taken together, PΔEY6 flies showed statistically significant reduction in their climbing abilities as well as in their memory retention similar to the reduction shown by flies of the AD model Aβ-42.

DISCUSSION

Both Mutants Can Perceive Odors

In Alzheimer's patients, decreased olfactory perception is often observed. This may be in part due to the fact that the nAChRs contribute to olfactory perception and are restricted from function in AD (3, 9). Previous studies confirmed that Aβ-42 and PΔEY6 mutants had no disability in olfactory perception (6, 8). However, to ensure that the flies would not be impaired by lack of olfactory perception and eliminate this as a contributing factor to the olfactory shock learning test, olfactory perception was tested. Both mutants (PΔEY6 and Aβ-42) and the WT performed at the same level of odor detection in the olfactory perception test, with no significant differences detected from one population to the next. This indicates that the differences from the olfactory shock learning/memory test are solely driven by the memory retention and learning abilities of the fly populations. The observed results for all three fly stains were similar to prior results from past work.

Locomotive Decline was Detected in Both Mutant, Aβ-42 and PΔEY6, but not in WT

Deteriorating locomotive ability is a key indicator of AD-like progression, which is why we examined Dα7 mutants' climbing abilities. Previous studies indicated Aβ-42 mutants have a locomotive decline of 40% in the climbing assay, and fly mutants lacking Dα7 have a behavioral defect in visually-mediated jump responses (6, 8). Since the authors tested the mutant visual abilities and found their vision was not affected, it was possible that the impact in jump responses could be related to locomotive abilities. Hence, we expected that the PΔEY6 mutants would show a similar trend or perform even worse in the climbing assay, than the Aβ-42 fly strain, dissimilar to the WT. The Aβ-42 and PΔEY6 mutants displayed similar locomotive decline in contrast with the WT populations (**Figure 2**).

Olfactory Learning and Memory Deficiencies Detected in Both Mutant Types, Aβ-42 and PΔEY6, but not in WT

Previous studies indicated Aβ-42 mutants have an average learning and memory retention deficit of 50% as evaluated by the olfactory shock learning Test (8). In this work, learning and memory retention abilities of the PΔEY6 mutants were tested using the same kind of test. PΔEY6 flies have reduced short- and long-term memory at the same level that was reported for Aβ-42, indicating that lacking a functional Dα7 receptor has a similar impact to the accumulation of Amyloid-β caused by Aβ-42 insertion (**Figure 3**).

These results showed that the lack of the nicotinic acetylcholine receptor α7 in fruit flies drives an Alzheimer's disease-like response, indicated by AD's primary symptoms: decline in memory retention and locomotive ability. This reinforces the suggested hypothesis that the α7 nAChR is involved in AD (3). However, the model is limited because essential elements of AD such as cognitive dysfunction caused by cell type-specific neurodegeneration cannot be tested in flies. Furthermore, several proteins including the aggregating Amyloid-β are not present in *Drosophila*. Further studies analyzing mutants of nAChR α7 in vertebrate model organisms like rats are needed to make more robust assertions about its participation in AD. Nevertheless, results in this work provide further motivation to study nAChR α7 and its potential as an AD therapeutic target.

Future Research

The relationship between the nAChR α7 and various G-proteins in the G-protein signaling pathway still remains misunderstood, hindering the study of G-proteins in the context of AD. G-proteins interact with the nAChR α7 and a loss in G-protein interaction of the nAChR α7 completely impedes its ability to modulate neural growth (10-11). Given this information, studying the nAChR α7 in conjunction with the G-protein pathway complex in the context of Alzheimer's disease poses as an interesting topic of exploration.

MATERIALS AND METHODS

Dα7 PΔEY6 (FBal0211001) and Amyloid-β Arc-42 (FBti0141192) fly stocks were obtained at Bloomington

Drosophila Stock Center. Carolina® Wild Type flies (obtained from Carolina® Biological Supply) were used as a negative control.

Fly Conditions

Flies were kept in fly vials with Instant Drosophila Medium. Vials were kept in indirect light inside of partially insulated plastic boxes and checked weekly for general wellbeing. Temperature and humidity were monitored weekly. Average temperature was kept at 24°C (18-25°C) and 60% (38-84 %) relative humidity. T-maze and olfactory perception tests were performed in a dark chamber to avoid light stimulation.

Olfactory Perception

This protocol was adapted from (12). Flies were placed in a non-odorous chamber that contained two 1 mL cut pipette tips upside down. One pipette tip held an attractive odor (apple cider vinegar) and the second one held a neutral odor (mineral oil). After two minutes, the number of flies that chose each trap was recorded. To reduce non-associative effects, three repetitions using separate populations were tested in the same environments, using at least 20 flies in each repetition. The behavior of the WT and mutant strains were compared.

Climbing Assay

Flies were transferred to a clean, empty vial and kept there for one minute in a dark chamber to allow them to acclimate to the environment. Following this, the flies were tapped down to the bottom of the vial and given 18 seconds to climb 5 cm. The number of flies that successfully reached the 5 cm line were recorded (13). The behavior of WTs and the mutants was tested using at least 3 repetitions composed of separate populations of at least 50 flies.

Olfactory Shock Learning (OSL)

OSL test was modified from (12) as well as (8) and (14). A training chamber was built to submit the fly populations to shocks of 60 V in intervals of 1.25 seconds every 5 seconds for 1 minute, while simultaneously allowing air to flow through carrying the corresponding odor. A T-maze was built using acrylic pipe, with two flexible plastic tubes attached to enable air flow and odor exposure, using an air pump. Octanol-3 (Sigma Aldrich 589-91-3) and methylcyclohexanol (Sigma Aldrich 5340-36-3) were used as the testing odors, diluted in mineral oil (MCH – 1:67, Oct-3 – 1:100) (14). Initially, we trained the flies by exposing them to an electrical shock while simultaneously exposing the fly population to the CS- (negative conditioned stimulus) odor. After one minute of rest, the same fly population was exposed to the CS+ (positive conditioned stimulus) odor in the same chamber without electricity. After one hour, the flies were tested in the T-maze offering the two odors to examine short-term memory and after one week, the same population was tested to examine long-term memory. Populations consisting of one- and two-week-old flies were used.

To reduce non-associative effects, we made sure to switch the direction in which odors were provided in the T-maze. And, tested both Octanol-3 and methylcyclohexanol acting as CS- and as CS+. No significant differences were found when analyzing odor direction or the reagent used as CS+ or CS-. Additionally, all the tests were performed in the same environment, using at least 50 flies in each population and at least 3 repetitions using separate populations. We compared the behavior of the WT and the mutants (using the WT populations as a control group), and worked in the dark to avoid light stimulation. A photographic record was used to determine the fraction of flies that chose the CS+ over the total flies tested.

Chi-Square Statistical Analysis

Chi-squared test for independence, also known as Fisher's test, was used to establish statistically significant differences between the effect of two treatments on each dependent variable. Using contingency tables, paired analysis was done to establish association between treatments. In particular, the association between the recorded behavior of a particular mutant and the WT or the two mutants under a particular experimental condition was tested. This was achieved by calculating the Chi-squared value and comparing it to the standard Chi-squared distribution to obtain the p-value. P-values smaller than 0.05 were used to reject the null hypothesis that there are no differences between the two treatments.

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