BEHAVIORAL CONSEQUENCES OF POINT MUTATIONS IN THE VESICULAR ACETYLCHOLINE TRANSPORTER

by

DANIEL WHITE

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This thesis is approved by the following members of the Final Oral Review Committee:

Dr. Hakeem Lawal, Committee Chairperson, Department of Biological Sciences, Delaware State University
Dr. Murali Temburni, Committee Member, Department of Biological Sciences, Delaware State University
Dr. Y. Hwan Kim, Committee Member, Department of Biological Sciences, Delaware State University
Dr. Dayan Knox, External Committee Member, Department of Psychological and Brain Sciences, University of Delaware

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ABSTRACT

The neurotransmitter acetylcholine (ACh) is involved in critical organismal functions as locomotion, learning and memory. Therefore, alterations in this neurotransmitter system is a key underlying factor in movement and cognitive deficits. The vesicular acetylcholine transporter (VAChT) mediates the packaging of ACh into synaptic vesicles for exocytotic release. Mutations in this protein ultimately diminish locomotion whereas complete loss of function of VAChT is fatal. The direct role of altered acetylcholine release and its association with impairment or enhancement of cognitive functions is still not fully understood**.** We hypothesize that point mutations in VAChT causes age-related deficits in cholinergic-mediated behaviors such as locomotion, learning and memory. Using *Drosophila melanogaster* as a model system, we have generated several mutations within VAChT and observed its effect on survivability, longevity and locomotive behavior. Here we report that VAChT point mutants cause defects in locomotion ability and an allele dependent deficit in lifespan. These results demonstrate that cholinergic release is important for the regulation of behavioral performance in *Drosophila*. In future studies, we will test methodologies to effectively rescue these deficits with implications for intervention strategies to treat cholinergic deprived disorders such as Alzheimer's disease. Future studies will turn to investigating the effects of VAChT overexpression to determine whether the deficits seen in the *Vacht* mutants can be decreased or reversed.

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COMMON ABBREVIATIONS

ACh ..... Acetylcholine
AD ..... Alzheimer's Disease
ChAT ..... Choline Acetyltransferase
cm/s ..... Centimeters per second
CSP2 ..... Cysteine String Protein 2
GFP ..... Green Fluorescent Protein
kb ..... Kilobases
kDA ..... Kilodaltons
MWT ..... Multi-worm Tracker
PVDF ..... Polyvinylidene Fluoride
TBST ..... Tris Base Saline with Titon-X-100
UTR ..... Untranslated Region
VAChT ..... Vesicular Acetylcholine Transporter
WT ..... Wildtype

CHAPTER 1: INTRODUCTION

Aging is an inevitable biological process that causes us to grow old and eventually pass away. Aging can be defined as a "progressive decline in physiological integrity and function" (He and Jasper, 2014). This is a broad definition but the biological systems behind aging is quite complex. As we grow older, the functionality and effectiveness of our molecular and cellular systems decline and as a result, tissues and organs are negatively affected (He and Jasper, 2014; Harman, 1981; Longo *et al.,* 2015). As we age, the probability of being diagnosed with aging related diseases such as cardiovascular disease, cancer, diabetes, and Alzheimer's disease significantly increases (Dilorito and Murphy, 2015; Liochev, 2015). Aging can have a negative impact on your livelihood as it causes hearing/vision loss, locomotive deficits, cognitive impairment, and financial issues.

The exact causes of aging still remains unknown, but a common theory of what causes the effects of aging is the progressive accumulation of cell, DNA, and protein damage due risk factors such as oxidative stress, the environment, and free radicals (Liochev, 2015; Zimniak, 2012). Even though the effects of aging leads to inevitable mortality, the rate of progression and how severe the consequences of aging have great variabilities between individuals and is alterable (Longo *et al.,* 2015). Studies have shown that many factors such as diet, exercise, stress, and genetics alter life expectancy in humans, invertebrates and rodents.

Aging is the greatest contributor to mortality and typical signs that suggest aging is a progressive decline in locomotor skills and strength, and the capacity of learning and memorizing (Muira, 1996). Previous studies show that a pathological feature in Alzheimer's disease is characterized in part by the death of cholinergic neurons (Ferreira-Vieira *et al.,* 2016; Lombardo and Maskos, 2015). Along with acetylcholine's (ACh) critical role in regulating

muscle contractions, investigating further into cholinergic system in an aging model could be ideal to determine potential regulators, triggers, and biomarkers for normal aging.

Acetylcholine, the first neurotransmitter to be identified (Loewi, 1921), is an organic chemical responsible for relaying signals involved in locomotion, learning and memory. It is synthesized in the cytoplasm of cholinergic neurons by the enzyme choline acetyl transferase using acetyl coenzyme A and choline (Kitamoto *et al.,* 1998). After synthesis it is then docked for transport by the vesicular acetylcholine transporter (VAChT).

VAChT's name describes its function, as it transports acetylcholine molecules into pre-synaptic vesicles. VAChT couples the influx of one acetylcholine molecule with an efflux of two vesicular protons using a counter-transporter (Martin and Krantz, 2014; Ferreira-Vieira *et al.,* 2016). Once vesicles are fully filled with ACh molecules, the vesicles travel to the membrane of the pre-synaptic neuron in ready releasable pools awaiting exocytotic release (Kitamoto *et al.,* 1998; 2000). The role of VAChT is of great importance for life and all downstream pathways of ACh, as without it, no ACh would be released to bind to its respective nicotinic or muscarinic receptors.

With increasing evidence that shows that decreased endogenous levels of acetylcholine plays a role in the consequences of normal aging, there is very little published work that investigates the regulator of ACh release, which is VAChT. An ideal model organism for an aging study involving VAChT is the *Drosophila melanogaster*. Powerful genetic tools can be used in *Drosophila*. It also has a short and inexpensive lifespan and has many genes that are either evolutionary conserved between humans and rodents or homologs (Sun *et al.,* 2013). The first aging study using *Drosophila* as a model organism began in 1916 (Loeb and Northrop *et al*., 1916), where it was discovered that the temperature has a significant impact on lifespan. Since

then, other significant findings include the relationship between genetics and lifespan (Clark and Gould, 1970), how some drugs could extend lifespan (Gardner, 1948), and how reproduction/fertility affects longevity in female flies (Luckinbill and Clare, 1985). Interest in *Drosophila* aging studies continues to increase.

VAChT, which is also the first vesicular neurotransmitter to be identified in *Drosophila* (Kitamoto *et al.*, 2000), is one of those genes that are evolutionary conserved with human VAChT. In this study, we aimed to determine the consequences of mutating VAChT and altering the amount of ACh molecules that were transported into secretory vesicles. Independent point mutations were inserted into the VAChT gene, resulting in a change of amino acid and overall effectiveness of VAChT expression (Kitamoto *et al.*, 2000). A previous experiment was conducted on *C. elegans* where 12 nonlethal point mutations in *Vacht* were used to show that there is a "direct relationship between the intrinsic parameters of vesicular acetylcholine transport *in vitro* and the storage and synaptic release of acetylcholine *in vivo*." (Zhu *et al.*, 2001). There have not been any reports on the effects of point mutations in VAChT in regards to aging.

Hypothesis

Due to our knowledge of VAChT and acetylcholine and the given data from published work cited relating to the relationship between aging and the cholinergic system, we hypothesize that the most severe mutation of VAChT causes the most severe deficit in survivability, lifespan, and locomotion in aging male *Drosophila* flies. Additionally, we hypothesize that the least severe mutation of VAChT will have the least negative consequences. Rather than using 12 non-lethal point mutations, we used four ($Vacht^{1}$, $Vacht^{2}$, $Vacht^{4}$, $Vacht^{8}$) and we found that mutations $Vacht^{1}$ and $Vacht^{4}$ caused the most deficits in the aforementioned factors when

compared to control flies and that *Vacht*$^{\delta}$ had minimal effects. In the near future we aim to analyze the learning and memory capacity of these mutant flies using the courtship assay (Ejima and Griffith, 2009). Additionally, we aim to investigate the effect of VAChT overexpression and whether or not these deficits observed in *Vacht* mutants can be rescued or reversed.

CHAPTER 2: LITERATURE REVIEW

2.1 Aging

In simple terms, aging is the process of growing old. Scientifically in regards to most species, aging could be defined as a progressive decline in the biological, chemical, and biochemical systems resulting in the loss of integrity and functioning of cells, tissues and organs (Harman, 1981). Inevitably, mortality will be a consequence of aging. The effects of aging have been well observed and documented, but the triggers and drivers that causes aging remain speculated. A common theory (damage theory) of the cause of aging is the collective progression of oxidative stress, free radicals, damage to DNA and mitochondria, ubiquitination, and many other factors are considered to contribute to the rate of aging. The damage and death of cells leads to higher susceptibility to pathological diseases such as cancer, diabetes, and cardiovascular diseases (Liochev, 2015; Gladyshev, 2014).

Although mortality due to the effects of aging is inevitable in most species, the progression of aging can be altered by several factors. Genetics, diet, the environment, exercise and personal lifestyle choices are among some of the factors that can either improve or decrease healthspan, thus affecting lifespan (Barnes, 2015; Diloreto and Murphy, 2015.) In regards to the environment, pollution, stress, and commercially available toxins such as insecticides have also been shown to accelerate molecular and cellular damage (Geller and Zanick, 2005; Janezic *et al.*, 2016).

The complexities, variabilities, and mysteries of the aging system make it difficult to identify any biomarkers for aging humans. To be a biomarker for aging, it must be able to predict how functional cells, tissue or organs will be at an older age better than chronical age (Wagner *et*

al., 2016) Common phenotypes caused by aging such as graying hair, loss of skin elasticity, and gradual visual/hearing loss cannot be good biomarkers for aging because even though they are clear signs that one is aging, they cannot show the functionality of possible mechanisms for aging in the future. However, recent and current biogerontological studies are giving rise to potential novel aging biomarkers such as the "epigenetic clock" (Horvath *et al.,* 2015; Lowe *et al.,* 2016), advanced glycation end products (AGEs) (Simm *et al.,* 2015), and metallothioneins (Malavolta *et al.,* 2008). In the near future, it may be possible to predict the biological age of our bodies better than knowing the chronological age and intervention strategies can be established to extend lifespan.

2.2 ACh and its Potential Role in Aging

Acetylcholine (ACh), the first neurotransmitter identified (Loewi, 1921) is an excitatory organic chemical and is used by all cholinergic neurons and it is essential for the central, peripheral, and autonomic nervous systems. In many vertebrates and invertebrates, cholinergic transmission regulates many neuronal systems such as muscle stimulation, learning, memory, sensory properties and sleep by relying on using acetylcholine as the signaling molecule (Ferreira-Vieira *et al.,* 2016).

Acetylcholine is synthesized by the enzyme choline acetyltransferase (ChAT) using choline and acetyl coenzyme A (acetyl CoA). After synthesis, it is then stored within secretory vesicles by the vesicular acetylcholine transporter (VAChT) (Kitamoto *et al.,* 1998; Varoqui and Erickson, 1996). (For further reading into the cholinergic locus, refer to section 4. 2)

Acetylcholine is thought to have an important role in the progression of aging as common phenotypes that suggest aging in elderly people is decreased mobility and strength, deficits in the

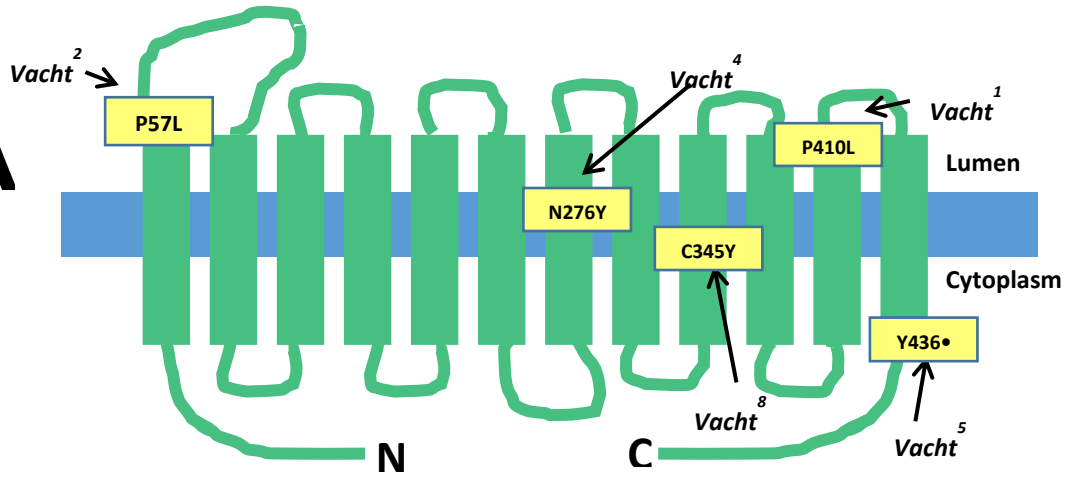
ability to learn, store and retrieve new memories. In fact, post mortem examinations in brains of people with Alzheimer's disease (AD) shows that there are is significant loss and damage to cholinergic neurons in the basal forebrain (Muira, 1996; Whitehouse *et al.*, 1981 and 1982). Additionally, studies have shown that there is also a significant decrease in the amount of ChAT that is expressed in patients with AD and other forms of dementia (Muira, 1996; Perry *et al.*, 1978). Drugs or genetic interventions that aim to prevent the decrease of acetylcholine synthesis or release could prove to have protective measures against aging and its effects.

2.3 The Cholinergic Locus

Along with choline acetyltransferase (ChAT), the vesicular acetylcholine transporter (VAChT) is essential in the cholinergic neurotransmission pathway. ChAT is responsible for the biosynthesis of acetylcholine (ACh), where it is then transported into secretory vesicles in the membrane of presynaptic cholinergic neurons (Kitamoto *et al.*, 1998; Varoqui and Erickson, 1996). Even though these two proteins can be regulated and expressed independently, they remain closely related genetically. The two genes that encode for ChAT and VAChT comprises the "cholinergic locus", whereas the VAChT gene (*Vacht)* is nested within the first intron of the ChAT gene (*Cha).* The structure of VAChT was shown to be of a 12 transmembrane domain after the *Vacht* gene was cloned in *Caenorhabditis elegans* (Alfonso *et al*., 1993) (Figure 1). This cholinergic locus and structure of VAChT was later shown to be evolutionary conserved in many species such as mice (Naciff *et al.*, 1997), rats (Erickson *et al.*, 1994), *Drosophila melanogaster* (Kitamoto *et al.*, 1998), and humans (Erickson *et al.*, 1994). VAChT has been shown to have sequence homology with the vesicular monoamine transporters and the functionality of VAChT has been determined ((Kitamoto *et al.*, 1998; Kitamoto *et al.*, 2000; Martin and Krantz, 2014). VAChT transports acetylcholine molecules in vesicles by relying on proton electrochemical

gradients created by a vacuolar-type H-ATPase (Rand and Russel, 1984; Varoqui and Erickson, 1996; Kitamoto, 1992). As one ACh molecule enters the vesicle, the gradient pushes out two vesicular protons (Ferreira-Vieira *et al*., 2016).

A



B

Allele	Nucleotide change	Amino acid change
Vacht^{1}	C→T (1229)	Pro→Leu (410)
Vacht^{2}	C→A (170)	Pro→Leu (57)
Vacht^{4}	A→T (826)	Asn→Tyr (276)
Vacht^{5}	T→A (1308)	Tyr→Stop (436)
Vacht^{8}	G→A (1034)	Cys→Tyr (345)

Figure 1. The structure of VAChT and the effect of the *Vacht* point mutations. A. After the *Vacht* gene was cloned in *C. elegans*, the evolutionary conserved 12 transmembrane structure of VAChT was identified. The location of the mutants *Vacht*^{1}, *Vacht*^{2}, *Vacht*^{4}, *Vacht*^{5}, and *Vacht*^{8} are labeled. B. The effectiveness of VAChT function is altered by single changes of amino acids due to point mutations in the *Vacht* gene.

Several mutants in of ChAT and VAChT in *C. elegans* including *cha-1* and *unc-17* were identified, respectively (Zhu *et al.*, 2001). Studies showed that complete loss of *cha-1* or *unc-17* resulted in no development but non-lethal mutations in either gene showed decreased movement coordination, resistance to ACh esterase inhibitors, and other behavioral deficits. Even though mutations in either ChAT or VAChT expression show similar results, one difference that

separates the two is the amount of ACh present in the cholinergic neurons. In *cha-1* mutants, the level of ACh is significantly reduced, whereas ACh levels in *unc-17* mutants are significantly increased (Kitamoto *et al*., 2000).

Mutant alleles for ChAT in *Drosophila* were isolated and characterized in 1980 by RJ Greenspan (Greenspan, 1980), but it wasn't until 2000 where mutant alleles for VAChT were isolated and characterized (Kitamoto *et al*., 2000). By studying these VAChT mutant alleles (*Vacht*^{1} *and Vacht*^{2}*),* it was determined that homozygous mutant alleles resulted in either death in the embryonic or larval stage. Heterozygous *Vacht* mutants allows for survivability for adult flies but with significant decrease in cholinergic transmission.

2.4 Drosophila as a Model System for the Study of Aging

There are many reasons to why *Drosophila melanogaster* is an ideal model organism for aging studies. *Drosophila* is a powerful genetic tool as its entire genome is fully sequenced. Sequencing its genome taught us that more than 50% of fly genes have homologs in human and that 75% of genes that causes known human diseases have fly homologs (Adams *et al*., 2000; Myers *et al*., 2000; Reiter, 2001). They also have several genes such as *Vacht* that are evolutionary conserved between several species such as mice, *C. elegans,* and humans (Kitamoto *et al.,* 1998). Additionally, compared to other model organisms, manipulating *Drosophila's* genome is fairly simple. With these genetic advantages in mind, investigating possible mechanisms of aging by knocking down/out or overexpressing genes of interest could prove beneficial when using *Drosophila* as a model. When it comes to average lifespan in aging studies, *Drosophila* has an advantage over most model organisms, even though *C. elegans* has an average shorter lifespan (20-30 days in 25 degrees Celsius) (Olsen *et al.,* 2006) than fruit flies (60-80 days in 25 degrees Celsius) (Sun *et al*., 2013), *C. elegans* do not possess the rich genetic

profile such as *Drosophila's.* Even still, with a short lifespan of roughly two months, it is easily able to have hundreds of flies in a single lifespan experiment. Lastly, the cost of maintaining flies in an aging study is significantly inexpensive when compared to other model organisms.

There are some challenges when using *Drosophila* as an aging model however, as the environment has a significant influence on fruit flies lifespan. When using this model, the temperature, humidity, and light/dark cycle must be consistent. Cooler temperatures have been shown to extend lifespan and warmer temperatures have been shown to accelerate aging (He and Jasper, 2014). As a standard, fruit flies are contained in a 25 degrees Celsius environment with 50% relative humidity.

The dietary restrictions provided to the flies also have an impact on lifespan (Clancy *et al*., 2002). The major caveat with this is that even though there are standardized food preparations available, it is not uncommon for a laboratory to create their own food because it is more cost effective. As a result, findings in aging studies have the potential to have high variability and lack of reproducibility because the standard contents and ratio of the fly food ingredients in one laboratory is not the same as another laboratory. Yeast is the main protein source for flies in fly food and along with other nutrients and water, the concentrations of each of these present in the food will alter the lifespan of the flies (Mair, 2003; Piper and Patridge, 2007). It has been shown that bad diet, genetics, and lifestyle, changes intestinal barrier dysfunction increases and thus driving mortality (Rera *et al.*, 2012; Clark *et al.*, 2015). Lastly in terms of diet, the storage of both used and unused food influences the integrity of the food, consequently influencing the lifespan of the flies. Performing separate aging studies on *Drosophila* based on sex is also ideal. For several reasons yet to be fully understood, there are significant differences in lifespan between male and females across many species such as *C.*

elegans, *Drosophila*, mice and humans (Tower and Arbeitman, 2009). It is proposed that the specific genetic makeup that separates male and female and how they respond to biochemical changes plays a role in lifespan differences. For example, it has been reported that male *Drosophila* increases their lifespan when introduced to mild heat stress early in life, whereas lifespan in females decrease (Sørensen *et al.*, 2007). Maternal effect is also theorized to have an influence on the differences of lifespan due to sex (Wolf and Wade, 2009).

CHAPTER 3: MATERIALS AND METHODS

3.1 Drosophila Food Stocks and Maintenance

Fly stocks *w^{1118}CS15* (w^{1118} outcrossed fifteen times to Canton-S) were raised in a 12 hour light/dark cycle on a sugar-yeast-corn meal diet which was produced in house and poured into clean plastic vials both as larvae and adults. All vials were kept in a 25 degrees Celsius incubator when not in use for the assays. Keeping fly food in 25 degrees Celsius for too long results in the food either becoming too soft and sticky or the food becoming too dry. Both circumstances results in premature death in the flies so to prevent that, all vials were swapped out for new vials containing food every six days.

3.2 Generation of V*acht* Point Mutations

Vacht point mutations were generated by T. Kitamoto using ethlymethane sulfonate as a mutagen. The protocol for the recovery of the *Vacht* point mutations are described in Toshimoto *et al.,* (2000) and Lindsley and Zimm, (1992).

3.3 Generation of VAChT Rescue Mutants

10-15 virgin females with Vacht/TM6B-GFP were placed in custom made mating chambers with 3-5 UTR/UTR; Df3R/TM6B-GFP males. The untranslated region (UTR) flies are a shorthand for [UTR/Cy; DF3R/TM6B-GFP]. These constructs were generated by fusing a 7.4 kilobases (kb) 5' untranslated region to a wildtype VAChT cDNA. The resulting VAChT overexpression constructed was then crossed into a construct with a deletion of the *Vacht* genomic locus and neighboring genes (referred to as *Df3R*). Once crossed, negative selection against the green fluorescent protein (GFP) marker was used to identify *UTR-VAChT/+*;

Vacht^{mutant}*/Df3R* larvae which are readily identifiable as non-GFP larvae. Phenotypic genetic screening was performed using the Nikon SMZ18 stereomicroscope, which illuminates GFP.

3.4 Developmental Survival Assay

Independently, first, second, and third instar larvae from *w*^{1118}*CS15* (control) and *Vacht1, Vacht2, Vacht4, and Vacht 8* rescue mutants were collected from their respective mating chambers and placed into vials containing standard corn meal media. *Drosophila* reaches adulthood (flies) after a process called holometabolism (or complete metamorphosis) where they go through four stages of transformation – embryo, larvae, pupae, and imago (Belles, 2011). Once the larvae became pupae, the percentages of the total number of larvae collected that progressed into the pupae stage was observed and recorded. Subsequently, the percentages of pupae that eclosed to adult flies was observed and recorded. Lastly, the overall percentages of larvae that survived into adulthood was calculated. All successful adult flies that eclosed were then removed from their respective vials and placed in new vials containing fresh food. These flies (males only) were then used for the lifespan and locomotion studies.

3.5 Lifespan Assay

For each genotype, all adult male flies that were collected from the developmental survival assay that eclosed within a week of each other were placed in cohorts such that the individuals were 2-9 days old. The total number of flies in each cohort was observed and recorded. The last day of the 7 day life-span became day 0 of the lifespan assay. Every three days from Day 0, the total number of flies that died was recorded and subtracted from the total amount of flies that were alive when the cohorts were established. This assay continued until all flies were dead. To prevent flies from dying prematurely due to bad food, the cohorts were

independently swapped into new vials containing food on every 6th day from day 0 or twice/week.

3.6 Locomotive Behavior Assay

To determine any effects of locomotive behavior in our *Vacht* rescue mutants we used the automated Multiworm Tracker developed by Scwierczek *et al*., (2011). This real-time computer vision system allows for rapid quantification of locomotive behavior of *C. elegans*, larval (Scwierczek *et al*., 2011) and adult *Drosophila* (Pizzo *et al*., 2013; Grygoruk *et al*., 2014) with minimal human effort. Briefly, this system comprises of a real-time image analysis software, the MWT (overhead camera), offline behavioral parameter measurement software, and choreography; that together analyze the behavior of multiple flies on one petri dish. The MWT is unable to record movement in a three dimensional field so to prevent the flies from jumping or flying, we poured Sylgard 170 silicone elastomer into the petri dish and lid and let it hardened. The amount of Sylgard poured left just enough space for the flies to walk freely but unable to jump or fly. At n=5, the MWT captured and recorded the flies speed (centimeters per second), and time in motion (s) during a 90 second window. To allow the flies to acclimate to the environment and slow down their movement due to agitation and stress, the first 30 seconds of recording was omitted. For full specifications of the MWT and its software please refer to Scwierczek *et al*., (2011). This assay was performed once a week during early morning hours until all the flies have passed. Early mornings are the preferred time for this assay because that is when flies are most active based on their circadian rhythm (Tataroglu and Emery, 2014).

3. 7 Western Blotting

To quantify protein expression in the various *Daughterless*-Gal4/UAS-VAChT fly lines of drosophila, western blot was performed as described previously (Boppana *et al*., 2017). One fly head for each fly-line was homogenized in sodium dodecyl sulfate lysis buffer in the presence of dithiothreitol as suggested by the manufacturer (New England Biolaboratories). Fly head homogenates were ran on polyacrylamide gels and transferred on to a polyvinylidene fluoride (PVDF) membrane. After blocking for 1 hour in Tris base saline with Triton-X-100 (TBST) milk, the blot was incubated with anti-VAChT (1:2000) and anti-CSP2 (1:2000) overnight at 4^0C.

After three washes for five minutes each with TBST, anti-rabbit and anti-mouse horseradish peroxidase conjugated secondary antibodies (Cell Signaling, Danvers, MA) were used at a dilution of 1:2000 for primary antibody detection at room temperature for two hours. Protein bands were visualized with an enhanced chemiluminescence substrate solution (BioRad, Hercules CA). Images were developed by exposing the PVDF membrane to a western blot scanner (LI-COR Biosciences Lincoln, NE).

CHAPTER 4: RESULTS

4.1 The Effect of the *Vacht* Rescue Construct on Drosophila

Genotype	% larvae survival to:	
	Pupal	Adult
Wildtype	83.4 +/- 7.3	65.5 +/- 10.5
Vacht^{1}	0	0
Vacht^{1} *rescue*	71.6 +/- 11.2	65.4 +/- 10.9
Vacht^{2}	0	0
Vacht^{2} rescue	93.1 +/- 2.5	88 +/- 3.9
Vacht^{4}	0	0
Vacht^{4} *rescue*	83.5 +/- 6.5	68 +/- 7.9
Vacht^{8}	0	0
Vacht^{8} rescue	93.6 +/- 3.4	83.9 +/- 4.0

Table 1. Comparing the survivability of larvae becoming adult flies. The percentages of larvae that became pupae and subsequently adult flies for each genotype. Homozygous mutants do not make it past embryonic development.

Our overall experimental goal was to observe whether or not there are any enhancements in the deficits of cholinergic-mediated systems seen during normal aging. To determine this effect we measured survivability, locomotion, and lifespan. First, we sought to generate adult *Vacht* mutants. Because a severe loss of *Vacht* is lethal (Table 1), we deployed some genetic manipulations to by-pass the developmental lethality. By expressing a wildtype copy of VAChT

driven by a 7.4 kb UTR sequence, we expressed VAChT in each of the mutant lines studied. Indeed, we recovered a high percentage of *Vacht* mutant survivors.

Genotypic appropriate larvae from each rescue mutant line were independently collected into vials containing food. The percentages of total larvae that reached the pupal stage and subsequently adulthood was observed and recorded (Table 1). With having the most severe mutation, *Vacht*^{1} mutant larvae had a success rate of 65% ± 10.9 of eclosing to adult flies. Vacht8 on other hand has the least severe mutation of the four studied and displayed an 84% ± 4.0 chance of larval survival which was slightly higher than wildtype. *Vacht*^{4} also shows a noticeable decrease in survivability, with just a 3% ± 7.9 increase in complete morphism than *Vacht*^{1}.

4.2 The Effects of the *Vacht* Rescue Construct on Drosophila Longevity

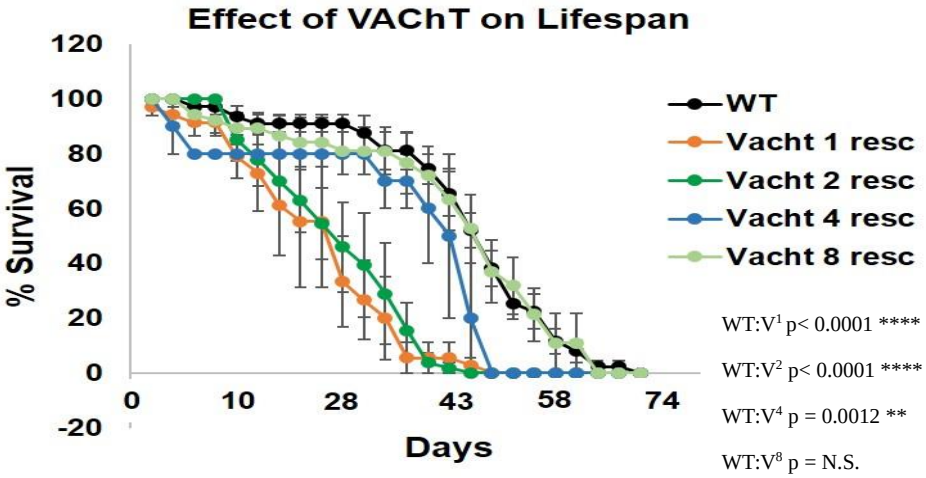


Figure 2. Average survival rate of the *Vacht* rescue mutants. The percentage of flies alive as time progresses between each *Vacht* rescue mutant and wildtype. Mentel-Cox log-rank test was used for statistical analysis. WT:V^{1} p< 0.0001 ****, WT:V^{2} p< 0.0001 ****, WT:V^{4} p = 0.0012 **, WT:V^{8} p = N.S.

Flies that successfully hatched from the survivability assay were used in the lifespan assay. Fly cohorts consisted of flies that hatched within a week from each other and the date the cohorts were established initiated the lifespan study. Wildtype flies lived an average of 62 days and comparatively *Vacht*^{8} mutants lived to an average of 58 days which was insignifcant after

Log-rank (Mentel-Cox) statistical analysis. Lifespan is significantly lower (Log-rank, p<0.0001) in *Vacht*^{1} mutants as they usually do not survive more than 45 days. Surprisingly, average lifespan of the *Vacht*^{2} mutants is significantly similar to *Vacht*^{1} (Log-rank, p<0.0001) about 37 days. This was not expected since its larval to adulthood survivability is 88%, on par with wildtype and *Vacht*^{8} mutants. Genetically, it is more severe than *Vacht*^{8} and less so than *Vacht*^{1}. The lifespan of *Vacht*^{4} mutants had a great variation between its cohorts. This type of variability is intriguing, as it was not seen in the other three mutant lines. For example, in one cohort the oldest flies lived up to 31 days but in another cohort flies lived up to 48 days. When looking at averages, however, *Vacht*^{4} had a p-value of 0.0012 meaning it was also significantly lower than the average lifespan of wildtype flies. Figure 2 represents the average percentage of flies alive as time progressed between all cohorts of each mutant rescue compared to wildtype. Overall, the data strongly suggest that the efficiency of acetylcholine release to the synaptic cleft has great importance in dictating the longevity of flies and mammals.

4.3 The Effects of *Vacht* Rescue Construct on Locomotion

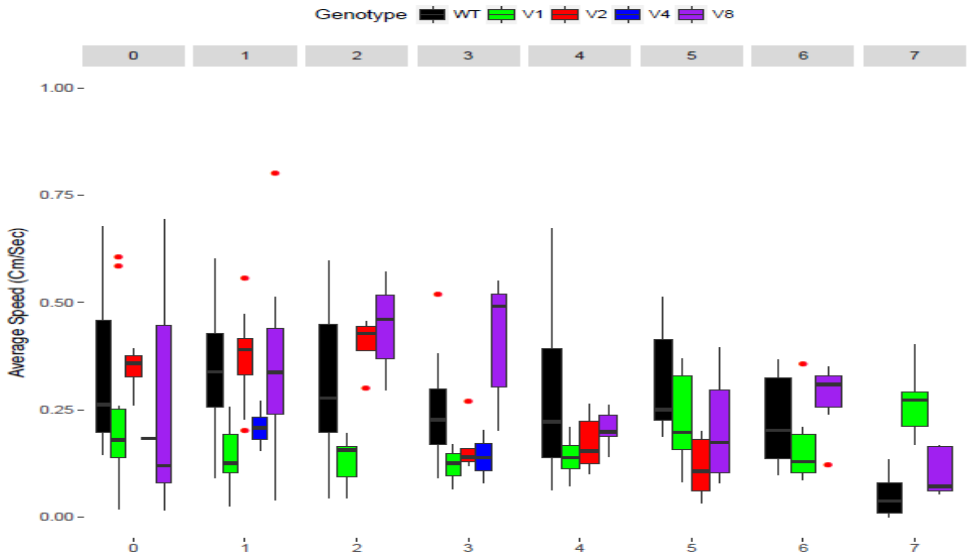


Figure 3. Effect of *Vacht* mutations on average locomotion speed. The average speed of WT, *Vacht*^{1}, *Vacht*^{2}, and *Vacht*^{8} were compared across their lifespan. (SA) The data analysis and the algorithm used to generate it were performed and developed by Dr. Raquel Abreu (UCSB).

Data analysis on MWT data provides several different types of quantitative data. The tracker was used to measure the average speed of up to n=5 simultaneously. Independently, the averages of all flies within a cohort in a given week was calculated each week until the end of their lifespan (Figure 3). In (Simon *et al*., 2006), it was reported that during the first two weeks of living, flies showed a significant progressive decline in locomotion. However in the current data set presented, this behavior was only clearly observed in *Vacht*^{2} and *Vacht*^{8} mutants. Representing just one set of experiments though, this data do not fully represent the pattern yet. In any event, we do still observe a noticeably reduced speed in *Vacht*^{1} and *Vacht*^{4} when compared to wildtype (Figure 3). Instead of the progressive decline that was predicted to occur with all of the genotypes, WT, *Vacht*^{1} and *Vacht*^{8} appeared to have a consistent speed throughout their lifetime. This was determined by looking at the median average speed per week. Once al data analysis is completed for this extensive study, it should provide a more vivid picture to how these VAChT mutations and the locomotive decline seen in aging are related.

At any given time during recording, MWT data analysis could pinpoint the speed of any fly (individual circles on speed plot graph). The data allow for us to see a pattern of locomotive behavior with each of our genetic lines (Figure 4). This result only compares wildtype with *Vacht*^{1} and *Vacht*^{8}. These two mutations are considered our most severe and least severe respectively, so we wanted to observe any drastic change of locomotive speed between the two ends of the spectrum. Wildtype flies show to have a random and sporadic locomotive behavior at any given time. For example, at 40 seconds some flies were moving above 0.6 centimeters per second (cm/s), whereas other flies moving under 0.2 cm/s. However, the top speed appears to decline as the time of recording progresses. Our least severe mutation *Vacht8* had a similar behavior compared to wildtype in terms of being sporadic. What was intriguing however was

that the mutants did not show a noticeable decline over time like wildtype flies. Top speeds increased until around the end of recording (90 seconds). Using a correlation and linear regression test, it was determined that the values of both these genotypes are strongly associated and do not have any significant difference (r^2 = .726). The locomotive behavior of our most severe mutation *Vacht*^{1} did not show a sporadic pattern as it did with both wildtype and *Vacht*^{8}. Throughout recording, *Vacht*^{1} flies usually stayed at 0.4 cm/s and below with majority of them having a speed around 0.2 cm/s. There was no significant relationship of locomotive speed between *Vacht*^{1} and wildtype (r^2 = .330). Overall, these results suggest that altered acetylcholine release has an impact on maximum and minimum speed that the flies can crawl at any given time.

Time-in-motion comparison between *Vacht*^{1} *and Vacht*^{8} *mutants.*

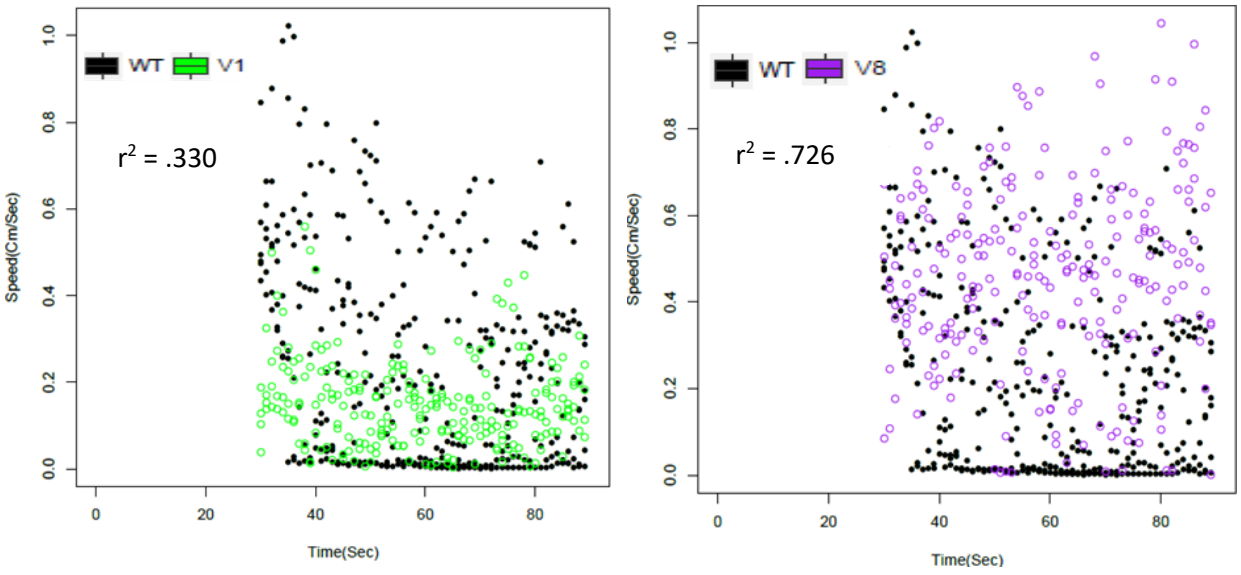


Figure 4. Time-in-motion Analysis of the two extreme *Vacht* mutations. At any time between 30-90 seconds *Vacht*^{1} does not usually crawl faster than 0.4 cm/s whereas *Vacht*^{8} has high variation of speed. Correlation statistical analysis was performed to measure correlation of WT and the *Vacht* mutant data.

4.4 *Vacht* Rescue Mutants Displays a Change in their Displacement Patterns

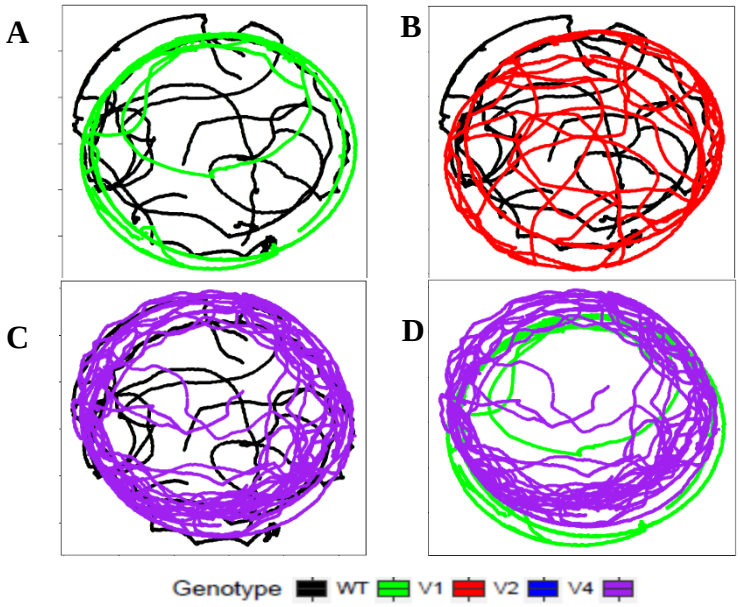


Figure 5. **Comparing the displacement pattern between genotypes.** The MWT was capable of tracing the path of the flies' movement in real time. When compared to the displacement of wildtype flies (black lines), each mutant had their own distinct pattern of movement. A. Wildtype was compared to *Vacht*^{1} (green lines). B. Wildtype compared to *Vacht*^{2} (red lines). C. Wildype compared to *Vacht*^{8} (purple lines). D. *Vacht*^{1} compared to *Vacht*^{8}.

The MWT traced the displacement of all of the flies it recorded. From this, we noticed that each mutant had their own distinct tracing pattern (Figure 5). During recording, the direction (clockwise or counterclockwise) that the flies preferred was observed, but there was not enough visual evidence that showed preference. Randomly, flies would crawl in either direction. Noticing a preferred rotation direction would have led to suggestions to mutations with VAChT influencing left or brain hemispheres activity, giving more insight to behavioral changes in our flies. Movement of wildtype appeared random as it was not possible to predict a pattern that they would follow. *Vacht*^{2} (Figure 5B) displayed a similar tracing pattern but *Vacht*^{1} (Figure 5A) and *Vacht*^{8} (Figure 5C) did not. The tracing pattern of *Vacht*^{1} and *Vacht*^{8} were similar however, but with different rates of speed (Figure 5D). Both of these mutants preferred to crawl along the

outer edges of the petri dish. By contrast, wildtype flies appeared to have a random displacement pattern. This data is interesting to us because it shows that when VAChT expression is not normal, there may be mechanistic changes in the neuromuscular system, effecting coordination. The displacement of all flies were recorded throughout their lifetime and the displacement pattern for each genetic line remained consistent until death (data not shown).

4.5 Identifying a suitable *Vacht* overexpression line for future studies (preliminary).

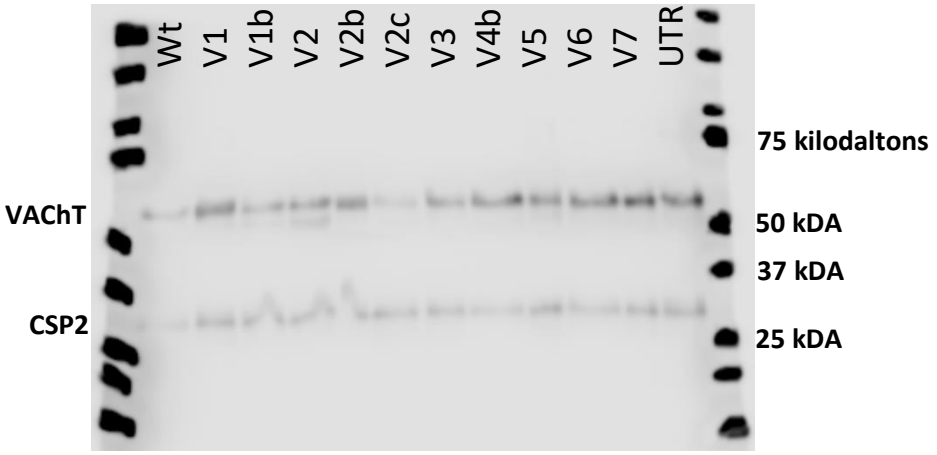


Figure 6. Western blots compares the different levels of VAChT overexpressed in fly heads. Preliminary experiments quantifying VAChT protein levels using homogenates from fly heads in each of the 10 VAChT overexpression lines. Observing the density and thickness of the overexpression protein bands and comparing it WT leads to a qualitative conclusion of the overexpression line that produces the most VAChT. Cysteine string protein 2 (CSP2) was used as the loading control.

In the near future, we plan to perform a similar study to the study presented here but instead of VAChT mutations, VAChT overexpression flies will be used. With VAChT overexpression, we predict that lifespan, survivability, and locomotion will be rescued and enhanced compared to wildtype. Additionally once we are able to conduct memory and learning experiments using the courtship assay (Ejima and Griffith, 2009) we predict that VAChT overexpression will improve the learning and memory capabilities compared to *Vacht* mutants and wildtype. Performing this experiment will therefore provide us with even more insight with

VAChT and its potential role in facilitating the progression of aging and its effects. Currently our laboratory has 10 VAChT overexpression lines but it is not determined yet which line expresses the most VAChT compared to wildtype so to determine that we isolated fly heads from each line and measured their VAChT protein levels using Western blotting (Figure 6). Fly heads were used to produce the homogenate being as though VAChT is strictly expressed in the brain. w^{1118}*CS15* and UTR-VAChT flies were used as controls. All genetic lines excluding the line labeled "V2c" shows higher expression of VAChT compared to wildtype but the band for "V7" (full genetic constructs are not presented here) appears to consist of the most VAChT protein between the other lines and wildtype. All conclusions based on the Western blots were based by just visually observing the density of the bands but we are going to measure the exact levels once this overexpression experiment is fully established. This overexpression experiment is preliminary and more western blots are being conducted to confirm if this line will be most appropriate in future aging studies.

CHAPTER 5: DISCUSSION

Aging is an inevitable biological process that leads to the breakdown of our physiological systems as time progresses. Decline of function in cholinergic mediated systems such as decreased locomotive ability, and learning/memory deficits are amongst the common effects seen in maturing individuals. There has been limited aging studies that investigated the role the vesicular acetylcholine transporter plays mechanistically in the processes that triggers or accelerates aging. It has been reported that VAChT expression is decreased in patients with Alzheimer's disease (Efange *et al.*, 1997; Ferreira-Vieira *et al*., 2016). It has also been reported that when VAChT is overexpressed in aging mice, there is significant improvement in spatial memory acquisition (Nagey and Aubert, 2015). On the contrary, it was reported that VAChT overexpression drives neuromuscular aging and cholinergic dysfunction in mice, increasing age and disease related decline in locomotion (Sugita *et. al*, 2016).

The aim of this study was to decrease expression of VAChT using point mutations within the *Vacht* gene to analyze any enhancement or decrease in locomotion and cognitive behavior (learning and memory) as *Drosophila* males aged. Additionally, with acetylcholine synthesis and release being essential for livelihood, we aimed to analyze any change of survivability for larvae to undergo complete metamorphosis and the average life span of the adult mutant flies. In the present study, we show that when VAChT expression is decreased the ability to go through complete metamorphosis, average lifespan and locomotive behavior is altered. Vacht8 is our least severe mutation and Vacht1 is our most severe and between all assays, there was significant difference between the data for these two. When compared to wildtype, there were no significant difference with Vacht8 mutants, showing that the amount the VAChT expressed and acetylcholine that is released is still sufficient enough for a normal life in this mutation. The

consequence of decreasing VAChT expression showed an interesting effect on how long the flies can live. In (Sugita *et al*., 2016), it showed that overexpressing VAChT decreases the lifespan of mice. In this study, we did not look at overexpression but decreased expression and also observed decreased lifespan, with the most decreased expression having the shortest lifespan (Figure 2). This suggest that VAChT expression needs to remain consistent throughout the entire life of an organism to have the longest lifespan.

It was reported that *Drosophila* will show a weekly progressive decline in locomotion after the first two weeks of life (Simon *et al*., 2006). Our data does not fully agree with that report as of now because wildtype, Vacht1, and vacht8 showed a consistent range of speed throughout most of their life (Figure 3). However, this data is not conclusive yet and both data collection and analysis is still a work in progress. The displacement pattern of the VAChT mutants provided us with interesting insight on the neuromuscular network (Figure 5). $Vacht^{8}$ and $Vacht^{1}$ mutants preferred to crawl in a circular path whereas wildtype flies moved randomly with no distinct pattern. It remains to be understood how two mutations on each end of the severity spectrum can have such a similar displacement pattern yet different pattern compared to wildtype but it may relate to the findings that was found in (de Castro *et al.*, 2009). It was reported that VAChT is required for proper neuromuscular development and function. The VAChT mutations could possibly be disrupting the neuromuscular networks of our flies.

Overall, this present study suggests that decreasing the amount of acetylcholine released into the synaptic cleft by hindering VAChT expression may have an additive role in progression of aging. However, this study is not conclusive and serves as stepping stone into an very extensive aging study When considering aging, one cannot focus entirely on one protein because of how complex and mysterious the biological processes that drives aging are. Even though we

predict that VAChT regulates the deficits in cholinergic mediated systems in an aging model, there are many other factors that could drive the causes and effects of aging that we have seen in this study. For example, studies in the near future will aim to measure the levels of oxidative stress in the brain and intestinal inflammation in our aging *Vacht* mutants. Measuring barrier intestinal dysfunction in *Drosophila* has an advantage because there is a noninvasive procedure called the Smurf assay (Rera *et al*., 2012), which we aim to use. These experiments along with the experiments reported here will give us better understanding with how essential VAChT is with in the process of aging.

Analyzing the consequences of VAChT overexpression is currently underway. Our lab possesses different 10 lines that uses the GAL4-UAS system to target VAChT overexpression. To identify which genetic line expresses the most VAChT, we measured the protein levels of individual flies from each line using Western Blotting (Figure 6). The line that is shown to produce the highest VAChT expression will be used in future aging studies. In aging mice, VAChT overexpression was shown to improve spatial memory (Nagy and Aubert, 2015) but also increase neuromuscular aging (Sugita *et al.,* 2016). We need to determine what overexpression does to aging *Drosophila* and if this aging mechanism is conserved.

Critiques: Genetically, acquiring $Vacht^{4}$ rescue were not as easy as it was for the other 3 mutants. We are not entirely sure what causes the low number of rescues but as a result, our $Vacht^{4}$ population was not as much as we intended. In future studies, we plan to have at least double the amount of fly stocks for $Vacht^{4}$ to possibly overcome this challenge. In the average speed of locomotion data, there was not a noticeable decline in speed as the flies progressed in life, as it was hypothesized and shown in (Simon *et al*., 2006). However, the data presented is not all data that we will have. Data analysis using data recorded by the MWT is quite complex and

must be done by a third party, Dr. Raquel Abreu located at University of California, Santa Barbara. There is much more data to be analyzed that will possibly give us a better idea of the relation of VAChT and locomotion.

The survival data showed that only 65.5% of wildtype survive into adulthood, a relatively low number for wildtype. This could have been caused by sample size issue, and to address this, more experiments of on wildtype is underway. Many variables could have affected the locomotion data of the flies. The MWT often lost tracking of the flies when they had a burst of speed or stopped. When placed in the Sylgard dishes, the flies were given time to acclimate to the environment to reduce stress and allow for grooming. Even after the acclimation time was over however, the flies often stopped moving in order to groom or interact with the each other. The MWT is stationed in a room temperature setting, so it is possible that the fluctuation of the temperature may have had an impact on the movement of the flies. To combat the facility's controlled temperature, a portable heater/cooler and humidifier could be stationed in the room with the MWT.

An aim that we desired to be a part of this current study was to test the learning and memory capacity of our male *Vacht* mutants using the courtship assay (Ejima and Griffith, 2009). This assay was unable to be conducted in house due to space limitations but we hope our collaborators at Drexel University will be enable to conduct this experiment on our *Vacht* mutants.

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