VILNIUS UNIVERSITY

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Science project report

Characterizing the interactions between HMGCR and acetylcholine system in the regulation of Drosophila melanogaster locomotion

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Contents

Abbreviations

DAMS – Drosophila Activity Monitoring System

EH – flies with *HMGCR* knockdown in neurons (Elav-GAL4 > UAS-Hmgcr^{RNAi})

EW – flies with normal Hmgcr levels, used as control (Elav-GAL4 > w1118)

WH – flies with normal Hmgcr levels, used as control (w^{1118} > UAS-Hmgcr^{RNAi})

RT-PCR – Real Time PCR

HMGCR – 3-hydroxy-3-methylglutaryl coenzyme A reductase

SAMS – Statin associated muscle symptoms

CK – Creatine kinase

AchE – vertebrate acetylcholine esterase

Ace – fly's orthologue of acetylcholine esterase

CG4757 – fly's orthologue of acetylcholine esterase

CG4382 – predicted fly's orthologue of acetylcholine esterase

nAchR – nicotinic acetylcholine receptor

EGR1 – human early growth response protein 1

Sr – Fly's orthologue of EGR1

ADAM10 – human α-secretase

Kuz – predicted fly's orthologue of ADAM10

Kul – predicted fly's orthologue of ADAM10

Nrx1 – Neurexin 1

Nlg - neuroligins

Aim and tasks

The **aim** of this report is to build and study a molecular model explaining the relationship between HMGCR and acetylcholine system in the regulation of Drosophila melanogaster locomotion.

Tasks:

1) Make fly crosses with *HMGCR* knockdown in neurons *Elav-GAL4* > *UAS-Hmgcr*^{RNAi}, (from now on known as EH) and controls - *Elav-GAL4* > *w*^{1118} (from now on referred to as EW) and *w*^{1118} > *UAS-Hmgcr*^{RNAi} (from now on referred to as WH).
2) Using the Drosophila Activity Monitoring System (DAMS), compare locomotory behavior between EH and controls – EW, WH.
3) Test if the cholinesterase inhibitor neostigmine recovers impaired locomotion of the flies with HMGCR knockdown in the neurons.
4) Select *D. melanogaster* orthologues of human acetylcholine esterase for RNA expression measurements.
5) Using STRING protein interaction database and R package "iGraph", build a model, explaining the relationship between *HMGCR* and the acetylcholine system. Then, select key genes from the model for RNA expression measurements.
6) With quantitative real-time PCR (RT-PCR), in the EH flies and control flies (EW and WH), measure RNA expression of the following genes: *rpl-32*, *Ace*, *CG4382*, *CG4757*, *nAchR*, *Sr*, *Nrx*, *Kuz* and *Kul*.

Summary

Statins are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR). This group of drugs is used to lower low-density lipoprotein cholesterol (LDL-C) and is one of the most effective drugs in decreasing the incidence of cardiovascular disease (Newman Connie B. et al., 2019). However, one of the major obstacles in preventing these diseases is discontinuation of statin treatment due to adverse effects. Statin-induced muscle symptoms (SAMS), which makes up to 72% of all statin adverse effects, is a Major major cause of treatment discontinuation (Ward et al., 2019). However, the mechanisms that relate statin treatment to SAMS is not clear.

To study statin side effects, our lab used the model organism *Drosophila melanogaster* (further referred to as fly). Previous studies found that statin treatment impairs fly locomotory behavior (Williams lab, unpublished data). Therefore, it was presumed that this impaired locomotory behavior mimics the SAMS phenotype. This study tested the locomotory behavior of the fly model where HMGCR is knocked down in all neurons, which showed the impaired locomotory behavior. The findings led an investigation to elucidate the molecular reasons of impaired locomotory behavior, which presumably mimics statin-induced muscle symptoms.

The impaired locomotion was partially recovered with the cholinesterase inhibitor neostigmine, therefore, I investigated the RNA expression of three fly homologues of human acetylcholine esterase (Ace, CG4757, CG4382), nicotinic acetylcholine receptor and an orthologue of human early growth response 1 - Stripe. In parallel, I used STRING protein interaction data to build a model that relates neuronal HMGCR to the acetylcholine system. This analysis provided a model that links neuronal HMGCR to acetylcholine signaling in neuromuscular junctions. Thus, in the third part of this study I measured RNA expression of three key genes in this model – *Neurexin1* (*Nrx-1*), kuzbanian (*kuz*) and *Kuzbanian-like* (*Kul*). Although the results didn't confirm the expectations about this model, they gave projections to what should be further investigated.

Santrauka

Statiniai tai 3-hydroksi-3-metilglutaril koenzimo A reduktazė (HMGKR) baltymo inhibitoriai. Ši vaistų grupė yra naudojami kaip cholesterolį mažinantys vaistai ir yra vieni efektyviausių vaistų mažinant širdies ir kraujagyslių ligų skaičių (Newman Connie B. et al., 2019). Tačiau viena pagrindinių kliūčių šių ligų prevencijoje yra gydimo statinais nutraukimas dėl šalutinių poveikių. Statinų sukelti raumenų simptomai (SSRS) yra viena pagrindinių priežasčių nutraukiant gydymą statinais, nes jie sudaro 72% visų statinų sukeliamų šalutinių poveikių. Tačiau mechanizmai, kurie paaiškintų šių šalutinių poveikių priežastis, nėra aiškūs.

Šių mechanizmų išaiškinimui mūsų laboratorija naudoja *Drosophila melanogaster* (toliau vadinamos kaip muselės) modelį. Ankstesni tyrimai nustatė, kad statinai sutrikdo muselių judėjimą (M. Williams laboratorija, duomenys nepublikuoti) ir tai buvo interpretuota SSRS fenotipo mėgdžiojimas. Šioje studijoje buvo naudojamos muselės su nutildytu HMGKR muselės neuronuose ir jose nustatytas ryškus muselių judėjimo sumažėjimas. Tai leido tęsti tolimesnius tyrimus, siekiant išsiaiškinti molekulinius mechanizmus, paaiškinančius sutrikusį muselių judėjimą, kuris galimai mėgdžioja SSRS fenotipą.

Sutrikęs judėjimas buvo dalinai atstatytas su acetilcholino esterazės (AchE) inhibitoriumi, neostigminu. Dėl to padariau prielaidą, jog HMGKR nutildymas padidina muselės AchE ortologų (Ace, CG4382, CG4382) raišką ir mažina acetilcholino kiekį sinapsėse. Taigi, ištyriau šių genų RNR raišką, bei kitų acetilcholino sistemos dalyvių raišką – nikotino acetilcholino receptorių, ir Sr (Stripe). Papildomai, naudojant STRING baltymų sąveikos duomenis, sukūriau modelį, kuris sieja HMGKR su AchE ortologais vaisinėje muselėje. Taigi, trečiojoje tyrimo dalyje patikrinau šio modelio esminių genų RNR raišką – Neureksinas 1 (Nrx1), Kuzbian (Kuz), Kuzbian like (Kul). Rezultatai nepatvirtino modelio, tačiau davė kryptį tolimesniems tyrimams.

1. Introduction

1.1.HMGCR and statins

HMGCR is localized to the endoplasmic reticulum, where it synthesizes mevalonate. Unlike in humans ("UniProt," 2019), *Drosophila melanogaster* HMGCR doesn't participate in cholesterol synthesis. Since HMGCR does not participate in cholesterol synthesis, *Drosophila melanogaster* (further referred as the fly) becomes a preferable model to study statin effects not related with cholesterol synthesis. Also, although the fly's HMGCR is does not participate in cholesterol formation, it has other branches of metabolism in common with humans.

The direct product of HMGCR, mevalonate, is a precursor of isoprenoids, which are very important in post-translational modifications of small GTPases and other proteins, that allow protein-protein interactions or protein interactions with the lipid membrane. (Novelli & D'Apice, 2012). HMGCR inhibitors reduce isoprenilation of small G proteins of the Ras and Rho families. This prevents their activation and reduces the number of active GTPases in the cytoplasm. Thus, HMGCR is of great importance for cellular signal transduction (Doren et al., 1998). Statins are also associated with reduced radical formation (Desai et al., 2014), cell cycle and apoptosis (Matusewicz et al., 2015). HMGCR inhibitors are studied in various contexts as well. Statins are associated with increased risk of diabetes, reduced risk of Alzheimer's disease (Desai et al., 2014) and are also studied in cancer research (Matusewicz et al., 2015). This shows that HMGCR may play a role in various cholesterol unrelated pathways that might be shared between humans and flies.

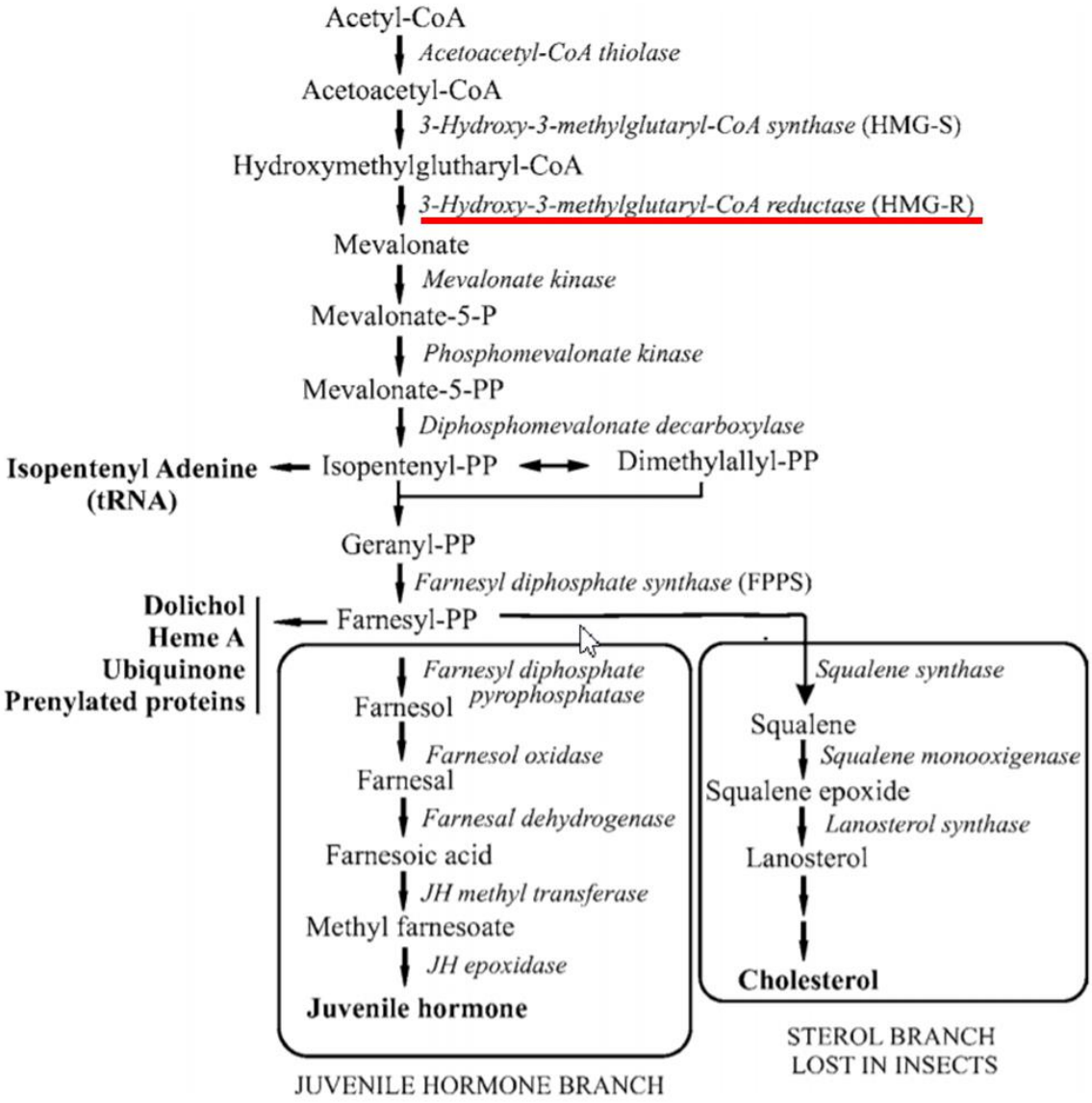

Figure 1 Insect HMGCR (underlined in red) does not participate in sterol synthesis. However it still has a role in mevalonate pathway and isoprenoid synthesis as in humans (Bellés et al., 2005)

1.2.Drosophila melanogaster model

Drosophila melanogaster is a popular model in modern biology and genetic studies. Findings often are done first in this model and later generalized in other animals or even humans. This model is still used in Nobel prize winning studies – the discovery of molecular mechanisms that control the circadian rhythm of *Drosophila*, won 2017 Nobel Prize in Physiology or Medicine (Yamaguchi & Yoshida, 2018). Comparison of human and fly genomes shows high homology (Yamaguchi & Yoshida, 2018). It is estimated that 75% of human disease related genes have orthologues in the fly (Reiter et al., 2001). Identity between human and fly orthologues is around 40% and conserved orthologues may have more than 80% of identity (Yamaguchi & Yoshida, 2018). Experiments with human or other animal models has its ethical and technical limitations. The fly overcomes these issues, since it has a very rapid life cycle, and its experiments don't require ethical permission. (Pandey & Nichols, 2011) In addition, the fly is a complex animal, regarding its organs and

behavior. The fly has similar structures to the vertebrate heart, gut, kidneys, lung and reproductive tract. Furthermore, the fly brain is composed of 100 000 neurons that form circuits, responsible for circadian rhythm, sleep, memory and learning, feeding, aggression, grooming, complex behaviors, *etc*. (Pandey & Nichols, 2011)

By knocking down neuronal HMGCR in the fly, we expected to mimic statin effects in humans. However, "the fly is not a miniature person". For complex phenomena, such as behavior or multifactorial human diseases, it takes only one gene to be absent in the organism and the results could be misleading. Potential differences in the pharmacokinetics, pharmacodynamics, metabolism and administration of the drug as well as physiological differences (such as blood brain barrier) must be considered (Pandey & Nichols, 2011). Therefore, the findings of this report will be associated more with observed phenotype of the knockdown flies, than with the statin side effects.

1.3.Statin side effects

Statins may cause a broad spectrum of side effects. These can be headaches, sleep impairments, nausea, arthritis (Banach et al., 2015), renal and liver toxicity or even novo-onset type 2 diabetes mellitus (Ward et al., 2019) as well as the most common side effects – statin induced muscle symptoms (SAMS). SAMS makes up to 72% of statin adverse events (Backes et al., 2017). It is a spectrum of symptoms from which most common are myalgia, rhabdomyolysis, myositis (Ward et al., 2019) These are further referred as myopathies (Desai et al., 2014).

Myopathies are neuromuscular disorders with the primary symptom of muscle weakness due to dysfunction of muscle fiber. Other symptoms include muscle cramps, stiffness, and spasms (*Myopathy Information Page | National Institute of Neurological Disorders and Stroke*, n.d.). Myalgia is muscle pain or weakness with no increase in creatine kinase (CK) expression. (Abd & Jacobson, 2011). Myositis covers muscle symptoms that have CK expression 10 times higher than the upper limit of normal range. (Abd & Jacobson, 2011) Rhabdomyolysis comprise muscle symptoms with marked CK elevation (typically greater than 10 times the upper limit of normal levels), creatinine elevation, brown urine and urinary myoglobin.(Abd & Jacobson, 2011). In addition, some studies show that statins may worsen myasthenia gravis – an autoimmune disorder caused by antibodies against nicotinic acetylcholine receptor (Gilhus, 2009).

Interestingly, HMGCR knockdown in different cells of the fly, results in a few phenotypes that may be related to statin side effects in humans – diabetes and muscle symptoms. HMGCR knockdown in a gland known as the *Corpus allatum* showed impaired dimorphic locomotor behavior and reduced body size (Belgacem & Martin, 2007). In our lab, researchers found that HMGCR knockdown in insulin producing neurons reduces fly size, as well (Williams lab, unpublished data). This reduced body size may be related to abnormal body mass index in humans that is related to a statin induced side effect - new-onset type 2 diabetes mellitus (Desai et al., 2014). In addition, the same team earlier found that Fluvastatin impairs male fly's locomotion,

as well (Belgacem & Martin, 2002). The observed impaired locomotion in Belgacem & Martin and our studies, may refer to another statin side effects – SAMS.

1.4. Acetylcholine system and Neostigmine

Acetylcholine

Acetylcholine is the chief neurotransmitter that passes signals from neurons to muscles. However, the cholinergic system is not restricted to neuromuscular junctions, it's also present in the central nervous system, as well. In addition to locomotion, acetylcholine is also important in memory and learning, therefore, it is significant in Alzheimer's disease. (The Editors of Encyclopaedia Britannica, 2019)

Acetylcholine is regulated via synthesis and release into the neuromuscular junction, choline uptake and binding. Release into the neuromuscular junction is triggered by incoming neural impulse that starts a Ca^{2+} influx, which triggers exocytosis of acetylcholine vesicles. In the neuromuscular junction, acetylcholine binds to its receptor (AchR) and is degraded by cholinesterases (e.g. AchE) (Taylor & Brown, 1999) . In muscle cells, activated AchR induces transcription of AchE, thus creating a negative feedback loop of acetylcholine signaling (Kammer et al., 1998). After AchE breaks down acetylcholine, neurons reuptake choline from neuromuscular junctions and choline acetyltransferase anabolizes it back to acetylcholine. Reuptake is a rate limiting step in acetylcholine synthesis (Taylor & Brown, 1999).

As mentioned before, in flies, statin treatment and HMGCR knockdown impairs locomotion. Our lab also ordered RNA sequencing of flies with neuronal HMGCR knockdown (Williams lab, unpublished data) that showed an increased expression of the *CG4757* transcript - an orthologue to human acetylcholine esterase (AchE) (Thurmond et al., 2019). Since, AchE breaks down acetylcholine in the synapse, we presume that *HMGCR* knockdowns elevate expression of AchE orthologues that decreases acetylcholine levels in neuromuscular junctions, which impairs fly locomotion. Therefore, part of this report, focused on AchE orthologues that participate or might participate in acetylcholine regulation.

AchE orthologues in Drosophila melanogaster

An orthologue search of the "Flybase" database (Thurmond et al., 2019) found that flies have at least 3 AchE homologues: Ace, CG4382 and CG4757. Ace was the best match and is referred as the acetylcholinesterase of the fly (Thurmond et al., 2019). CG4382 is 2nd best match and has carboxylic ester hydrolase activity and is expressed in wing disc (Butchar et al., 2012). The 3rd homologue, CG4757, of Ace participates in Glycerophospholipid metabolism, by breaking down acetylcholine (KEGG database) (M. Kanehisa & Goto, 2000). Further predicted orthologues were ignored, due to their cellular location (Nrt is membrane protein, Jhe is mitochondrial protein) or due to insufficient information (α-Est1, α-Est2, α-Est6). In this report, the chosen orthologues have been studied for RNA expression.

Transcription factors of AchE

We were also curious about the reason of elevated *CG4757* RNA expression when *Hmgcr* is knocked down in the nervous system. Various experiments on human and mouse cell lines showed that the *AchE* promotor has a binding site for the early growth response (EGR1) transcription factor and its expression dependence on this transcription factor (Nitsch et al., 1998), (Mutero et al., 1995). Furthermore, a positive correlation between *EGR1* and *AchE* RNA expression was found in human and mouse frontal cortex (Y.-T. Hu et al., 2019). Therefore, we looked for orthologues of EGR1 that could explain increased expression of the CG4757 esterase.

BLAST (Altschul et al., 1997) and "Flybase" (Thurmond et al., 2019) tools showed that the best fly EGR1 orthologue, Stripe (Sr), has 76,3% identity in Zn finger DNA binding domain. These findings, led us to hypothesize that neuronal *Hmgcr* knockdown could induce *Sr* expression, which would elevate expression of the AchE orthologues, thus reducing acetylcholine levels in the pre-synapse – leading to impaired fly locomotion.

Neostigmine

When AchE expression is elevated, acetylcholine levels can be increased by inhibiting its breakdown in neuromuscular junction. Neostigmine is a reversible AchE inhibitor that indirectly increases acetylcholine levels in the synapse. It was one of the most used AchE inhibitors in clinical practice, in 2009 (Srivastava & Hunter, 2009). One of its applications is the treatment of myasthenia gravis – one of the statin associated muscular symptoms (Mantegazza et al., 2011).

Neostigmine was used in experiments with the flies (Rajaram et al., 2005). Here researchers treated flies with 0.168 mM Neostigmine concentration, to study their visual system. This was helpful in the experiments of recovering the impaired locomotion in flies with neuronal *HMGCR* knockdown.

2. Methods

2.1.Fly maintenance

Flies were maintianed on "Drosophila fly food Jazz-mix "(Fisher Scientific, Göteborg, Sweden) food, mixed with baker's yeast (0,8%). The environmental conditions were: 12h:12h dark: light cycle, 20% humidity, 25°C temperature.

Three fly lines were used to make experimental and control crosses. The strain description and gender are denoted in Table 1**.**

Strain name	Description	Source
Elav-Gal4	Has a Gal4 protein gene with a neuron specific promoter - Elav.	Bloomington Stk #458
UAS-HMGCR	Has a HMGCR siRNA gene, with UAS enchanter sequence	Bloomington Stk #50652
w^{1118}	Has a w^{1118} mutation in *white* gene. It is used to make control crosses.	Bloomington Stk #5905

Table 1 Fly lines used to make control crosses and crosses with neuronal HMGCR knockdown.

2.2.Fly crosses and maintenance

System of neuronal HMGCR knockdown

To make flies with neuronal *HMGCR* knockdown, we used UAS/Gal4 binary system. We crossed flies containing *Elav-Gal4* DNA sequences with flies containing UAS-$RNAi^{HMGCR}$ DNA sequences. Elav is a protein that is exclusively expressed in neurons (Yao & White, 1994). Right next to *Elav*, the gene of *GAL4* is inserted. Gal4 is a yeast transcription factor that has a specific target sequence – upstream activation sequence (*UAS*) (Brand et al., 1994). The $RNAi^{HMGCR}$ is RNA that interferes with *HMGCR* trenscript thus knocking down the expression of HMGCR protein. In F1 generation, when neuron specific *Elav* is expressed, so is *Gal4*. Consequently, Gal4 binds to its target sequence of *UAS* and starts the trancription of $RNAi^{HMGCR}$ (St Johnston, 2002). The transcript interferes with *HMGCR* transcript and the the expression of neuronal HMGCR is knocked down and protein expression is reduced.

In addition, the expression of Gal4 is temperature dependent, therefore the expression of target gene can be changed in different developmental stages (Brand et al., 1994). Since neuronal HMGCR knockdown is lethal, we used this property to knockdown HMGCR specifically in adult stage.

In addition, two control crosses were made and used in all experiments: Elav-Gal4 > W^{1118} (EW) and UAS-HMGCR > W^{1118} (WH). The further details are described in the Table 2**.**

F1 generation names	P generation	Phenotype of male F1 progeny
EH	♀ Elav-Gal4 x ♂ $RNAi^{HMGCR}$	Neuronal HMGCR knockdown, Red eyes, Straight wings.
EW	♀ Elav-Gal4 x ♂ W^{1118}	Red eyes, Straight wings.
WH	♀ W^{1118}-Gal4 x ♂ $RNAi^{HMGCR}$	White eyes, Straight wings.

Table 2 Description of F1 generation. Short name, sex and genotype of parents (P generation) and phenotype of male progenies.

To make crosses, we collect female virgins – ones that are no older than 2 hours – and incubate them in the vial with food for 3 days. If larva does not show up, their virginity is confirmed. Then, 30 females and 15 males are transferred to propylene square bottles (170ml) with food and closed with cellulose acetate plug. These bottles were transferred to +18° incubator where flies mated and laid eggs for 6-10 days. (After it, P generation was transferred to a new bottle and left to mate in the same conditions). The F1 generation hatched after 18 days and were collected every 6 days (to make sure that flies are no older than 6 days).

For all experiments we used male flies of all 3 strains, with the phenotypes described in the Table 2. EH strain had neuronal HMGCR knockdown and EW, WH were used as controls.

2.3.Fly treatment

UAS-Gal4 system's activity is temperature dependent. All crosses, that were grown in 25 °C died, therefore, P and F1 generations were incubated in 18°C. Male adults, with EH,EW and WH genotype, no older than 6 days, were collected and incubated in 29 °C for 2 full days. This incubation decreased HMGCR expression in EH flies that have *UAS-Gal4* and $RNAi^{HMGCR}$ genes.

Flies for DAMS experiment, were incubated with food, containing (or not containing) 0,1ml neostigmine bromide (0,1mM)(Merck, Darmstadt) for 72h. Part of the flies were incubated with DAMS system to monitor their locomotory activity. Another part of flies, after incubation in 29 °C, were frozen in -80 °C for further use in RT-PCR experiments.

2.4.DAMS

Drosophila activity monitoring system (DAMS) is a tool to monitor circadian rhythms, sleep behaviors and general locomotory activity. The system is composed of DAM2 monitor (TriKinetics ®) with 32 slots for 32 plastic tubes (Figure 2A) . A fly is placed into each tube, which is sealed with food in one end and cotton in another one (Figure 2B). The prepared tubes are placed in the monitor, so their center intersect with monitor. Here, the monitor beams infrared laser and when the fly passes the infrared laser, DAMS registers it as one move (Pfeiffenberger et al., 2010). Schematics of DAMS is depicted in.

In my experiments, I sealed tubes with food, containing 100 µL neostigmine bromide (0,1mM) or 100 µL of water, instead. Then, placed them into monitors and incubated in the 29 °C for 4 days. After 4 days I collected the data of their activity, which later was processed by manufacturers program and MatLab 2018.

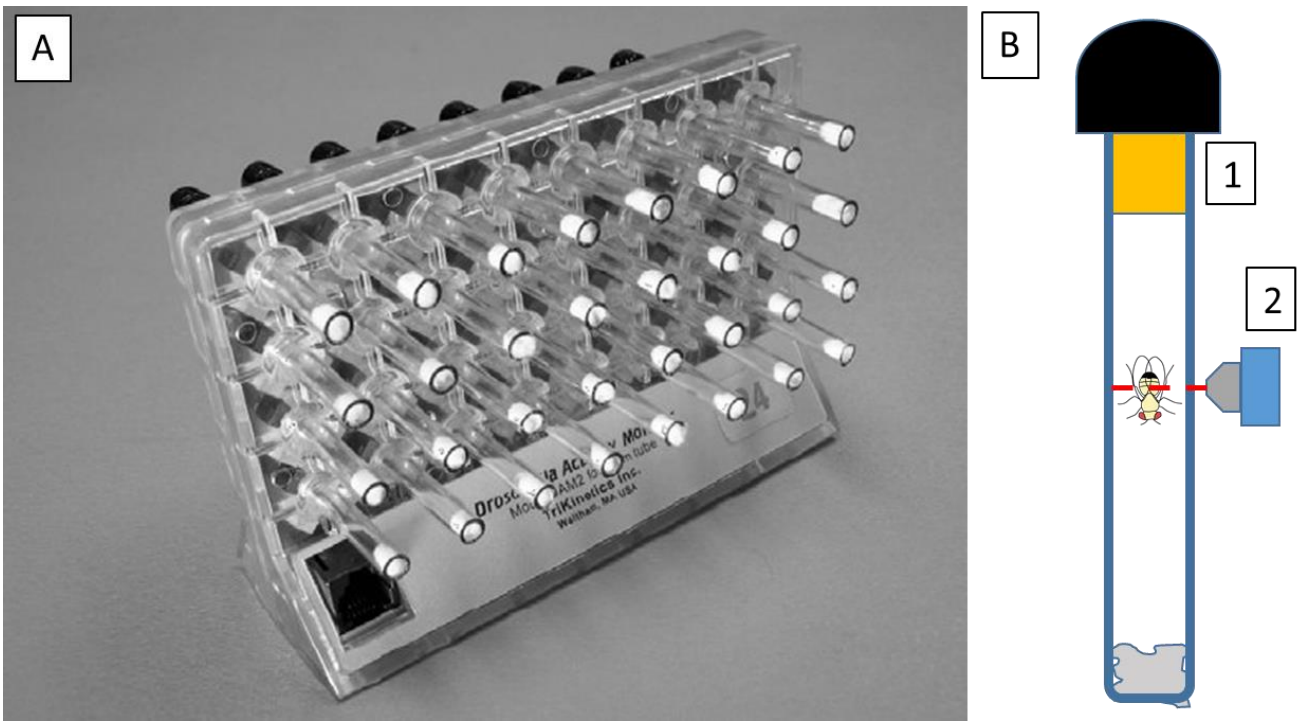

Figure 2Drosophila activity monitoring system (DAMS). (A) DAMS monitor to record fly's activity. (B) system components: (1) Tube sealed with food and cotton, (2) infrared beam detector in the monitor. When fly passes the beam, DAMS records it as one move.

2.5.Sample preparation and PCR

Adult F1 males, no older than 6 days, was frozen in -80ºC. On the day of RNA extraction, frozen flies were vortexed in 50ml vial to separate heads from the body. Bodies and heads were sorted on dry ice and transferred to ependorf tubes.

For RNA extraction, we use Trizol protocol, optimised for fly samples:

1. For 10 bodies (or 25 heads), we add 60uL Trizol and homogenize with automatic pestle and add additional 650 uL Trizol (for heads - 400 uL). The homogenate is incubated in room temperature (RT) for 5 min.
2. Then we add 160 uL of chloroform, vortex, and incubate for 5 min at RT and centrifuge for 12 min (14,000 rpm, 4 °C)
3. The upper fraction of contents (~200 - 250 µL) is transfered to a new tube. We add 450 uL isopropanol and incubate in -20 °C for 30 min.
4. We centrifuge the tubes for 15 min (14,000 rpm, 4°C), remove supernatant, and the precipitate is washed with 900 uL ice cold 75% ethanol, by repeating this step 3 times
5. We dry the pellet for 20 min in (RT) and add 20 uL DEPC-water and vortex to resuspend the RNA.
6. Finally, the RNA concentration and purity is measured.

The cDNA is synthesised with “High-Capacity RNA-to-cDNA™ Kit” (Applied Biosystems, Vilnius) by manufacturer’s protocol. Finally, the samples are diluted in water with (ratio 1:30).

In the end, for each line I made 6 body and 6 head samples.

2.6.RT-PCR

RT-PCR was per performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Vilnius), using manufacturers protocol. *Rpl32* was used as reference gene. All samples were repeated 3 times.

Primers were modeled by FlyPrimerBank (Y. Hu et al., 2013) and are listed below in the Table 3:

Gene name	Fw primer 5'-3'	Rv primer 5'-3'	Determined Ta
*Rpl32**	AGCATACAGGCCCAAGATCG	TGTTGTCGATACCCTTGGGC	60
Hmgcr	CCTGAATGTGAGCAATAATC	CACCAAATAGTTGCCATT	56,9
Ace	AGGTGCATGTCTACACGGG	CGTCACGTAGGATCATCACCC	55.7
nAChR beta 1	TGGAGTCTTCCTGCAAATCCT	CGAAGTAGCTCATCGGATGTG	55.7
CG4757	CCAAGACGGTCAAAAACGA	GATGTTGGCTCCCGAGATA	55.7
CG4382	TCTATGCCTTTCGTGGAATACCA	TCCAGTGTATCAAACCATTGCTC	59.5
Kuz	ACAATGGGGTATCGAGGGTA	CCGTGCGATTTGTGGGAAA	59.5
Kul	GGCAGAAGAGAGAGGTGACC	CTGTCGTGGGCGGAGAAAT	55,7
Nrx-1	TTACTAATACAGCCGACTC	CTTAATGTTCAGCACCTTC	56,9
Sr	AAGGGCTTGAAACCCTGGTG	CGAAGCTCAGCACATTGAAGTG	59.5

*Table 3 Genes studied for RNA expression, primer pairs and optimized annealing temperatures (Ta). *Rpl32 was used as a reference gene.*

For RT-PCR we use "iQ5 Multicolor Real-Time PCR Detection System" (Bio-Rad ®). Optimal annealing temperature (Ta) for listed primers was found by running manufacturers RT-PCR program using mixes of EH, EW, WH samples. Ta was picked based on melting curve and lowest CT value. The optimal Ta temperature is further added to the RT-PCR program to measure cDNA levels of target gene in 3 replicates of each sample.

2.7.Network analysis

STRING database integrates data of direct (physical) and indirect (functional) protein-protein interactions that collectively are called "functional associations". These interactions are defined by seven "channels": co-expression, text mining, biochemical/genetic data, pathway databases; and predictions of 3 genomic contexts: neighborhood, fusion and gene co-occurrence. Each channel is subdivided in two groups of evidences – ones from the organism of interest and ones transferred from other species, called "interlogs". All evidences evaluated by score, ranging from 0 to 1. These scores represent STRING's confidence that about the existence of given interaction. (Szklarczyk et al., 2019)

To find shortest possible molecular pathway between HMGCR and CG4757, I used "Protein-Protein Interaction Networks Functional Enrichment Analysis" tool (further referred as STRING online tool) and iGraph R package to analyze STRING interaction dataset.

From https://string-db.org/ I downloaded STRING dataset (7227.protein.actions.v11.0.txt.gz (5.8 Mb)) that contains possible functional interactions between fly's proteins. This dataset contains 7 columns where 2 columns define STRING identifiers of interacting proteins, and 5 columns describe the interaction:

- mode – type of interaction ("reaction", "expression", "activation", "ptmod"(post-translational modifications), "binding", "catalysis")
- action – describes outcome of interaction (inhibition or activation)
- a_is_acting – states the direction of interaction.
- is_directional – states if interaction is directional
- score – states STRING's best combined score of all interactions.

The dataset contains STRING names for proteins, which is very inconvenient. Therefore, I used 7227.protein.aliases.v11.0.txt.gz dataset, to change STRING names into official gene symbol in interaction dataset. The code is provided below:

```
# Changing names in interaction dataset (all_paths_STRING); from STRING ID to Official gene name. As a source of Official gene names, I used STRING dataset, where STRING names are related to Official gene name (7227.protein.info.v11.0.txt.gz (688 Kb).

string_ids_a<-string_ids %>% rename(item_id_a = protein_external_id, preferred_name_a=preferred_name)
string_ids_b<-string_ids %>% rename(item_id_b = protein_external_id, preferred_name_b=preferred_name)

all_paths_names_a<-merge(as.data.frame(string_ids_a),as.data.frame(all_paths_STRING), by = c("item_id_a"))
all_paths_names_b<-merge(as.data.frame(all_paths_names_a),as.data.frame(string_ids_b), by = c("item_id_b"))

all_paths_names_b<-all_paths_names_b[,c(1,2,3,9,4,5,6,7,8)]

# Renaming the output dataframe to more convenient name - all_paths
all_paths <-all_paths_names_b
```

The output dataset (all_paths_STRING_full) I used as input for network analysis with iGraph package in R.

```
library(igraph)
# Creating an edgelist - dataframe that contains only interaction data. This is required by iGraph.
g<-graph_from_edgelist(as.matrix(all_paths[,3:4]))

# Code to find shortest pathway between proteins of interest (Hmgcr and CG4757)
 g_shortest<-get.all.shortest.paths(g, from="Hmgcr", to= "CG4757", mode="all")
 sub_graph_i<- induced.subgraph(g, g_shortest)
# Code to plot the output pathway
plot(sub_graph_i)

#Attaching full interaction data (all_paths_STRING_full) to the output pathway data table (the_path).
```

```
the_path<-as_edgelist(sub_graph_i, names=TRUE)
colnames(the_path)<-c("preferred_name_a", "preferred_name_b")
the_path_STRING<-merge(the_path, all_paths_STRING_full,
by=c("preferred_name_a","preferred_name_b"))
```

The output dataset contains information about interactions but does not contain references, nor details about interaction. Therefore, I pasted all proteins from the output file to online STRING online tool. This tool provided references, describing interactions provided by iGraph and STRING online tool.

Finally, to build a model, additional literature analysis was made. Since most of the interactions had evidences from other organisms, I made additional literature analysis, to confirm that orthologues in the fly have similar function. With this information, I formed a model, explaining HMGCR relationship with acetylcholine system as well as results, from behavior data.

2.8. Data processing and statistics

DAMS

DAMS data was extracted using manufacturers program. The data of last 4 days was extracted in 30 min intervals averaged and further processed with manufacturers package in MATLAB. Flies that were found dead were excluded.

Statistics was calculated with GraphPad Prism 6. One way ANOVA (Tuckey post-hoc, Games-Howel correction) was used to calculate significant differences between EH experimental group and EW, WH controls. To measure differences between neostigmine treated and not treated groups, Wilcoxon matched pairs signed rank test was employed.

RT-PCR

RT-PCR data was processed with MyIQ 1.0 software (Bio-Rad, CA, USA). If the Ct difference between one replicate and other two was greater than 0.5, this replicate was removed. For further calculations, I used at least two replicates from each gene. Means of these replicates were used to calculate expression with Pfaffl method (Pfaffl, 2001). EW control was used as reference sample for EH experimental group and WH control. Rpl32 was used as reference gene. Finally, the expression results were tested for outliers using Grubbs' test.

To compare expression in EH, EW and WH samples I used R package “FDA” and non-parametric Kruskal-Wallis method. If this method showed significant difference between groups ($p<0.1$), Dunn’s post hoc test with Holm’s procedure was applied – this revealed which groups had significant difference. Graphs were generated using GraphPad Prism 5.

3. Results

3.1.Locomotory activity measurements

Locomotion impairment

HMGCR knockdown in the neurons, reduceses fly's locomotion. One way ANOVA (Tuckey post-hoc, Games-Howel correction) confirmed that Elav>UAS-HMGCR ^{RNAi} (EH) flies had lower locomotory activity during whole experiment ($p<0.01$, N=45) than controls: UAS-Hmgcr^{RNAi}>w1118 (WH), N=43 and Elav-Gal4>w1118 (EW), N=46 (Figure 3A). Same statistical model also showed that EH had lowest total movement count ($p<0.001$, ***) (Figure 3B).

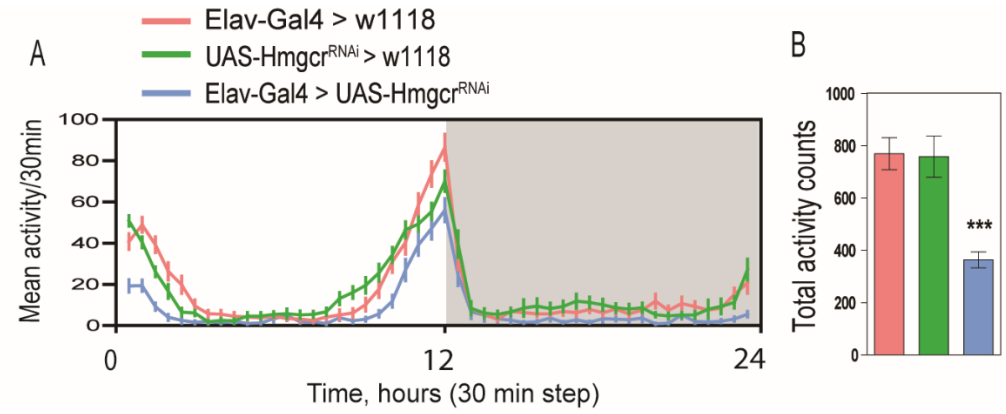

*Figure 3 Neuronal HMGCR knockdown decreased fly's locomotion. Elav>UAS-Hmgcr^{RNAi} is experimental line with neuronal HMGCR knockdown (blue, N=45) and controls (UAS-Hmgcr^{RNAi}>w1118, N=43; Elav-Gal4>w1118, N=46). (A) Average fly movement counts each 30min. Average of total moves from 3 experiment repeats (3 days each). White area (0-12h) – light time, grey area (12-24h) – dark time. One way ANOVA (Tuckey post-hoc, Games-Howel correction) showed EH has lowest activity during days and nights ($p<0.01$) (B) Average and SEM of total count of fly moves from 3 experiment repeats (3 days each). One way ANOVA (Tuckey post-hoc, Games-Howel correction) showed EH flies did least moves ($p<0.001$, ***) during the time of experiments.*

Locomotion recovery with neostigmine

When moves per hour were compared (Wilcoxon matched pairs signed rank test) between neostigmine treated EH flies (N=48) and non-treated EH flies (N=45), former had significantly higher locomotion ($p<0.001$,***). In EW group, neostigmine treated group (N=44) had significantly lower ($p<0.001$, ***) locomotion than non-treated group (N=45). In WH group, treatment resulted in no significant difference in locomotion.

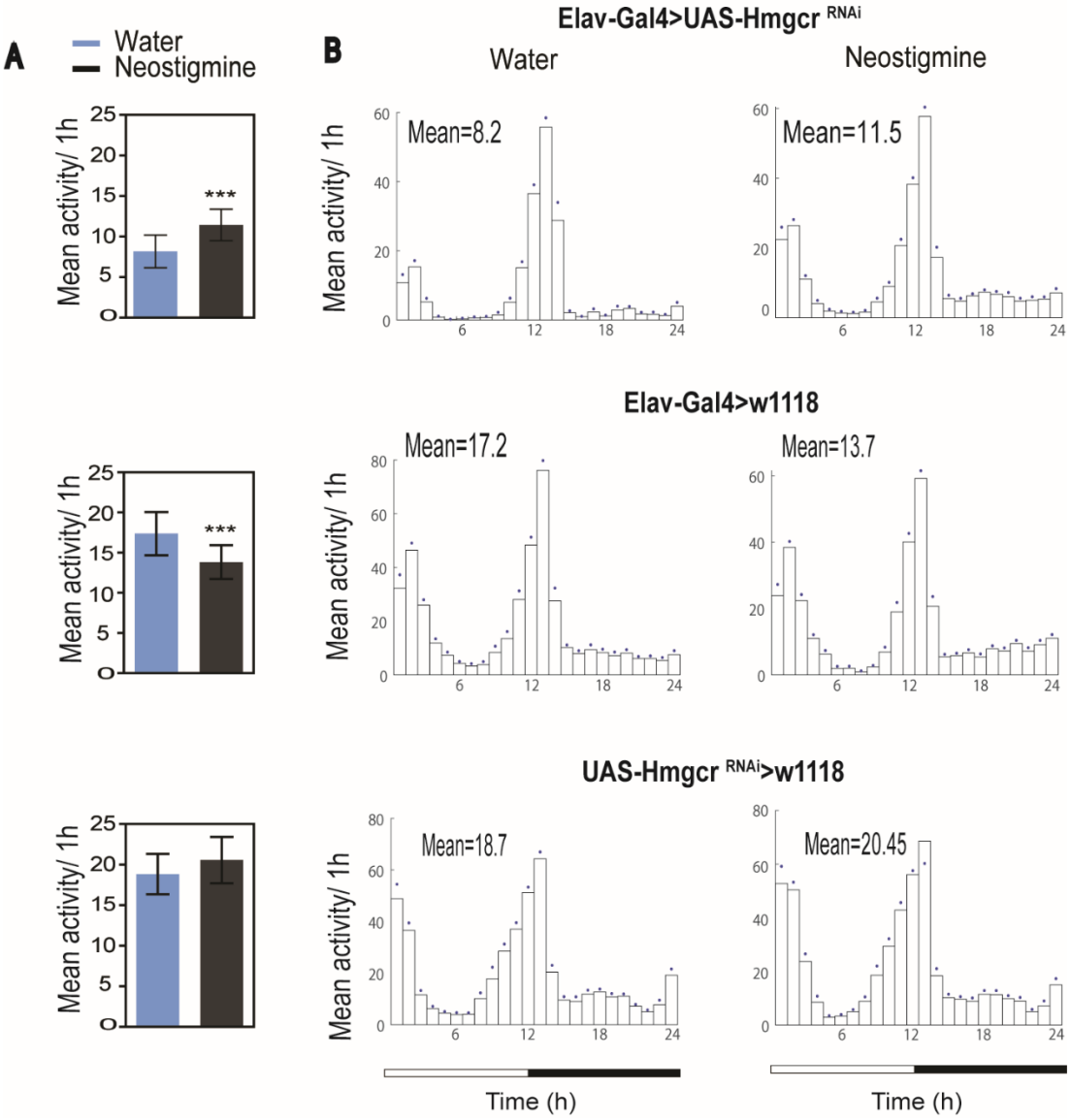

Figure 1 Locomotion monitoring of each hour showed partially recovered locomotion in neostigmine treated flies. Non-treated group (Water): knockdown flies Elav>UAS-Hmgcr^{RNAi} (EH, N=45) and controls, Elav-Gal4>w1118 (EW, N=46), UAS-Hmgcr^{RNAi}>w1118 (WH, N=43). Neostigmine group: EH (N=48), EW (N=39), WH (N=44). ***(A)*** *Averages from 3 experiments of moves per hour (mean and SEM). Wilcoxon matched pairs signed rank test showed that neostigmine partially recovered impaired locomotion of EH flies (p<0.001,***),reduced locomotion in EW flies (p<0.001,***) and made no significant change in WH fly locomotion.****(B)*** *Average locomotory activity each hour. White and black lines at the bottom of the figure represent light and dark periods (day and night). Mean states an average of hourly activity count that is depicted in Figure A.*

3.2.RT-PCR: HMGCR expression

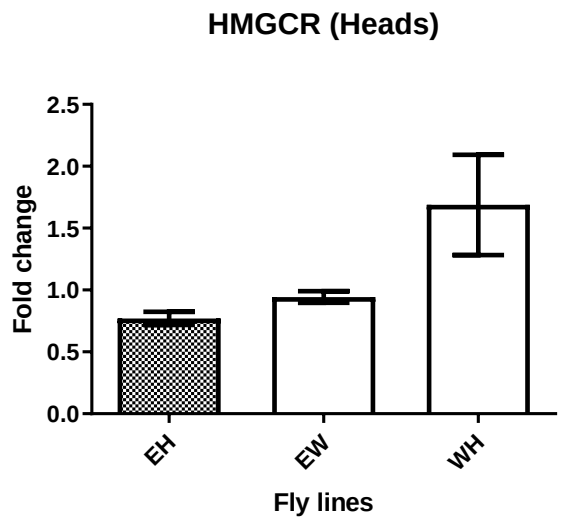

Figure 4 RT-PCR data. HMGCR RNA expression difference (Mean and SD)in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). HMGCR expression is not significantly lowest in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 16) = 4.88,$$
$$p = 0.087$$

Dunn's post-hoc analysis with Holm method showed no significant difference between experimental line (EH) and controls (Table 4)

Comparison	Z	P.unadj	P.adj
EH - EW	-1.860	0.063	0.126
EH - WH	-1.989	0.047	0.140
EW - WH	-0.046	0.963	0.963

Table 4. Dunn's post hoc analysis results of RT-PCR data from heads of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	0.770	0.118	0.726	0.120
EW	5	0.943	0.105	0.942	0.038
WH	6	1.688	0.992	1.597	1.703

Table 5. Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR)

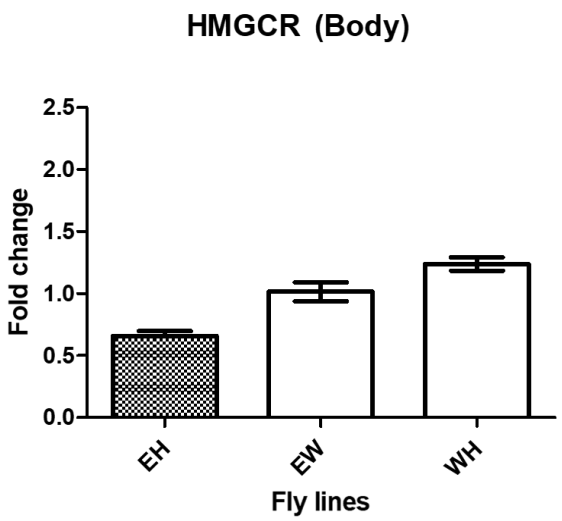

Figure 5 HMGCR RNA expression difference (Mean and SD)in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). HMGCR expression is significantly lowest in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 17) = 11.39,$$
$$p = 0.003$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and controls (Table 6).

Comparison	Z	P.unadj	P.adj
EH - EW	-2.006	0.045	0.090
EH - WH	-3.368	0.001	0.002
EW - WH	-1.429	0.153	0.153

Table 6. Dunn's post hoc analysis results of RT-PCR data from bodies of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	0.660	0.090	0.653	0.060
EW	6	1.015	0.188	1.044	0.322
WH	6	1.239	0.133	1.230	0.069

Table 7. Descriptive statistics of RT-PCR data from bodies of the flies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR)

3.3.RT-PCR: AchE orthologues, nAchR subunit β and Sr

Acetylcholine esterase (Ace) expression

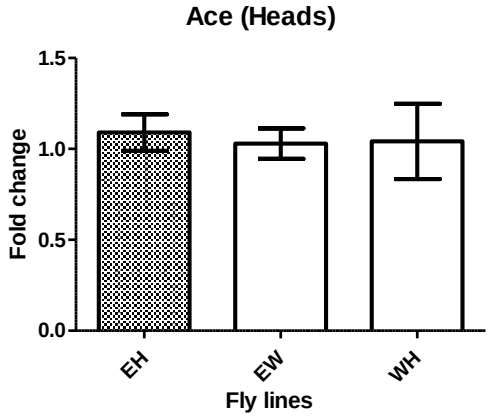

Figure 6 Ace RNA expression difference (Mean and SD)in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). Ace expression is not significantly different in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc)

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 16) = 5.8,$$
$$p = 0.055$$

Dunn's post-hoc analysis with Holm method did not show significant difference between experimental line (EH) and controls (table 5).

Comparison	Z	P.unadjusted	P.adjusted
EH - EW	-0.332	0.740	0.740
EH - WH	1.862	0.063	0.125
EW - WH	2.208	0.027	0.082

Table 8. Dunn's post hoc analysis results of RT-PCR data from heads of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	1.004	0.267	0.867	5
EW	5	1.010	0.160	1.006	5
WH	6	0.619	0.304	0.664	6

Table 9 Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

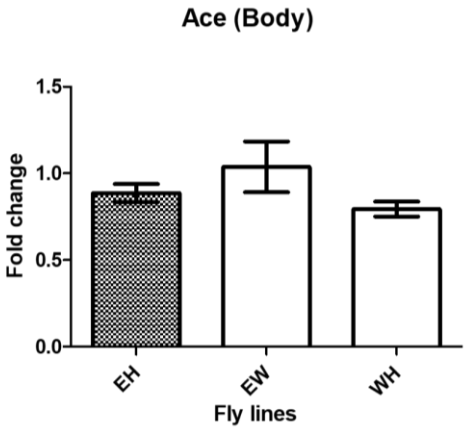

Figure 7 Ace RNA expression difference (Mean and SD)in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=6 and WH, n=6). Ace expression is not significantly different in EH samples (p>0.1, Kruskal – Wallis).

Differences between groups are statistically insignificant (p>0.1).

$$Kruskal - Wallis, \chi^2(2, n = 17) = 3.84,$$
$$p = 0.15$$

Fly line	n	mean	sd	median	IQR
EH	5	0.886	0.115	0.920	0.150
EW	6	1.032	0.294	0.971	0.150
WH	6	0.782	0.091	0.743	0.066

Table 10. Descriptive statistics of RT-PCR data from bodies of the flies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

CG4382 carboxylic esterase expression

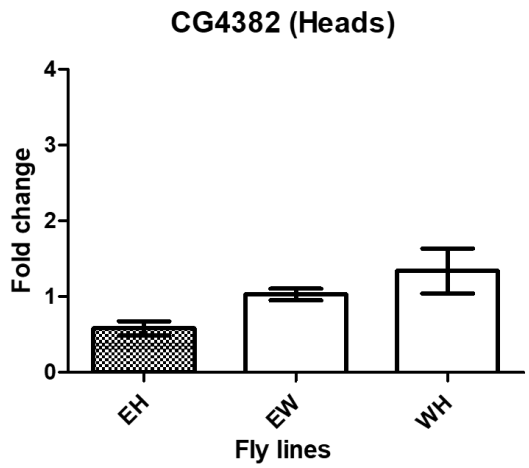

Figure 8 CG4382 RNA expression difference (Mean and SD) in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=6 and WH, n=6). CG4382 expression is significantly lowest in EH samples (p<0.1, Kruskal – Wallis, Dunn's post-hoc)

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 17) = 6.81,$$
$$p = 0.033$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and controls. (table 8).

Comparison	Z	P.unadj	P.adj
EH - EW	-2.47428	0.013	0.040
EH - WH	-2.04431	0.041	0.082
EW - WH	0.450956	0.652	0.652

Table 11. Dunn's post hoc analysis results of RT-PCR data from heads of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	0.580	0.303	0.596	0.242
EW	6	1.027	0.265	0.929	0.191
WH	6	1.338	1.025	1.141	1.600

Table 12 Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

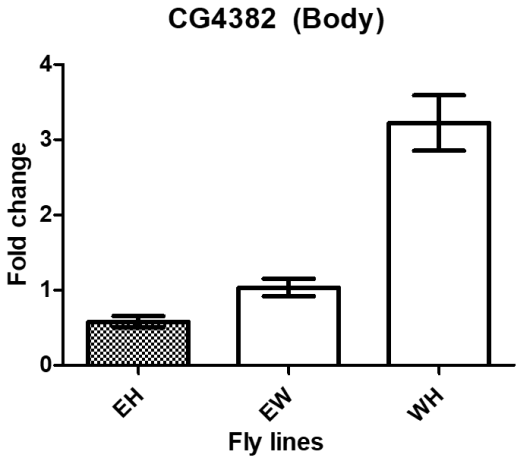

Figure 9 CG4382 RNA expression difference (Mean and SD) in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=4). CG4382 expression is not significantly lowest in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 14) = 10.06,$$
$$p = 0.002$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and WH control (table 10).

Comparison	Z	P.unadj	P.adj
EH - EW	-1.436	0.151	0.151
EH - WH	-3.172	0.002	0.005
EW - WH	-1.817	0.069	0.138

Table 13 Dunn's post hoc analysis results of RT-PCR data from bodies of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	0.576	0.167	0.650	0.200
EW	5	1.032	0.263	1.070	0.149
WH	4	3.221	0.738	3.397	0.662

Table 14 Descriptive statistics of RT-PCR data from bodies of the flies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

CG4757 carboxylic esterase expression

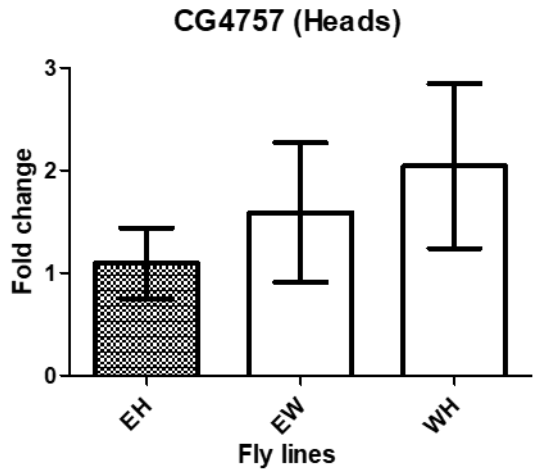

Figure 10 CG4757 RNA expression difference (Mean and SD) in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=6 and WH, n=6). CG4757 expression is not significantly different in EH samples (p>0.1 Kruskal – Wallis).

Differences between groups are not statistically significant. (p>0.1)

$$Kruskal - Wallis, \chi^2(2, n = 16) = 0.16, p = 0.921$$

Fly line	n	mean	sd	median	IQR
EH	5	1.094	0.772	0.984	0.833
EW	6	1.591	1.521	0.729	2.720
WH	6	2.044	1.966	1.743	2.341

Table 15. Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

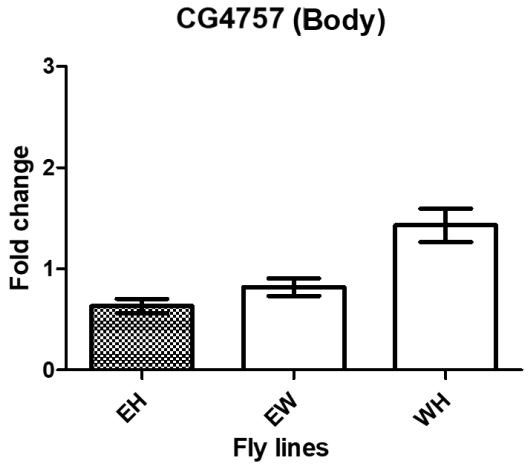

Figure 11 CG4757 RNA expression difference (Mean and SD) in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=3) and controls (EW, n=4 and WH, n=5). CG4757 expression is not significantly lowest in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant. (p<0.1)

$$Kruskal - Wallis, \chi^2(2, n = 12) = 8.79, p = 0.012$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and WH control (table 13).

Comparison	Z	P.unadj	P.adj
EH - EW	-0.847	0.397	0.397
EH - WH	-2.785	0.005	0.016
EW - WH	-2.067	0.039	0.077

Table 16 Dunn's post hoc analysis results of RT-PCR data from bodies of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values

Fly line	n	mean	sd	median	IQR
EH	3	0.632	0.125	0.646	0.124
EW	4	0.820	0.175	0.865	0.154
WH	5	1.431	0.367	1.401	0.364

Table 17 Descriptive statistics of RT-PCR data from bodies of the flies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

Nicotinic acetylcholine receptor (nAchR) expression

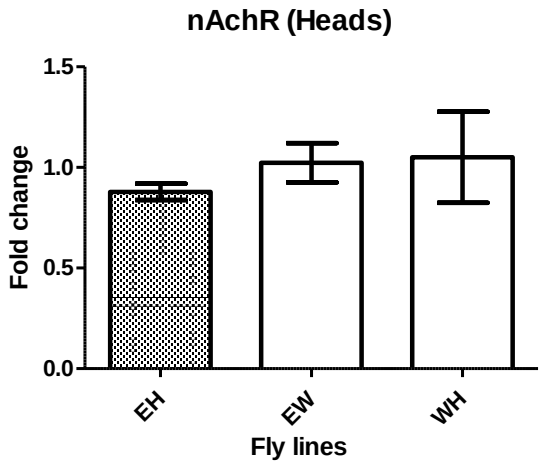

Figure 12 nAchR RNA expression difference (Mean and SD) in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=6 and WH, n=6). nAchR expression is not significantly lowest in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically insignificant.

$$Kruskal - Wallis, \chi^2(2, n = 17) = 0.44,$$
$$p = 0.825$$

Fly line	n	mean	sd	median	IQR
EH	5	0.878	0.091	0.844	0.092
EW	6	1.024	0.238	1.013	0.392
WH	6	1.050	0.554	0.949	0.886

Table 18. Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

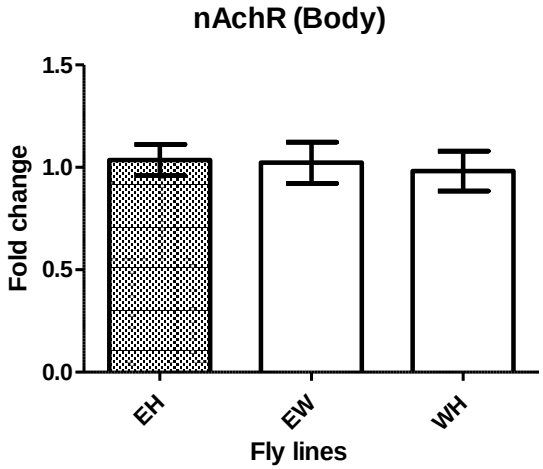

Figure 13 nAchR RNA expression difference (Mean and SD) in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=4) and controls (EW, n=6 and WH, n=6). nAchR expression is not significantly different in EH samples (p>0.1, Kruskal – Wallis).

Differences between groups are statistically insignificant.

$$Kruskal - Wallis, \chi^2(2, n = 16) = 0.89,$$
$$p = 0.63$$

Fly line	n	mean	sd	median	IQR
EH	4	1.036	0.151	1.032	0.100
EW	6	1.023	0.246	0.960	0.174
WH	6	0.982	0.239	0.893	0.090

Table 19. Descriptive statistics of RT-PCR data from bodies of the flies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

Stripe (Sr) expression

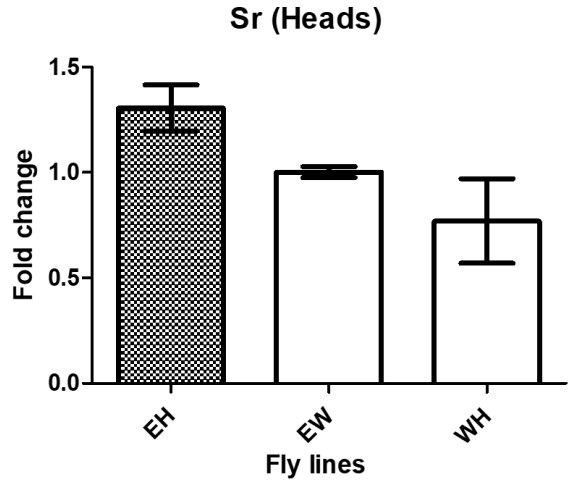

Figure 14 Sr RNA expression difference (Mean and SD) in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). Sr expression is not significantly highest in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc)

RT-PCR of body samples, didn't show Sr RNA expression. Experiment was repeated twice.

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 16) = 5.44,$$

$$p = 0.066$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and WH control. (table 17).

Comparison	Z	P.unadj	P.adj
EH - EW	1.860	0.063	0.126
EH - WH	2.174	0.030	0.089
EW - WH	0.231	0.817	0.817

Table 20 Dunn's post hoc analysis results of RT-PCR data from Heads of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	1.306	0.245	1.183	0.247
EW	5	1.001	0.059	0.996	0.047
WH	6	0.770	0.492	0.635	0.815

Table 21 Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

3.4.Building a model from STRING interaction data

STRING online tool for protein interactions showed no connection between HMGCR and CG4757 (Figure 15A) and after increasing number of nodes 4 times (Figure 15B).

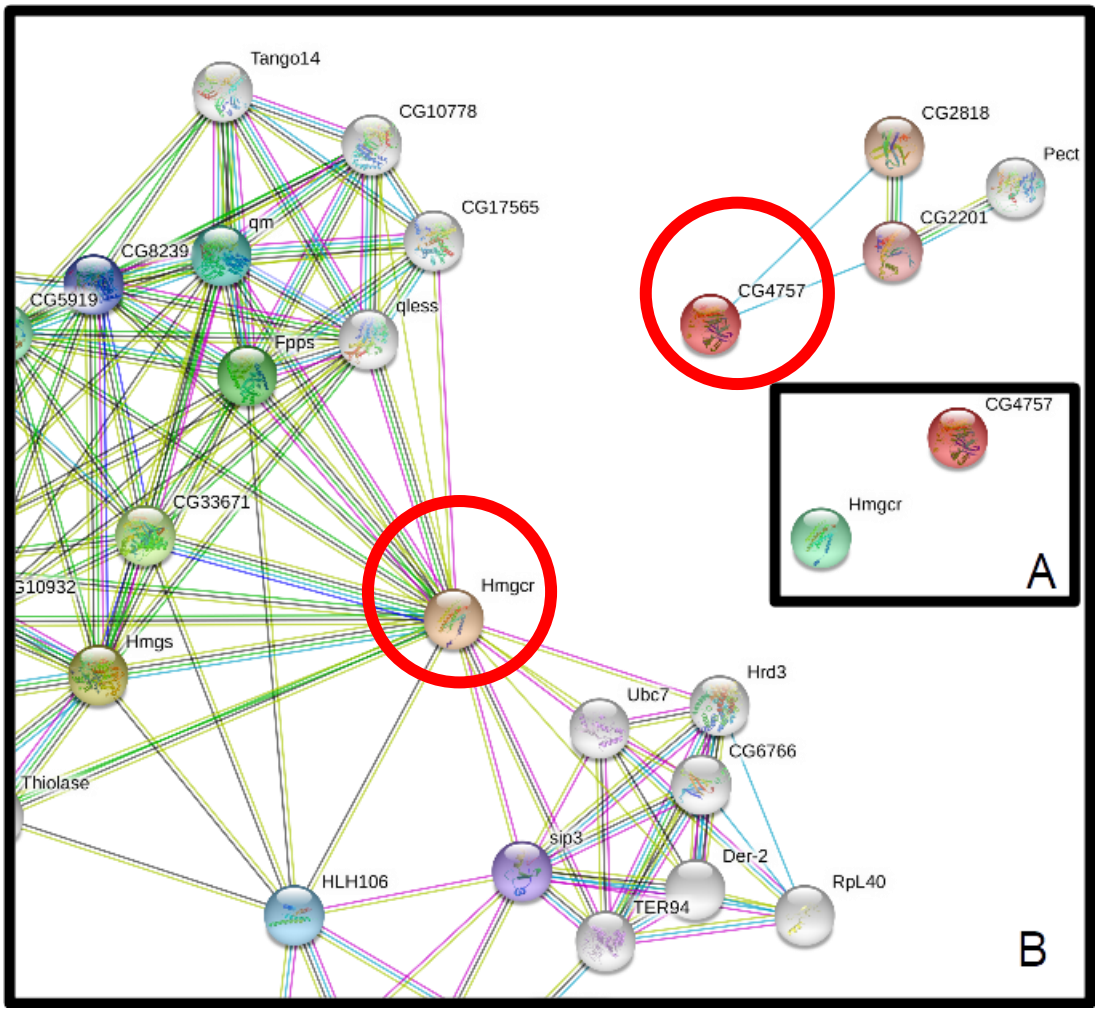

Figure 15 Protein interaction network, generated by online STRING protein interaction tool. (A) When only HMGCR and CG4757 proteins are entered as input, STRING does not find any connection between them. (B) After increasing the number of nodes, STRING online algorithms still could not find any interactions between HMGCR and CG4757, as well.

From STRING interaction data the iGraph package constructed a network and found the shortest chain of protein interactions (Figure 17):

HMGCR, Kul, Nrx1, CG4757.

After pasting these proteins to STRING online tool, I received a broader network (Figure 16), with some interactions (Kul,Nrx1 and Nrx1-Ace) connected via intermediate proteins, such as, Dlg1 and Appl. The new network showed connection between HMGCR and CG4757, as well.

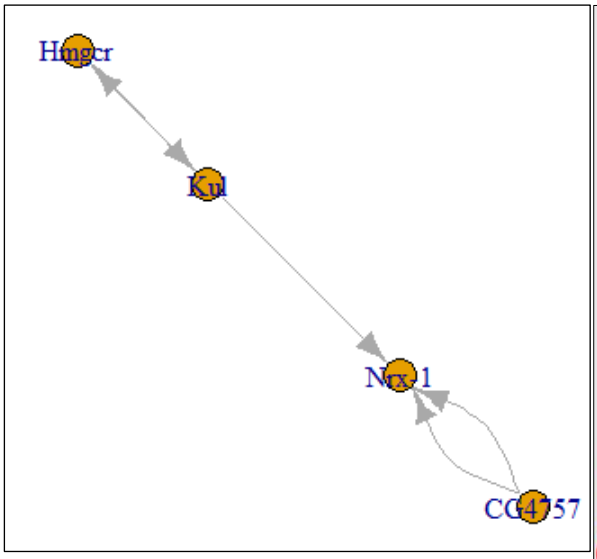

Figure 17 With input data from STRING, iGraph generated shortest pathway between HMGCR and CG4757 - HMGCR, Kul, Nrx1, CG4757. These for proteins were further pasted to String online tool.

STRING online tool provided references for the interactions in this network (Figure 16). These references, as well as additional literature analysis explained these interactions in greater detail. This information is summarized in the Table 22 . Proteins in the grey area of Figure 17, are irrelevant to the iGraph network, therefore are not included in the table of references (Table 22).

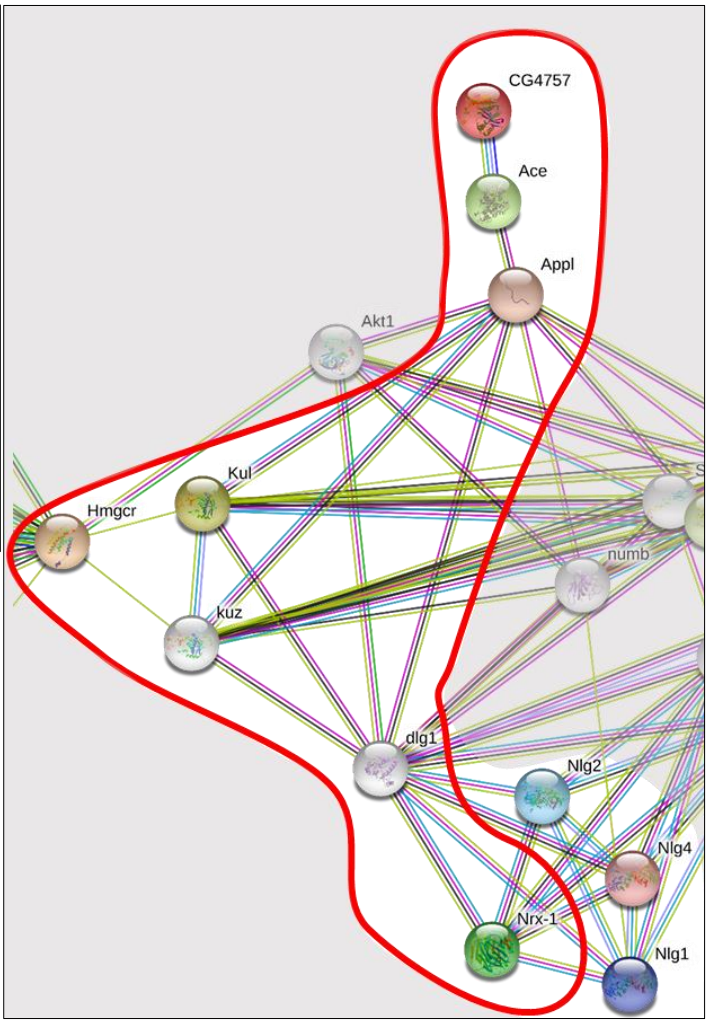

Figure 16 After pasting list of proteins to STRING online tool and increasing the number of nodes, the network of interactions was received. However, few proteins were connected via intermediate nodes (Dlg1, Appl and Akt1). Network also provided additional proteins , neuroligins (Nlg2, Nlg4, Nlg1). STRING online tool for each interaction provides reference. These references and further literature analysis revealed that Nrx1 and neuroligins regulate acetylcholine signaling. Nodes in the grey area were considered as irrelevant.

Protein of flies (Protein of humans)	Description	Relationship with next proteins
HMGCR (HMGCR)	A rate limiting enzyme in mevalonate pathway.(Rodwell et al., 1976)	In cell lines, statins, the inhibitors of HMGCR, increase expression of ADAM10 in 4 neural, 2 kidney (Kojro et al., 2001) and epithelial brain cell lines (Zandl-Lang et al., 2018)
Kul, kuz (ADAM10)	The orthologues of human metallopeptidase ADAM10. (Thurmond et al., 2019).	In human neurons, ADAM10 cleaves neurexin-1 ectodomain (Trotter et al., 2019), and prevents its nanoclustering in the pre-synapse. These nanoclusters are important for neurexin-1 functioning (Trotter et al., 2019)

Dlg1 (SAP97)	According to STRING and Uniprot, Dlg1 is the orthologue of Synapse Associated Protein 97 (SAP97) (Szklarczyk et al., 2019), (Morgat et al., n.d.). SAP97 is responsible for protein transport from ER to pre-synaptic membrane. (Marcello et al., 2007). Dlg-1 is important for synaptic development (Mendoza et al., 2003)	In rat neuron cell line, SAP97 binds to ADAM10 and transports it to synapse.(Marcello et al., 2007) Drosophila studies suggest, that full length Nrx-1 and neuroligins (Nlg) are necessary for Dlg1 colocalization (Banerjee et al., 2017)
Nrx1 (Neurexins)	There are 4 neurexins in the fly (Larkin et al., 2015).Neurexins are important for development and maintenance of synapse, including NMJ (neurotransmitter vesicle clustering and release). (Sun et al., 2009) Suppression of Nrx1 resulted in sleep impairments (Tong et al., 2016) and circadian rhythm impairments. Conversely, flies over-expressing Nrx1 show less fragmented sleep and "evidence of synaptic development".(Larkin et al., 2015).	Neurexins bind to Neuroligins and form a bridge between pre-synapse and post-synapse Thus, they align neurotransmitter release in pre-synapse with receptors in the post-synapse. (Kawaguchi & Gotoh, 2019). This bridge increases neurotransmitter release and receptor expression in post synapse. (Trotter et al., 2019) For further interactions, see Dlg1, Neuroligins and CG4757 proteins.
Neuroligins (Neuroligins)	Neuroligins (Nlg) - a cell adhesion molecule of post synapse. They are important in NMJ development, synaptic pruning, and neurotransmitter release. (Sun et al., 2011)	Binds to Nrx1 and bridge post-synapse with pre-synapse. This bridge aligns presynaptic vesicles and postsynaptic receptors. (Trotter et al., 2019) Nlg3 null mutants, has reduced synaptic transmission (Xing et al., 2014).
CG4757 (AchE)	According to KEGG database, CG4757 with acetylcholine esterase (Ace) participates in Glycerophospholipid metabolism. Here they metabolize acetylcholine to choline and acetic acid. (Minoru Kanehisa, 2019)	Nrx1 and acetylcholine esterase expression is related. Mice, overexpressing human AchE, had reduced neurexin Iβ expression in spinal cord neurons. (Andres et al., 1997) From cultured rat neurons, AchE was co-precipitated with neurexin-1β. (Xiang et al., 2014)
Ace (AchE)	Acetylcholine esterase orthologue.	Ace and Dlg1 expression might be related, since both have common translational regulator – Pum. After memory training in the fly, both were upregulated in the brain (Chen et al., 2008).

Table 22 Summary of references received from STRING online tool. These references explain interactions of HMGCR-Kuz, Kul - Dlg1 - Nrx1 – Neuroligins - CG4757 pathway and explain how HMGCR might be related to acetylcholine system .

STRING online tool and iGraph provided a model, linking Hmgcr to acetylcholine system. Based on this chain of interactions and references, the following model was formed (Human orthologues are depicted in brackets):

1. *HMGCR* knock down mimics statin inhibition and increase expression of Kuz or Kul (ADAM10).
2. Dlg1 (SAP97) transports Kuz or Kul (ADAM10) to pre-synaptic membrane.
3. In presynaptic membrane, Kuz or Kul (ADAM10) cleaves extracellular domain of neurexin. The cleavage disrupts neurexin-neuroligin bridge and impairs acetylcholine release.
4. Reduced acetylcholine levels in neuromuscular junction causes impaired locomotion in flies. Reduced acetylcholine levels are recovered with neostigmine – hence recovered locomotion after treatment.

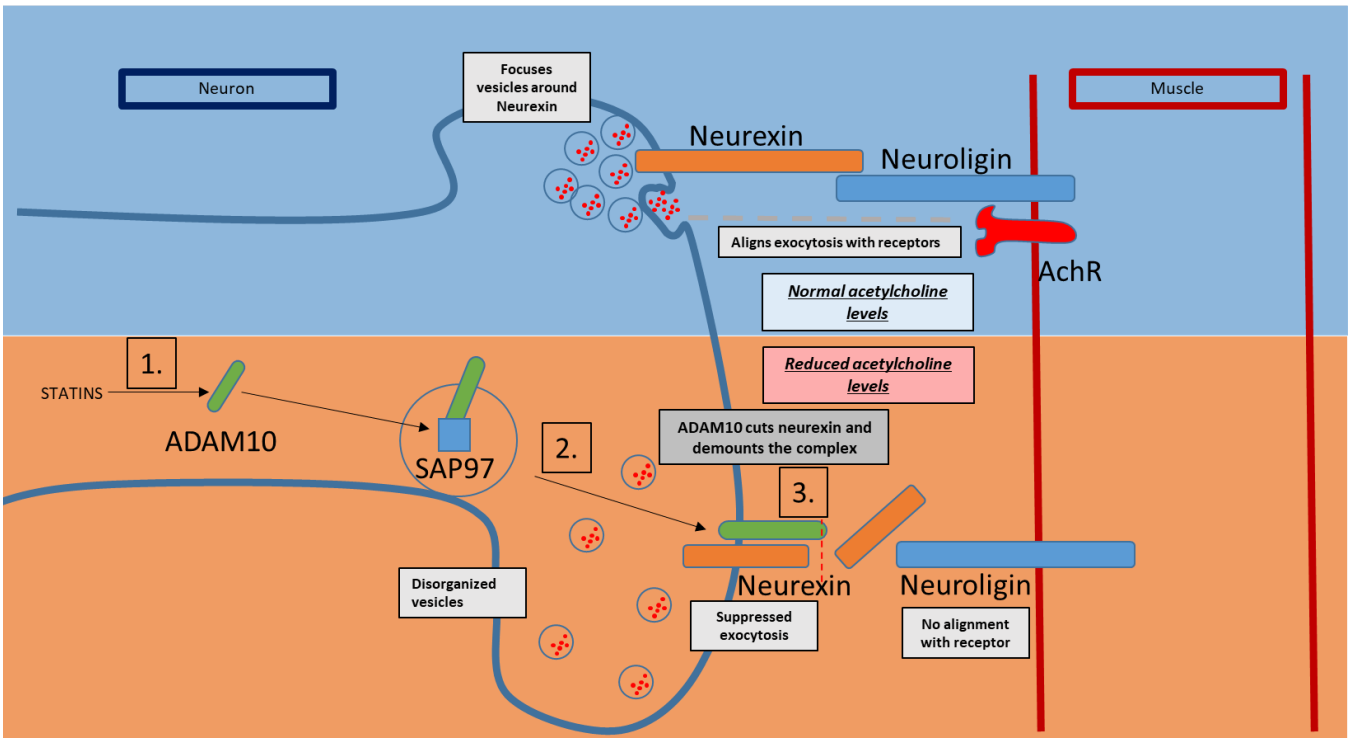

Figure 18 (Blue field) Neurexin-neuroligin bridge and its effects on acetylcholine signaling. The bridge, focuses acetylcholine vesicles around neurexin endodomain and aligns exocytosis with acetylcholine receptors. (Orange field) Model, generated from STRING interaction data. (1.) Statins increase expression of ADAM10 peptidase which (2.) is transported by SAP97 to presynaptic membrane. (3.) Here, ADAM10 cuts neurexin ectodomain and demounts neurexin-neuroligin bridge. This disruption, reduces acetylcholine release to the synapse and impairs exocytosis alignment with acetylcholine receptors (AchR).

3.5.RT-PCR: Nrx1, Kuz, Kul

Neurexin-1 (Nrx1) expression

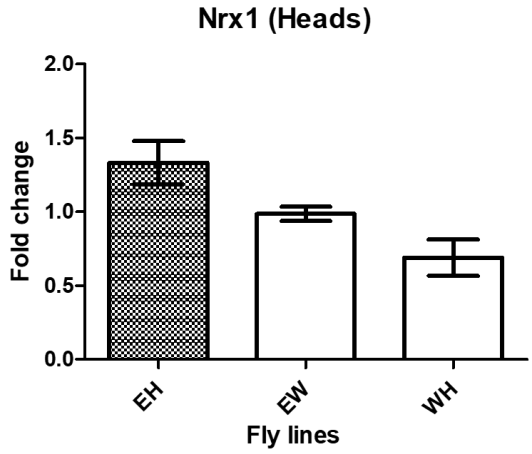

Figure 19 Nrx1 RNA expression difference (Mean and SD) in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). Nrx1 expression is not significantly highest in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc)

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 16) = 11.71,$$
$$p = 0.003$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and WH control. (Table 23).

Comparison	Z	P.unadj	P.adj
EH - EW	1.502	0.133	0.133
EH - WH	3.407	0.001	0.002
EW - WH	1.838	0.066	0.132

Table 23 Dunn's post hoc analysis results of RT-PCR data from heads of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	1.229	0.359	1.146	0.179
EW	5	0.986	0.154	1.019	0.224
WH	6	0.691	0.423	0.636	0.453

Table 24 Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

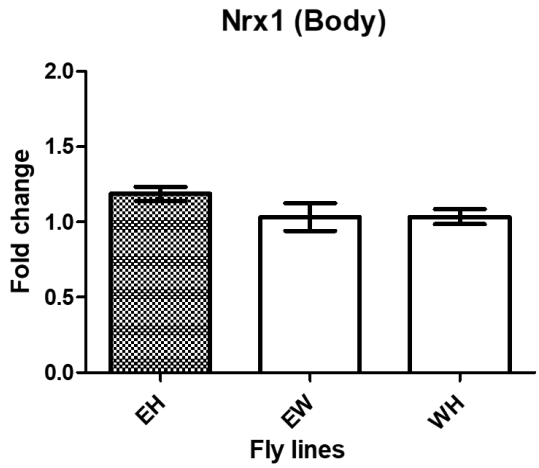

Figure 20 Nrx1 RNA expression difference (Mean and SD) in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=5). HMGCR expression is not significantly different in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 15) = 4.985,$$
$$6. \quad p = 0.083$$

Dunn's post-hoc analysis with Holm method did not show significant difference between experimental line (EH) and controls (table 22).

Comparison	Z	P.unadj	P.adj
EH - EW	2.090	0.037	0.110
EH - WH	1.744	0.081	0.162
EW - WH	-0.396	0.692	0.692

Table 25 Dunn's post hoc analysis results of RT-PCR data from bodies of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	1.187	0.147	1.186	0.211
EW	5	1.033	0.290	0.899	0.361
WH	5	1.035	0.162	1.011	0.169

Table 26 Descriptive statistics of RT-PCR data from fly bodies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

Kuzbian like (Kul)

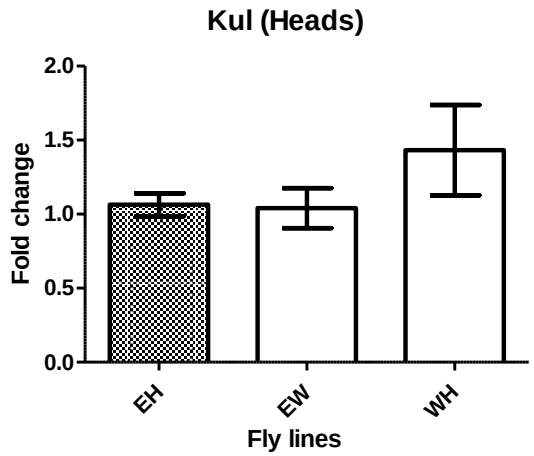

Figure 21 Kul RNA expression difference (Mean and SD) in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=6 and WH, n=6). Kul expression is not significantly different in EH samples (p>0.1, Kruskal Wallis)

Differences between groups are statistically insignificant (p>0.1).

$$Kruskal - Wallis, \chi^2(2, n = 17) = 0.51,$$
$$p = 0.773$$

Fly line	n	mean	sd	median	IQR
EH	5	1.064	0.173	1.029	0.121
EW	6	1.041	0.330	0.980	0.435
WH	6	1.433	0.747	1.365	1.255

Table 27 Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

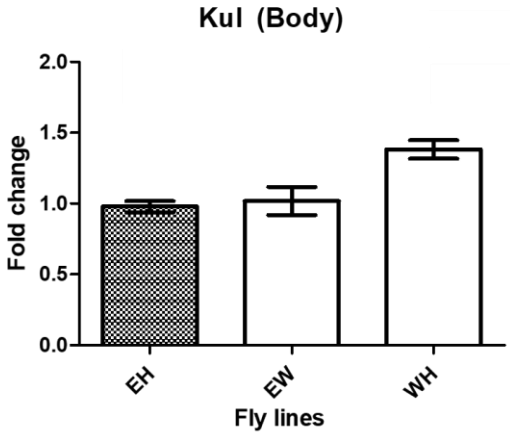

Figure 22 Kul RNA expression difference (Mean and SD) in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). Kul expression is not significantly different in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant (p<0.1).

$$Kruskal - Wallis, \chi^2(2, n = 16) = 4.985,$$
$$p = 0.019$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and WH control. (table 27).

Comparison	Z	P.unadj	P.adj
EH - EW	-0.066	0.947	0.947
EH - WH	-2.440	0.015	0.044
EW - WH	-2.370	0.018	0.036

Table 28 Dunn's post hoc analysis results of RT-PCR data from bodies of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values.

Fly line	n	mean	sd	median	IQR
EH	5	0.979	0.087	1.007	0.074
EW	5	1.018	0.222	0.971	0.084
WH	6	1.383	0.161	1.341	0.141

Table 29 Descriptive statistics of RT-PCR data from fly bodies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

Kuzbian (Kuz) expression

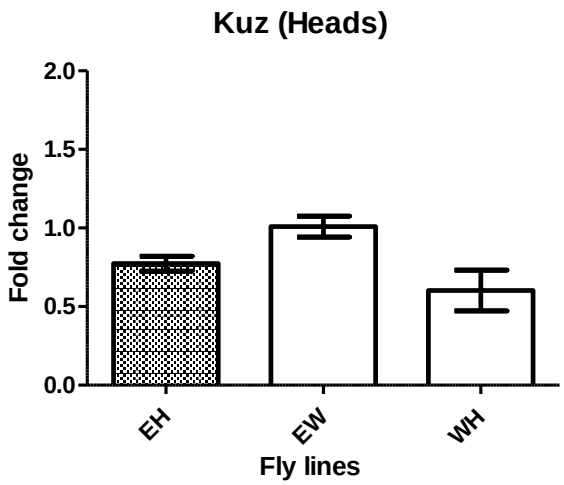

Figure 23 Kuz RNA expression difference (Mean and SD) in the heads of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). HMGCR expression is not significantly different in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant.

$$Kruskal - Wallis, \chi^2(2, n = 16) = 6.54,$$
$$p = 0.038$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and EW control (table 29)

Comparison	Z	P.unadj	P.adj
EH - EW	-2.059	0.039	0.079
EH - WH	0.220	0.826	0.826
EW - WH	2.370	0.018	0.053

Table 30 Dunn's post hoc analysis results of RT-PCR data from heads of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values

Fly line	n	mean	sd	median	IQR
EH	5	0.774	0.106	0.748	0.076
EW	5	1.009	0.150	0.993	0.065
WH	6	0.603	0.318	0.554	0.516

Table 31 Descriptive statistics of RT-PCR data from fly heads. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

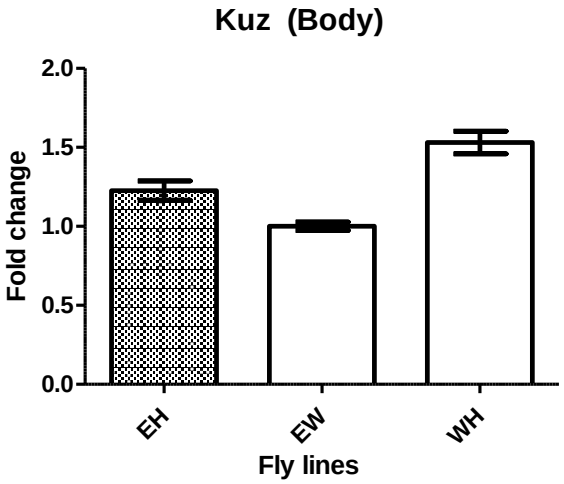

Figure 24 Kuz RNA expression difference (Mean and SD) in the bodies of fly lines with neuronal HMGCR knockdown (EH, n=5) and controls (EW, n=5 and WH, n=6). Kuz expression is not significantly different in EH samples (p>0.1, Kruskal – Wallis, Dunn's post-hoc).

Differences between groups are statistically significant.

$$Kruskal - Wallis, \chi^2(2, n = 16) = 11.981,$$
$$p = 0.003$$

Dunn's post-hoc analysis with Holm method showed significant difference between experimental line (EH) and EW control **(table)**.

Comparison	Z	P.unadj	P.adj
EH - EW	1.661	0.097	0.097
EH - WH	-1.723	0.085	0.170
EW - WH	-3.457	0.001	0.002

Table 32 Dunn's post hoc analysis results of RT-PCR data from bodies of the flies. Comparison between groups and its Z, P unadjusted, P adjusted values

Fly line	n	mean	sd	median	IQR
EH	5	1.227	0.135	1.291	0.210
EW	5	1.001	0.060	0.994	0.019
WH	6	1.531	0.177	1.508	0.234

Table 33 Descriptive statistics of RT-PCR data from fly bodies. Sample size (n), mean, standard deviation (sd), median and interquartile range (IQR).

4. Review of results and conclusions

The HMGCR knock down in the fly heads was not confirmed as its expression difference not significant enough. However, EH body samples showed significantly lowest *HMGCR* RNA expression. In addition, the flies used for PCR samples and used for DAMS experiments were from the same batch. These flies had impaired locomotion as in other experiments studies, where HMGCR knockdown in neurons was confirmed (Belgacem & Martin, 2007), (M.Williams lab, unpublished data). Therefore, the insufficient significance in head samples, might be due to insufficient sample size.

Our DAMS experiments supported other researchers results where *HMGCR* knockdown in neurons impairs locomotion. Previous experiments showed *HMGCR* knockdown in *Pars intercerebralis* (M. Williams lab, unpublished data) and in *Corpus allatum* (Belgacem & Martin 2007) impairs locomotion as well. Therefore, we investigated further, to find reasons of this impairment.

Initially we presumed that to decreased acetylcholine levels due to its breakdown was the reason of impaired locomotion. First, acetylcholine is the chief neurotransmitter in passing nervous impulse to muscles (The Editors of Encyclopaedia Britannica, 2019). Second, previously done RNA sequencing of EH flies showed increased expression of *CG4757* esterase which, as KEGG database shows, participates in acetylcholine breakdown. Therefore, to support this hypothesis, we applied neostigmine treatment which partially recovered impaired locomotion in EH flies. These results supported the hypothesis that impaired locomotion is due to elevated acetylcholine esterase expression, therefore RT-PCR experiments were employed further.

However, the RT-PCR results did not support this hypothesis, since *Ace* and *CG4757* didn't show increased RNA expression. But no change in RNA levels of *Ace* and *CG4757* does not mean protein levels. For example, translation of fly, human and rat acetylcholine esterase is regulated by Pumilio proteins (Chen et al., 2008), (Marrero et al., 2011). Therefore, although *Ace* and *CG4757* RNA levels didn't have significant change, protein levels might be changed. Nevertheless, these results set us to look for alternative hypothesis, explaining impaired locomotion in EH flies.

To create alternative model, STRING interaction data was employed. Although STRING online tool and iGraph used same interaction data, their output was different. These differences could be explained by the different nature of each tool. The output of STRING online tool is based on the confidence score of interaction, therefore, interactions with lower confidence score will be filtered out and not shown (Szklarczyk et al., 2019). Meanwhile, iGraph algorithm is based distance (Csárdi & Nepusz, 2006) – it looks for the shortest pathway in the network, between two proteins. Therefore, its output excludes intermediate proteins, such as Dlg1. Nevertheless, after reading papers, that explain these interactions, the different outputs turned to be supplemental to each other. Thus, the second hypothesis was formed - locomotion impairments are

caused by insufficient acetylcholine release and neostigmine treatment increases acetylcholine levels in the neuromuscular junction.

The new model proposes that *HMGCR* knock down impairs Neurexin-Neuroligin functioning and this reduces acetylcholine release. However, the RT-PCR experiments did not present solid evidences to support this model, therefore supportive and unsupportive points will be discussed further.

STRING interaction analysis showed relationship between neurexin and acetylcholine esterase. Andres et al. (1997) showed that neurexin expression has inverse relationship with *AchE* expression, and our results suggest the same inverse relationship between *Nrx1* and *CG4382*. The elevated Nrx1 expression could be explained as a response to Nrx1 proteolysis, as Larkin et al. (2015) proposed that there is a compensatory mechanism which elevates *Nrx4* expression when Nrx1 expression is impaired. Further, proteolyzed neurexin is unable to form complex with neuroligin and the acetylcholine release is suppressed (Trotter et al., 2019). This reduces acetylcholine signaling that, consequently, reduces acetylcholine esterase expression (Kammer et al., 1998). This could explain the reduced CG4382 expression.

However, the RT-PCR results are insufficient to support this interpretation. First, RNA expression increase of *Nrx1* in EH samples and *CG4382* decrease in EH body samples was not significant enough and they require bigger sample size. Second, the model doesn't explain why transcription of other two esterase genes (*Ace* and *CG4757*) is not changed. Additionally, it is very little known about CG4382 as only two articles about this protein were found and none of them studied CG4382 role in acetylcholine signaling (Lepennetier & Catania, 2016),(Pérez-Lluch et al., 2015). Finally, the *Kuz* and *Kul* genes did not show significant expression difference in EH flies so there are no evidences to support the hypothesis of Nrx1 proteolysis. Therefore, more investigation on these genes is necessary to support the model.

The results from behavior experiments and STRING interaction model proposed that HMGCR knockdown in the brain reduces acetylcholine levels in neuromuscular junction. DAMS results proposed it is due to increased acetylcholine esterase expression while STRING online tool and iGraph proposed that it is due to poor release of acetylcholine. In the end, RT-PCR results were insufficient to confirm these hypotheses. Therefore additional research is needed to confirm or disconfirm that HMGCR knockdown in the brain reduces acetylcholine levels in a neuromuscular junction due to acetylcholine breakdown or its release.

For further research, RNA expression experiments needs an increased sample size. Also, the proteins should be studied for expression, localization in neuromuscular junction, proteolysis and participation in acetylcholine system. *HMGCR*, *Nrx1* and *CG4382* RNA expression must be studied with increased sample size, since the expression differences are promising but not significant enough. Second, Ace and CG4757 proteins should be studied for expression and CG4757 localization in neuromuscular junction should be proven as well. The increased proteolysis of Nrx1 in EH flies should be studied first and if confirmed, Kuz or Kul co-

localization with Nrx1 in neuromuscular junction should be tested further. Finally, CG4382 reactivity to acetylcholine and neostigmine as well as its localization in neuromuscular junction has to be studied as well.

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