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The use of the model organism *Drosophila melanogaster* to investigate the role in metabolism and behavior of obesity-linked genes *Nrx-1*, *Oscillin* and *Hmgcr*

Matteo Libero Baroni

Degree project in biology, Bachelor of science, 2014
Examensarbete i biologi 15 hp till kandidatexamen, 2014
Biology Education Centre and Department of Neuroscience, Uppsala University
Supervisors: Michael J. Williams and Philip Goergen

Abbreviations:

Central Nervous system (CNS); double strand RNA (dsRNA); Drosophila Activity Monitoring System (DAMS); ELAV (Embryonic Lethal, Abnormal Vision); Genome Wide Association Studies (GWAS); Low Density Lipoprotein (LDL); Quantitative Polymerase Chain Reaction (qPCR);

Abstract

Multiple eating disorders have a poor biological and biochemical understanding and only few treatments options are currently available to mitigate the effect of such conditions. A better comprehension of the factors characterizing the food intake of an individual may put the basis for improving the efficiency of the therapies commonly in use. Analyzing the complex human nervous and endocrine system is such a complex and time-consuming task, therefore, as a first line investigation, the fruit fly *Drosophila melanogaster*, can be used as model organism to explore the genetic and molecular pathways influencing the metabolism of an organism and the related behavioral outcomes. Genome-Wide Association Studies on humans (GWAS) have identified a number of genes associated with human body weight, and meanwhile some of them have a well-established role in affecting weight, others don´t present a clear function in food intake regulation and metabolism. For most of them, it has been shown that they have orthologues in distant species like *Danio rerio, Gallus gallus and Drosophila melanogaster*, suggesting their relevance for the homeostasis of an organism. The initial assumption considers the food intake, starvation resistance and activity level in *Drosophila melanogaster* to be affected by the expression of the following set of genes: *Nrx-1, Oscillin* and *Hmgcr*. To test this hypothesis, bipartite *GAL4*/UAS system was employed to reduce the expression of genes in a spatial pattern and the flies were screened under different conditions, at both molecular and behavioral levels. By using a *Gal4*-driver specific for neurons (Elav) the contribution that these genes give to metabolism, activity and sleeping time has been investigated. Since the genes mentioned have been shown to be orthologues in *Homo sapiens*, understanding the role they exert upon metabolism and regulatory functions may lead to further accomplishments in treating human obesity and related disorders

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Research aim

The goal of this project was to investigate the correlation between differential gene expression and regulation of feeding behavior, activity level and circadian rhythm in *Drosophila*. More in detail this work is part of a wider research, conducted at the neuroscience department, where a wide repertoire of obesity-linked genes are screened and tested from different angles, aiming to understand how they influence different aspects of the metabolism. This section focuses on: interaction between different diets and gene expression level, starvation and gene expression level, gene silencing in different strains and behavioral assay on knockdown flies.

Introduction

Pathological obesity is defined as a medical condition in which excessive body fat is accumulated to the extent that it has a harmful effect on health. Several cardiovascular and respiratory diseases are related to the onset of this disorder (1), consequently obesity can potentially reduce the life expectancy. Generally speaking body weight is determined by the excessive energy dietary supply, paired with a lack of physical activity, but besides the common understanding of the problem, studies on this topic (2) proved the existence of a non-negligible genetic component contributing the establishment of this condition. Twin studies (3) provided a substantial help for obtaining a clearer and more complete picture of the framework. In accordance with the data acquired, between 1996 and 2005 it has been possible to create a "human obesity gene map", showing the loci contributing to body weight or adiposity (4). This cluster of obesity-linked genes codifies for a wide repertoire of proteins such as receptors, ligands, signal transductors, transporters and transcription factors, thus making even more intriguing and challenging to understand how these gene could potentially promote the onset of this condition.

Genome Wide Association Studies on humans (GWAS) have recently found the presence of a subgroup of 33 candidate genes directly involved or linked to the onset of obesity(Williams, Almen *et al.* 2012). Surprisingly, signaling cascade pathways and transcription factors are enriched among these obesity genes, and several of them show properties that could be useful for future drug discovery (5). Within this group, by using NCBI HomoloGene, EMBL-EBI databases (6) and protein sequence similarity searches (7), it has been possible to identify few potential members of this set that could have a direct effect on human obesity: *NRXN3, GNPDA2* and *HMGCR*. Despite the characterization of their products, their influence upon obesity and metabolism is not yet understood, and the knowledge about the behavioral repercussion that they have is poor. A closer view on their role and function may lead to a better understanding of their contribution to obesity and related conditions.

Gene Background and Orthologies

As first step of this investigation it's required to obtain a deeper understanding of the processes in which the up-listed genes are involved, a brief overview follows here:

Homo sapiens

NRXN3

Neurexin 3 encodes a member of a family of proteins that function in the nervous system as receptors and cell adhesion molecules. Extensive alternative splicing and the use of alternative promoters results in multiple transcript variants and protein isoforms for this gene, but the full-length nature of many of these variants has not been determined yet. Genetic variation at this locus has been associated with a range of behavioral phenotypes, including alcohol dependence and autism spectrum disorder (8)

GNPDA2

Glucosamine-6-phosphate deaminase 2 encodes an allosteric enzyme that catalyzes the reversible reaction converting D-glucosamine-6-phosphate into D-fructose-6-phosphate and ammonium. Variations of this gene have been reported to be associated with influencing body mass index and susceptibility to obesity. Alternative splicing results in multiple transcript variants that encode different protein isoforms (9)

HMGCR

3-hydroxy-3-methylglutaryl-CoA reductase encodes the rate-limiting enzyme for cholesterol synthesis. Normally in mammalian cells this enzyme is suppressed by cholesterol derived from the internalization and degradation of low density lipoprotein (LDL) via the LDL receptor. Competitive inhibitors of the reductase induce the expression of LDL receptors in the liver, which in turn increases the catabolism of plasma LDL and lowers the plasma concentration of cholesterol. Alternatively spliced transcript variants encoding different isoforms have been found for this gene (10)

Drosophila melanogaster

Nrx-1

Similarly to the human "*NRXN3"*, this gene appears to encode an apolipoprotein that functions as a cell adhesion molecule interacting selectively and non-covalently with neuronal cell surface proteins that mediate synapse formation. It appears to be involved in the following biological processes: associative learning, neuromuscular synaptic transmission, synapse assembly, organization, growth and transmission, vitamine "A" transport (11)

Oscillin

Same as the human "*GNPDA2"* by speciation events, this gene encodes for the glucosamine-6-phosphate deaminase, an allosteric enzyme that catalyzes the reversible reaction converting D-glucosamine-6-phosphate into D-fructose-6-phosphate and ammonium. It is involved in the amino-sugar and nucleotide sugar metabolism (12)

Hmgcr

Like the human *HMGCR*, it encodes hydroxymethylglutaryl-CoA reductase. In the fruit fly it has been linked to several biological functions such as: coenzyme A metabolism, biosynthetic processes, pole cell migration and locomotory behaviors (13)

In both fruit fly and human, they participate in important metabolic and homeostatic processes and for almost all of them a reasonable explanation regarding their relation body weight can be provided. Differently from the other two genes (*HMGCR* and *GNPDA2*), to propose a possible mechanism explaining how *NRXN3* and *Nrx-1* affect body weight seems to be a more complicated task. Meanwhile *GNPDA2, HMGCR* (with the related orthologues in Drosophila) are directly involved in processing-transporting nutrients, *NRXN3* and *Nrx-1* don´t show this peculiarity. According to previous literature among this topic (14), *NRXN3* appears to be implicated in addiction and reward behavior, lending further evidence that some, but not all, forms of obesity may be a central nervous system-mediated disorder. Coherently with the findings in human, fruit fly *Nrx-1* has been found to affect the behavior. A previous study (15) demonstrated how *Nrx-1* null mutants have shortened lifespan. The synapse number is decreased in the central nervous system in *Nrx-1* null mutants. In addition, *Nrx-1* null mutants exhibit associative learning defect in larvae. This aspect can somehow have a detrimental effect in the overall feeding behavior of the animal, underlying how the central nervous system (CNS) carries out a crucial role in determining the food intake of an organism.

To understand the how the gene activity characterizes the behavioral outcome, the *Gal4*-UAS system has been employed.

The aim behind using this method is to selectively control and down-regulate the activity of the genes of interest. This, according to the initial hypothesis, should alter the feeding behavior and the general activity level of the flies.

Gal4-UAS and RNA interference

Gal4 codifies for a 881 amino acids transcription factor found in the yeast *Saccharomyces cerevisiae,* functioning as genes regulator. The DNA binding activity of GAL4 is located in the first 74 residues, while its transcriptional activation function is found into two regions, residues 148–196 and 768–881. *Gal4* expression is then able of promoting the transcription of a so called "reporter gene" under an Upstream Activating sequence (UAS) in *Drosophila.* This technology allows the expression of any gene that lies downstream of the UAS, making it a very powerful tool in targeting gene activity and control (16).

To activate the transcription of our target sequence in the off spring (UAS followed by the gene of interest), "Responder lines" are mated to "Driver lines" flies (fig.1), expressing *Elav-Gal4* (a construct containing the RNA-binding protein gene *ELAV*, that will drive the expression of *GAL4* in all neurons). The resulting progeny will hopefully show a reduced expression for the target genes in the CNS. This is due to the fact that downstream the UAS element, an artificial construct has been strategically inserted. The set-up is made of two copies of the same gene placed consecutively where the second copy is inverted compared to the first. As soon as the expression is activated the gene will express sense and antisense RNA that will fold into a stem-loop structure, made of two complementary strands of RNA. Since double strand RNA (dsRNA) is a characteristic genetic material of several RNA-virus taxa, higher eukaryotes have the enzymes for detecting and cleaving these potentially harmful

structures (17). When the dsRNA is exogenous (coming from infection by a virus with an RNA genome or laboratory manipulations), the RNA is imported directly into the cytoplasm and cleaved to short fragments by an endoribonuclease (acting on double strand RNA) (18) . The RNA interference mechanism (RNAi) is then really helpful for silencing genes activity post-trascriptionally. Within this experiment this mechanism will be exploited to knockdown the genes of interest, therefore a reduction in their expression is expected.

It has to be remembered that by employing this technology we will assist to a reduction of the overall expression level of a particular gene, thus meaning that the gene is still active once the transcription mechanism starts.

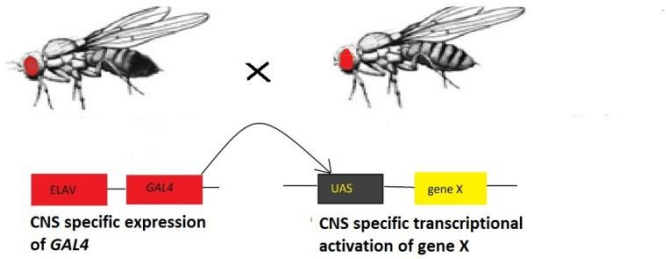

Fig.1
Graphical depiction of how Gal4 protein binds to UAS sequence inducing the transcription of the gene of interest. Driver and Responder carry the two elements separately; meanwhile in the offspring resulting from this cross will have them together in the genome. In this project this technique is employed to obtain a reduction in the overall mRNA expression of the genes of interest

The gene-silencing approach should indeed provide us a better understanding of how the genes affect the general level of activity and feeding, compared to control flies.

ELAV

Embryonic-Lethal Abnormal Visual is a RNA binding protein, which regulates and mediate the development of CNS cells. It is involved in RNA processing and coordinates alternative splicing of mRNA, leading to different isoforms of the same protein (19). Since ELAV is involved in regulating neurons differentiation, it is present across the CNS and in "Glial Cells" as well, making it a good candidate for selectively driving the expression of *GAL4* in the brain. Therefore in the Driver lines, the *GAL4* is coupled with *Elav*, ensuring their mutualistic expression within all neurons.

Materials and methods

Flies husbandry

All flies were raised and kept at 25°C on a standard yeast-corn meal/agar medium (Fisher-Scientific® Jazz-Mix* Drosophila Food), under 12:12 h light: dark conditions. The experimental and control samples (25 males for each replicate) have been kept at 29 °C on standard medium enabling the optimum expression of *Elav-Gal4/UAS* system

Virgin collection and experimental set-up

As initial step of this work, it was necessary to set up the fly crosses between different strains. The aim of this procedure is to ensure that unmated females carrying *Elav-Gal4* can breed with males carrying the UAS system that is linked to the gene of interest. In parallel to each experimental line, two control groups were arranged. These were made to confirm that the gene manipulation did not cause any detrimental effect on the offspring and for checking that the overall fitness is unchanged; a brief scheme can be set as follow:

Experimental line	Control n.1	Control n.2	Parental line
Elav-Gal4/UAS-Nrx-1	*Elav-Gal4/White^{118}*	*White^{118}/Nrx-1*	10 females, 8 males
Elav-Gal4/UAS-Oscillin	*Elav-Gal4/White^{118}*	*White^{118}/Oscillin*	10 females, 8 males
Elav-Gal4/UAS-Hmgcr	*Elav-Gal4/White^{118}*	*White^{118}/Hmgcr*	10 females, 8 males

Table 1) Experimental and control groups genotype are presented, together with the number of flies used for arranging the different crosses

Within the following two weeks, once the first generation hatched, 125 males of each strain were placed in separate vials at 29 ° C and aged for 5 days. This environmental condition allows the optimal expression of *Gal4*, consequentially increasing its regulatory activity. The reason leading us to collect only the males is based on the fact that females undergo profound hormonal changes after mating, and these could alter the normal level of genes expression and modify the behavior during the different stages of life. Since one of the requirements for this test is to eliminate, as much as possible, all the factors influencing genes expression, the hormonal stability of flies represents a key element to focus attention on. By mixing the two genders we would not be able to detect any significant difference in expression level between experimental and control groups.

RNA extraction, cDNA synthesis and qPCR analysis

As first step, once the males aged for 5 days, they were frozen and stored at - 80°C. Then, since the interest is focused on neuronal cells, the heads have been removed and placed separately from the rest of the body. The RNA extraction has been performed according to the "mini-Rneasy 96 kit" protocol from Qiagen$^{(R)}$. RNA concentration was measured using the

"Nanodrop 2000" spectrophotometer from Thermo Scientific(R). Here the sample absorbance at 260nm is measured, providing the RNA concentration (ng/μl).

Once the RNA concentration had been obtained, the cDNA synthesis took place. The reverse transcription was performed according to "High-Capacity cDNA Reverse Transcription Kit Protocol", provided by Applied Biosystem(R).

To confirm the synthesis of cDNA a PCR assay with the experimental samples, positive and negative controls has been run, according to the following protocol: 95,0°C 3 min, 95,0°C 30sec } x 35 59,4°C 30sec, 72,0°C 45 sec} x 35, 72,0°C 5min. Therefore an electrophoresis gel assay has been set up for both the experimental and control samples, where it has been shown that there were no contaminations among them. To check the cDNA concentration another set of spectrophotometric measurements with "NanoDrop 2000" has been performed, therefore the cDNA have been be diluted and employed for the qPCR assay, where the gene expression of Knocked-down flies is compared to control groups.

Assays for Knocked-down *Elav-Gal4 > UAS- Oscillin* and Knocked-down *Elav-Gal4 >UAS-Nrx-1* flies have been arranged. Here the aim is to verify and quantify the reduction in these two experimental lines and compare their expression profile to the control groups, *White> UAS- Oscillin*, $White^{118}$ *> UAS- Nrx-1* and *Elav-Gal4 >* $White^{118}$ flies

Locomotor activity

The Drosophila Activity Monitoring System (DAMS) was employed to measure the activity of individuals flies. In brief, individual flies were put in 65 × 5 mm glass tubes and introduced in the DAMS set-up (TriKinetics). One side of the tube was plugged with cotton and the other with food and capped with a plastic lid. Flies were acclimatised to the set-up for 24 hours before the 48 h recording started. Every time, a fly breaks the infrared beam by crossing the middle of the tube, it is recorded thus giving a general representation of the activity pattern of the fly represented as an activity index and sleeping time. The data were analysed using the

DAMFileScan Software provided by TriKinetics. A total of thirty replicates were performed per genotype.

Gene expression in flies on starvation and different diets

This section of the research takes into account the possible change occurring in the gene expression profile when the fly undergoes 24 hours starvation or is fed with food presenting a different carbohydrate- protein ratio.

One-day old male flies, in groups of 25, were starved for 24 hours and fed for 5 days on five different diets before being quick-frozen on dry ice. The diets were made up of a nutrient low diet, medium diet, nutrient rich diet, carbohydrates rich diet, and sugar rich diet with the sugar and yeast content ranging from 2.5 g/dl over 10 g/dl to 40 g/dl, respectively.

The recipe has been adapted from William W. Ja *et al* and is as follows:

Ingredients					
	Nutrient low diet 2.5:2.5	**Medium diet 10:10**	**Nutrient rich diet 40:40**	**Yeast rich diet 10:40**	**Sugar rich diet 40:10**
Water (mL)	100	100	100	100	100
Agar (g)	1.5	1.5	1.5	1.5	1.5
Sugar (g)	2.5	10	40	10	40
Yeast extract (g)	2.5	10	40	40	10
Tegosept (20%)(ml)	1.5	1.5	1.5	1.5	1.5

Table 2) Composition of the 5 dietary regimes employed in the test

Statistical analysis

All experiments have been performed with 5 replicates with a minimum of 25 flies for each genotype in each replicate, except for the locomotor activity assay, where thirty replicates (1fly each) were used. The statistical analysis was done with GraphPad Prism 5.0 and non-parametric statistic tests have been employed. The 24 hours starvation results were analyzed with the "Mann-Whitney test". The different diets assay, the activity assay (DAMS) and the quantitative time PCR (qPCR) results were analyzed using the "Kruskal –Wallis" followed by a "Dunn´s Multiple Comparison" as post-hoc test.

The statistical test results are given as p value < 0,05 (*), p value < 0,01 (**) and p value < 0,001 (***). All data were given as Mean ± Standard Error of the Mean (S.E.M)

Results

mRNA expression level under starved and non-starved condition

Since *Nrx-1* and *Oscillin* are linked to obesity, here the aim is to verify how their expression is affected under starving condition.

Nrx-1 and *Oscillin* expression levels appear not be significantly influenced by 24 hours starvation (Mann-Whitney test p.value < 0,05). The transcript level of *Nrx-1* and *Oscillin* in starved Wilde type [*Nrx-1* Starved (0.899±0.06991 n=5); *Nrx-1* Control: 0.932±0.099 n=4 *Oscillin* Starved (0.666 ± 0.108 n=5); *Oscillin* Control (0.842 ± 0.056 n=4)] was compared to non-starved Wilde type control groups, leading to the conclusion that there is no relevant change occurring within the two experimental groups and the two controls.

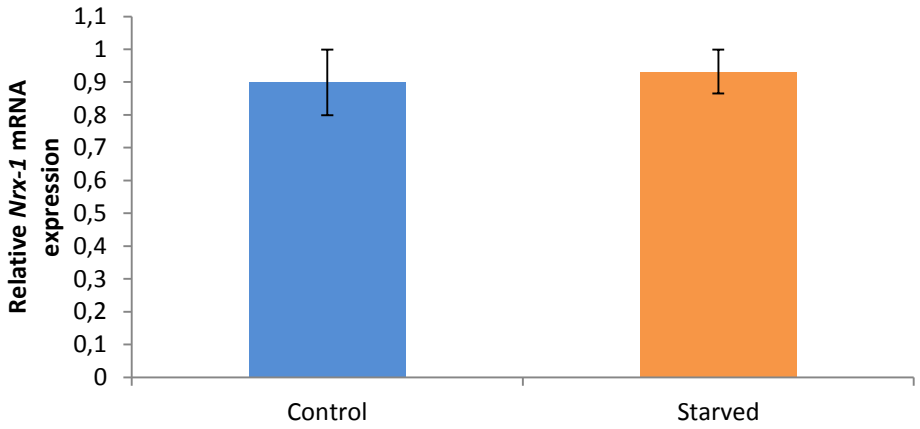

Fig.2) *Nrx-1* transcript levels are not influenced by starvation
Nrx-1 transcript level has been calculated under 24 h starvation and non-starved condition. No significance difference between the two groups has been detected. The results have been calculated on five samples run in duplicates, applying non parametric Mann-Whitney test with a confidence range of 95% (Mann-Whitney p value= 1.00) All values are given as Mean relative expression ± SEM

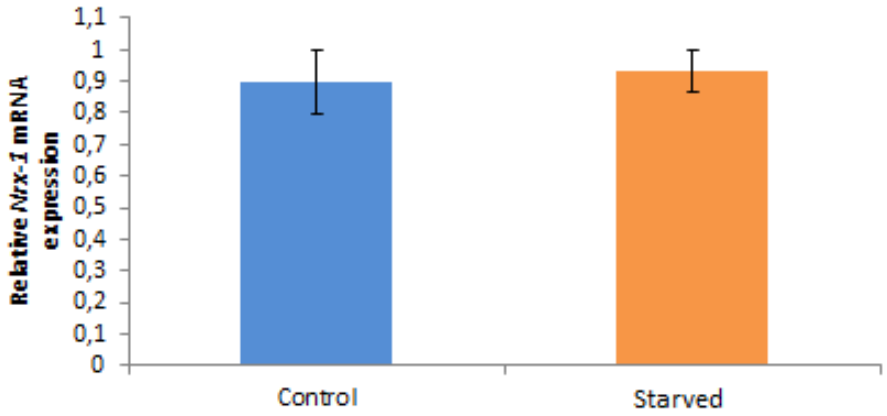

Fig.3) *Oscillin* mRNA expression is not influenced by starvation
Oscillin transcript level has been calculated under 24 h starvation and non-starved condition. Performing the Mann-Whitney test (p.value=0.4127) no significance difference between the two groups has been detected. Values are given as Mean relative expression ± SEM.

mRNA expression level among different dietary composition

Nrx-1 and *Oscillin* have been proven to play a role in the onset of obesity, therefore here the interest is focused on seeing whether or not the expression of these two genes is affected by different diets. *Nrx-1* and *Oscillin* transcript level has been determined for flies fed on five different dietary regimes. A restricted diet (2,5:2,5carbohydrates/ protein), a control diet (10:10carbohydrates/ proteins), a nutrient enriched diet (40:40carbohydrates/proteins), a protein rich diet (10:40 carbohydrates/protein) and a carbohydrates rich dietary regime (40:10carbohydrates/proteins). After 5 days on the different diets the flies heads have been chopped off and a quantitative PCR assay have been performed. The results collected showed that there was no significant difference within mRNA expression level for different diets compared to the standard 10:10. Nevertheless, as shown in fig.4, there was a numerical difference in the 40:40 group that did not reach a statistical relevance compare to the control group.

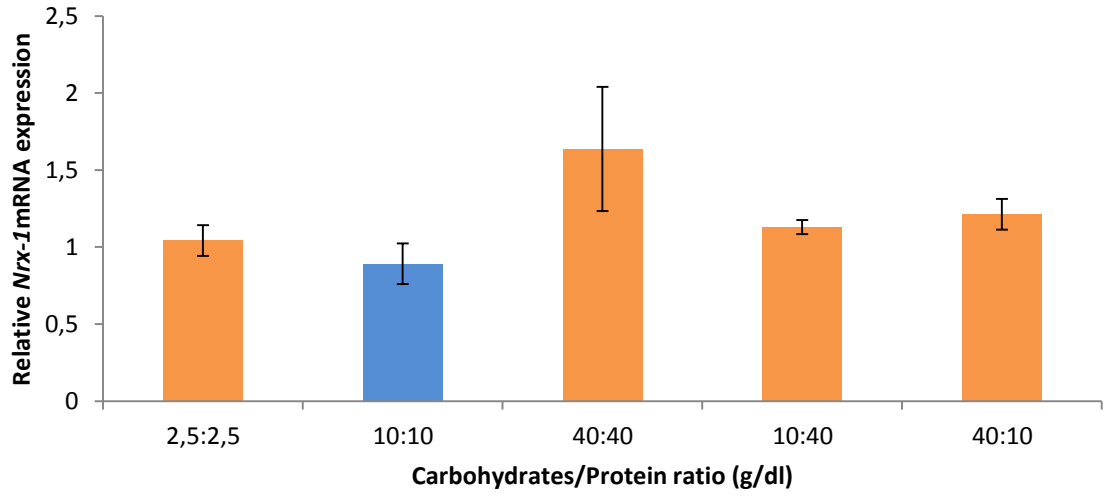

Fig.4) *Nrx-1* expression level of Wilde type flies is not influenced by variations in the diet composition
The data showing the relative *Nrx-1* transcript level have been calculated on five samples run in duplicates for each dietary regime. The control composition is 10:10 carbohydrates/protein (shown in blue), and by performing a Kruskall Wallis test (p.value= 0,3838) followed by Dunn's Multiple Comparison test (p.value > 0,05) no significant difference between groups has been detected. The results are given as Mean relative expression ± SEM

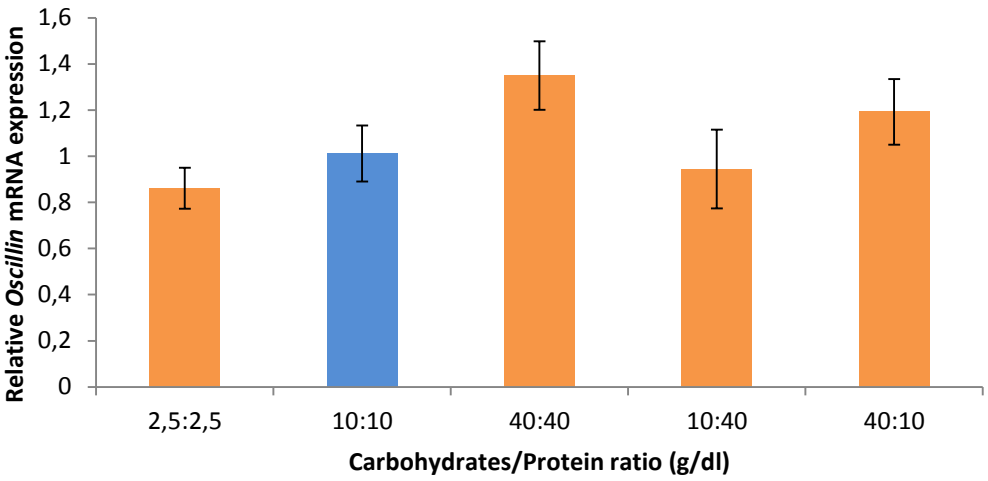

Fig.5) *Oscillin* expression level of Wilde type flies is not influenced by variations in the diet composition
The data collected show the relative *Oscillin* mRNA expression level. The control composition (shown in blue) is 10:10 carbohydrates/protein. By performing a Kruskall-Wallis test (p.value=0,2140) followed by Dunn's Multiple Comparison test (p.value > 0,05) no significant difference between groups has been detected. The results are given as Mean relative expression ± SEM

Oscillin	**Mean±SEM**	**N. of replicates**
2,5:2,5	0,87±0,09	5
10:10	1,02 ±0,13	4
40:40	1,35±0,15	5
10:40	0,95 ±0,18	5
40:10	1,20±0,15	5
Nrx--1	**Mean±SEM**	**N. of replicates**
2,5:2,5	1,05±0,10	5
10:10	0,90 ±0,14	4
40:40	1,64±0,41	5
10:40	1,13±0,05	5
40:10	1,22±0,10	5

Table 3 Expression values of *Nrx*-1and *Oscillin* are presented. These values have been graphically represented in figures number 4 and 5. The food composition is determined by the carbohydrates/protein ratio given as grams carbohydrates/grams protein per each deciliter of solution.

qPCR in Elav/Gal4>UAS-Nrx-1 and Elav/Gal4>UAS-Oscillin

To investigate if the Gal4-*UAS* system successfully induced a reduction in the *Nrx-1* and *Oscillin* transcript levels, the RNA was extracted, converted to cDNA and amplified. The

expression levels of the two experimental lines, *Elav/Gal4>UAS-Nrx-1*(Mean±SEM= 0,648± 0,118) and *Elav/Gal4>UAS-Oscillin*(Mean±SEM=1,106±0,147) were quantified and compared to control lines: *Elav/Gal4>White*^{118} for *Nrx-1* (Mean±SEM= 1,053± 0,094), *Elav/Gal4>White*^{118} for *Oscillin* (Mean±SEM= 1,821± 0,092), *White*^{118}*>Nrx-1*(Mean±SEM= 0,948±0,217) *White*^{118}*>Oscillin*(Mean±SEM=1,946±0,835).

The results show that there has been an overall mRNA reduction in the experimental lines, and, as shown in figures 6 and 7, the trend is well established among both *Nrx-1* and *Oscillin.* Nevertheless the trend observed within the two groups, by performing the Kruskal-Wallis test (p value> 0,05) and the Dunn´s Multiple Comparison test (p value>0,05) the experimental expression profile wasn´t found to be significantly different than controls. The data have been provided as Mean±SEM.

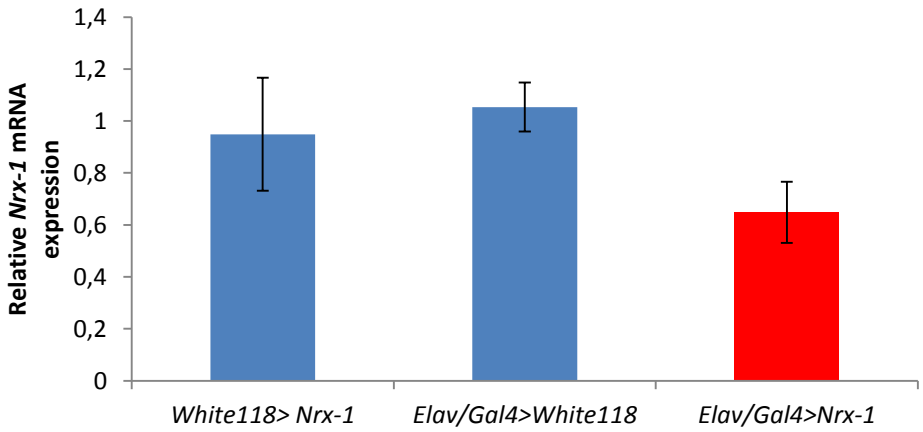

Fig.6) *Nrx-1* expression level has been reduced in the experimental line
After a 5 days aging period at 29 C° males flies have been quickly frozen in dry ice and RNA has been extracted and the expression profile has been quantified and compared to control groups (*White*^{118}*>Nrx-1; Elav/Gal4>White*^{118}). Kruskall-Wallis p value did not reach the statistically significant threshold (p value=0,0935) and Dunn´s multiple comparison did not underline any significant difference. The results are shown as Mean relative expression ± SEM

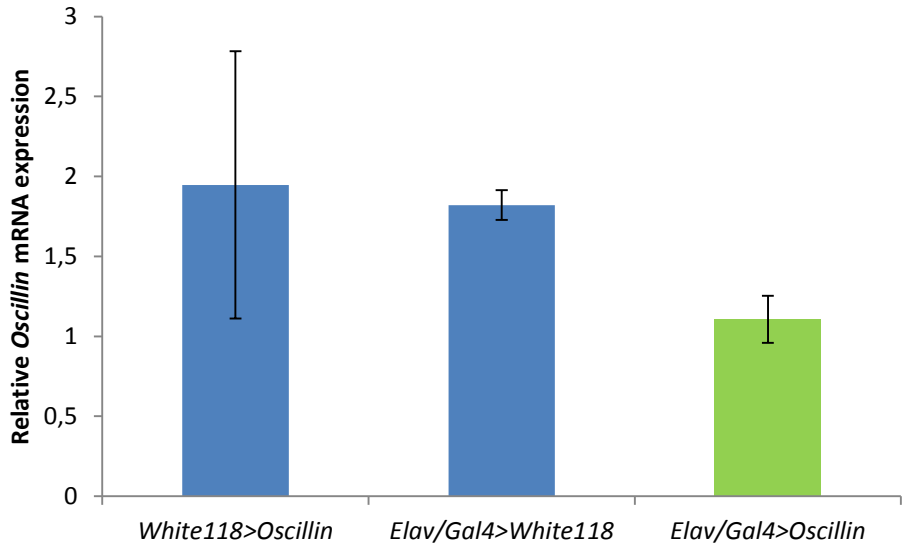

Fig.7) *Oscillin* expression level has been reduced in the experimental line
After a 5 days aging period at 29 C° males flies for each genotype have been quickly frozen in dry ice and RNA has been extracted and the expression profile has been quantified and compared to control groups(*White*^{118}*>Oscillin; Elav/Gal4>White*^{118}) .Kruskall-Wallis p value did not reach statistically significant value (p value= 0,0750) and Dunn´s multiple comparison did not underline any significant difference. The result are given as Mean relative expression ± SEM

Measurements of activity in flies with reduced expression in *Nrx-1, Oscillin* and *Hmgcr* in the central nervous system

This behavioral assay aimed to show how the overall activity and sleeping time of flies was affected by *Nrx-1*, *Oscillin* and *Hmgcr*, over a time lapse of 48 hours. The results obtained have shown that *Elav/Gal4>UAS-Nrx-1* flies are more active compared to *Elav/Gal4> White*^{118} control flies (p value< 0,01 **) meanwhile there is no significant difference regarding the activity index of *Elav/Gal4>UAS-Nrx-1* and the second control group *White*^{118}*> Nrx-1*. The sleeping time appears to be influenced as well by *Nrx-1*: *Elav/Gal4>UAS-Nrx-1* had a significantly higher sleeping time over the 48 hours than *White*^{118}*> Nrx-1* (p value< 0,05 *). For both control groups a significant difference in sleeping time and activity index was detected (p value <0,001 ***). Despite this finding, no significant difference in sleeping time was found between the experimental line and the other control *Elav/Gal4> White*^{118}. Regarding the influence of *Oscillin* upon activity and sleeping time, the data acquired showed

that *Elav/Gal4>UAS-Oscillin* flies sleep significantly more compared to both control groups: *White*^{118} > *Oscillin* and *Elav/Gal4> White*^{118} (p value< 0,001 ***), but the overall activity index has been shown not to be changed in the experimental line. *Hmgcr* affect both activity index and sleeping time in a statistically significant manner. Knocked-down experimental *Elav/Gal4>UAS-Hmgcr* flies have been demonstrated to sleep significantly more compared to both control *White*^{118}*>Hmgcr* (p value< 0,001 ***) and *Elav/Gal4>White*^{118} (p value< 0,05*). The two control groups, as shown in fig.8 and 9, appear to be different in both sleeping time and activity index (p value respectively < 0,001*** and 0,01**) The 48 hours activity index is also significantly modified in the experimental line: these flies in fact are generally less active compared to the control group *White*^{118}*>Hmgcr* (p value< 0,05*). Despite this finding, no significant difference was found between the experimental line and the second control *Elav/Gal4> White*^{118}

Sleeping time and activity index for all the three genes have been graphically represented in figures 8 and 9:

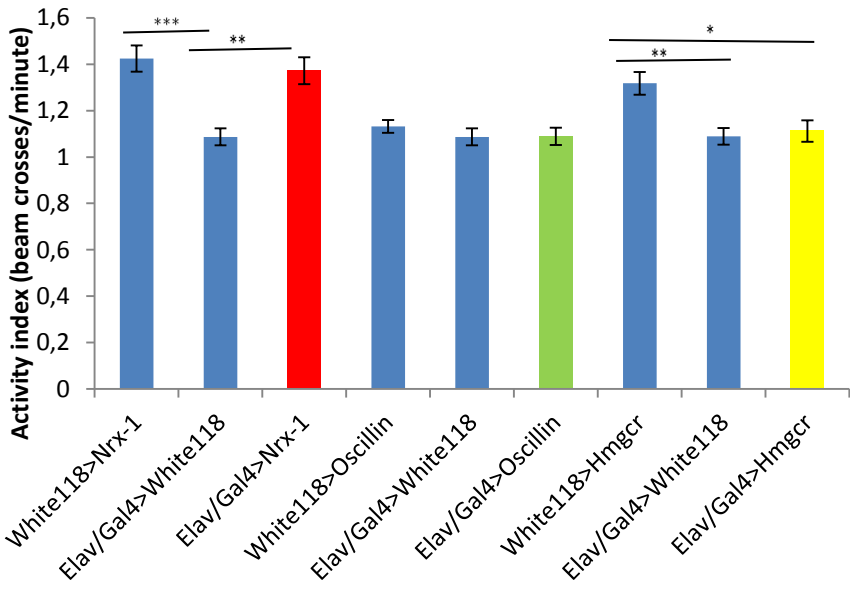

Fig.8) Activity index over different strains.
After a 5 days aging period at 29 C° males flies for each genotype have been collected and placed in sealed tubes for 72 hours (24 for acclimatize+ 48 hours as experimental time lapse).Kruskall-Wallis test has been used followed by Dunn's Multiple Comparison Test. p.value< 0,001(***), p.value<0,01(**) and p.value<0,05(*) denote significant difference within experimental and control groups(marked in blue). All values are given as Mean±SEM

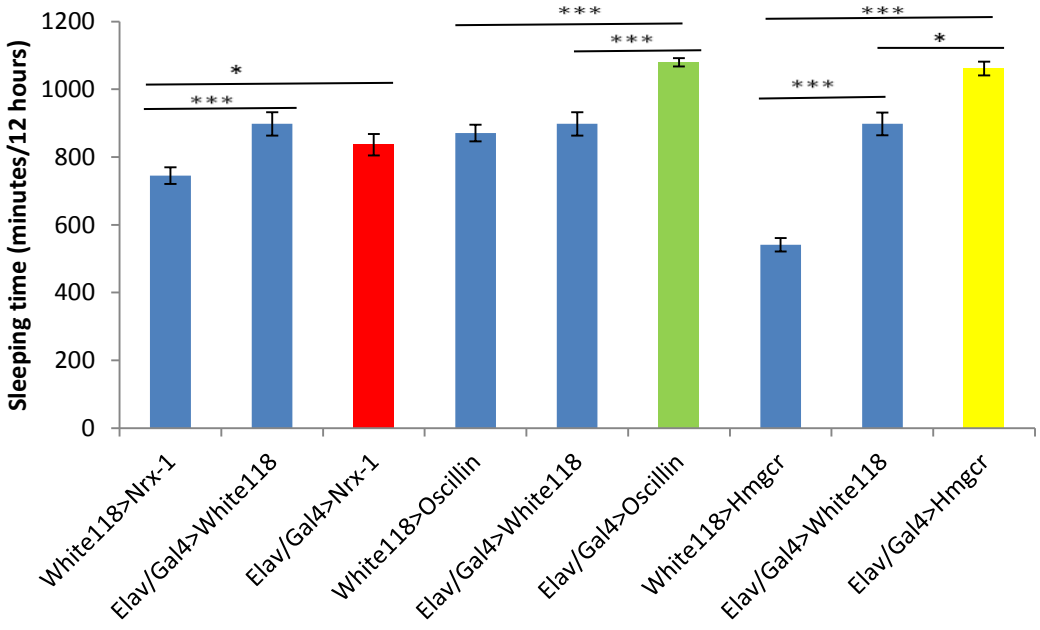

Fig.9) Sleeping time over different strains

After a 5 days aging period at 29 C° males flies for each genotype have been collected and placed in sealed tubes for 72 hours (24 to acclimatize+ 48 hours as experimental time lapse). Sleeping time, given as minute of sleep over 12 hours, for the three genotypes are presented. Kruskall-Wallis test has been used followed by Dunn's Multiple Comparison Test and p.value< 0,001(***), p.value<0,01(**), p.value<0,05(*) denote significant difference within experimental and control groups(marked in blue). All data are provided as Mean±SE

Genotype	Activity index (Mean±SEM)	Sleeping time (Mean±SEM)
Elav/Gal4>UAS-Nrx-1	1,38±0,06	840±40
White^{118}> Nrx-1	1,43±0,06	750±30
Elav/Gal4> White^{118}	1,09±0,04	900±40
Elav/Gal4>UAS-Oscillin	1,09±0,04	1110±20
White^{118} > Oscillin	1,14±0,03	880±30
Elav/Gal4> White^{118}	1,09±0,04	900±40
Elav/Gal4>UAS-Hmgcr	1,12±0,05	1100±20
White^{118}>Hmgcr	1,32±0,05	550±20
Elav/Gal4>White^{118}	1,09±0,04	900±40

Table 4)

Summary of the genotypes analyzed in the DAMS assay with the relative activity index and sleeping time values

Discussion, limitations and future prospects:

This work has shown that environmental elements such as 24 hours starvation and different dietary supply do not change significantly the expression of *Nrx-1* and *Oscillin.* Nevertheless these negative results a trend can be highlighted for both genes: they appear to be up-regulated when the flies undergo to an enriched dietary supply. As shown in figures 4 and 5 the relative mRNA expression levels are higher compared to both control and the other three experimental groups. It has to be underlined that also the variance within the 40:40 group is higher (table 3), thus leading to the conclusion that the occurring changes are not relevant upon the overall expression profile of the gene. Such a high variance among the 40:40 groups may be explained by the limited number of samples employed in the analysis. By increasing the number of replicates a decrease in the variance may be observable, leading indeed to a more accurate evaluation of the effects that these specific environmental conditions exerted upon *Nrx-1* and *Oscillin* expression.

The second part the project focused on, aimed to prove that *Nrx-1*and *Oscillin* were Knocked-down effectively, and the outcome seems promising. Even if the reduction in the expression profile was not statistically different from the controls, indeed the expected outcome has been obtained. What is relevant in this perspective is that the transcript levels of both genes were affected. Following this trend, now the number of samples and the incubation time at 29 C^{o} can be increased, thus improving the effectiveness of this approach and the reliability of the statistics behind the analysis. Notwithstanding the results of this section the behavioral assay conducted upon the *Nrx-1*, *Oscillin* and *Hmgcr* Knocked-down flies has been the most promising of the three tests conducted within these weeks. All the experimental lines showed modified parameters compared to the controls. *Nrx-1* and *Oscillin* affect the sleeping behavior, and their post-trascriptionally inactivation induced the flies to sleep differently over the 48 hours of recording. This can be seen as a partial elucidation for why these genes are

related to obesity. Their activity is somehow affecting the amount of time slept over a day, thus influencing the amount of calories required. *Nrx.1* affects the overall activity as well as the sleeping, but *Oscillin* unfortunately does not show this peculiarity, since the activity levels appear not to be changed in the experimental Knocked-down line. On the other hand *Hmgcr* affects both parameters, but in a different manner: Knocked-down *Hmgcr* flies sleep more than controls and also show the tendency to be less active over the entire length of the test. Further tests to verify the interaction between these genes, activity, sleeping and feeding behavior could indeed provide a wider understanding regarding how the calory intake is regulated or influenced by these obesity-related genes. Future research with a better set-up (longer incubation time, higher number of samples and cross testing between different behavioral assays) can indeed represent a key element for explaining the interconnection between genes activity and specific behavioral phenotypes.

Acknowledgments

I want to thank my supervisor Micheal J. Williams for the opportunity he gave me to work in his research group, and for support that he provided me during these ten weeks. A special thanks goes to Philip Goergen, the PhD student that followed me and thought me the techniques and working methods employed over the entire length of this project. Last but not least I want to truly thank all the members of the Fly laboratory that made these ten weeks project an extremely valuable and enjoyable experience under both professional and social perspective.

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