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Analyze Neural Circuits Underlying Key Insect Behaviors Using Advanced Neurophysiological Techniques

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Abstract

Every year, more than 50,000 people lose their lives to mosquito bites, which spread diseases like dengue, chikungunya, and yellow fever. It is necessary to construct domains of DNA-binding proteins that confer genomic sequence specificity in order to use TALENs, ZFNs, and homing endonucleases to accomplish mutagenesis in Ae. aegypti. In this article, we detail how to modify Aedes aegypti at specified sites using the Cas9-CRISPR machinery. This approach utilizes RNA-DNA base-pairing to give targeted specificity, resulting in flexible and efficient genome-editing tools. We examine the efficacy of injection mix compositions, show that CRISPR-Cas9 may produce diverse mutations via distinct repair processes, and disclose persistent germ-line alterations in many genomic locations. Using Aedes aegypti as a model, this study delves further into the usage of CRISPR-Cas9, which might open the door to genetic alteration in non-model species. In the tested setup, it failed to function as anticipated. No amount of hsp83, UAS, or tetO could prevent tTAV from upregulating the selected NIPP1 killer gene. A fatal positive-feedback loop was produced by tetO and tTAV2. When controlled by the tetO-tTAV system, the fusion proteins Gal4Groucho and Lexa Groucho, which had been used as corepressors in the past, proved to be lethal.

Keywords: Lexa Groucho, hsp83, UAS, or tetO, animals, tTAV2, RNA-DNA

Introduction

The study of insect behavior entails examining every action an insect does in connection to its surroundings. The field known as behavior genetics investigates the inherited causes of behavior. For a long time, qualities defined by "many" genes were studied using quantitative genetic approaches, whereas traits dictated by one or two genes were studied using Mendelian genetic studies.

Genetic studies of insect behavior are undergoing a paradigm shift due to the advent of molecular genetics and whole genome sequencing. But there is a catch, according to a new finding regarding the difference between lab and field behavior in the extensively studied fruit fly (Drosophila melanogaster): to avoid drawing incorrect conclusions, it is important to compare experimental settings that mimic natural habitats as closely as feasible.

An individual's behavior may be defined as any action they do in reaction to external stimuli or their immediate surroundings, with a focus on actions that are observable and comprehensible. On the other hand, insects may act without external influence. Research on insect behavior therefore include investigations of the insect's sensory input, processing of that data, and subsequent actions. Integrating data across time, including cues like hormones from inside the bug, may contribute to the way in which data is processed by the brain and spinal cord.

However, there may be a tangential relationship between the two and delayed. Compared to analyzing morphological or anatomical features, genetic study of behavior is perhaps more complicated. The challenge of providing a precise definition of the behavior is one obstacle to genetic analysis of behavior. It is easy to become confused about the number of genes involved when "a behavior" has numerous components. Particularly challenging is the task of differentiating between physiology and behavior.

There are at least four ways to look at the same behavior:

(1) the control or immediate cause; (2) the behavior's evolution throughout time; (3) the behavior's purpose; and (4) the behavior's evolutionary history.

Literature review

Arena, Paolo & Li Noce, Ale & Patané, Luca & Taffara, Salvatore. (2022) [2]. The perception-action loop is fundamental to how living things behave, but it is also one of the most difficult processes to describe because of the intricacy involved on two levels: the sensory system that is input and the behavioral repertoire that is output, which are both shaped by the actual limitations of the surrounding environment.

A vast array of actions is included by insect behavior. An individual's behavior may be defined as any action they do in reaction to external stimuli or their immediate surroundings, with a focus on actions that are observable and comprehensible. On the other hand, insects may act without external influence. A mix of commonsense practices, Integrated Pest Management (IPM) is a method for controlling pests that is both successful and kind on the environment. A variety of methods are included in integrated pest management (IPM), including cultural management, managing host resistance, behavioral control, pesticides, and biological control treatment, and some genetically-based management measures. which is discussed in this chapter along with signaling chemicals that convey information between living organisms and cause behavioral changes. Therefore, via integrated pest control, we can deduce the dynamics of insect behavior from the data it collects from its surroundings, analyzes, and acts upon.

Barbero, Francesca & Mannino, Giuseppe & Casacci, Luca. (2023) [4]. We investigate the potential function of biogenic amines like dopamine, tyramine, serotonin, and octopamine in regulating social behavior in insects, with a particular emphasis on ants. Various groups of Hymenoptera use amines to regulate mating behavior, movement, learning, memory, aggression, orientation affected by geomagnetic fields, and interspecific interactions, among other aspects. In addition, we conducted a bibliometric analysis to extract themes and patterns from the literature on biogenic amines and their potential role as social behavior modulators. Research on the aminergic regulation of behavior in social insects has shown encouraging results, suggesting that biogenic amines may play a pivotal role in the emergence of insect sociality. As a group, insects exhibit a greater level of interaction known as eusociality. Colony members are able to respond to the requirements of the society as a whole thanks to a multimodal communication system that keeps this intricate social structure running smoothly. Theoretically, neuromodulation of chemicals like biogenic amines allows colonies to exhibit flexibility, but the exact processes by which these regulatory substances do this are still not completely understood. In this article, we take a look at how the main bioamines-dopamine, tyramine, serotine, and octopamine-may influence the behavior regulation among the most significant groups of social insects, especially ants. Because functional roles vary across species and contexts, it is exceedingly difficult to directly correlate changes in behavior with changes in biogenic amines. In order to compile a summary of the current state of knowledge about biogenic amines in social insects, we used both quantitative and qualitative synthesis methods. Insect social evolution may be better understood with the help of new information on the aminergic regulation of behavioral responses.

Barr, Christina & Driscoll, Carlos. (2013) [5]. Depending on the circumstances, aggressive conduct may be adaptive; yet, it might backfire if it's too excessive or mishandled. Anxiety, reward sensitivity, and impulsivity are all genes that might influence violent behavior, according to neurogenetic research in apes and monkeys. Human and rhesus macaque studies have shown that the following genes-OPRM1, CRH, MAOA, DRD4, and SLC6A4-contribute to individual variances in aggressiveness: Mu-Opioid Receptor, Dopamine D4 Receptor, Monoamine Oxidase A, and Serotonin Transporter. An aggressive gene variant may have boosted evolutionary success but also increased the risk of psychopathology in contemporary humans, according to this corpus of studies.

Barron, Andrew et al. (2015) [6]. A stable unitary choice may be achieved by coupling pathways that accumulate data via inhibitory connections, as highlighted in both computational and theoretical models of decision-making. This coupling enables effective discrimination between competing alternatives. Additionally, theoretical research demonstrates that there are several techniques for implementing route coupling, which significantly enhances the efficiency of decision-making systems. It seems that the basal ganglia of vertebrates accomplish sustained action selection by serving as a hub for various inhibitory and excitatory inputs, allowing for the selection of a single response and the suppression of all others. It seems like the insect brain works on the same principles. At least for olfactory information, At the lateral protocerebrum (LP), a number of inhibitory and excitatory channels of olfactory data, allowing for stable decision-making and action selection. Despite its potential importance for efficient action section resolution, the LP has received very little attention from researchers studying insect brains. We propose that, while thinking about action selection in the invertebrate realm, it would be useful to draw inspiration from models built to study the vertebrate brain's function. In addition to laying the groundwork for future experimental investigations into the mechanisms by which insects make decisions and choose between possible courses of action, this method may also make it easier to propose new ideas.

Materials and Methods

For further in-depth instructions, see the Supplemental Experimental Procedures.

- **The Cas9 messenger RNA and its associated protein:** We used the mMessage mMachine T7 Ultra Transcription kit (AM1345, Life Technologies) to transcribe Cas9 mRNA from pMLM3613 (Addgene #42251). A recombinant Cas9 protein with the coding CP01 was acquired from PNA Bio.
- **Designing and building sgRNA:** Genomic areas were manually searched for Protospacer-adjacent motifs (PAMs) with the sequence NGG-where N may be any nucleotide-are used to create small RNAs (SgRNAs). To ensure proper transcription by T7 RNA polymerase, we listed the following requirements for sgRNA sequences: one or two 5′ terminal guanines, and a

length of 17–20 bp (not including the PAM).

- **Generation of DNA sequences:** Mosquito genomic DNA was extracted using a 96-well plate extraction method using the DNEasy Blood and Tissue Kit (69581, Qiagen) from both individual and group samples.
- **Analysis and sequencing of mutations produced by CRISPR-Cas9:** Primers were used in a two-step polymerase chain reaction (PCR) to amplify genomic DNA from G0 or G1 mice. procedure encompassing the probable CRISPR-Cas9 cut site. Libraries were subjected to Illumina MiSeq sequencing, followed by alignment to the wild-type reference sequence. Any polymorphisms, such as insertions or deletions, were then investigated. Go to https://github.com/bnmtthws/crispr_indel to get the scripts used to analyze this data.
- **Building donor cells for homology-directed cancer treatments:** 200 base pair 'Ultramers' (IDT Inc.) were used for the synthesis of single-stranded DNA oligodeoxynucleotides (ssODNs). Genomic DNA from LVP-IB12 was PCR-amplified and cloned Using the use of In-Fusion HD cloning from Clontech as the homology arms for the plasmid sources, into either PSL1180polyUBdsRED or pSL1180-HR-PUbECFP, which are both available on Addgene. The plasmid and oligonucleotide sequences utilized in homology-directed repair are identified in Supplemental Data S2.
- **Stable germ line alleles molecular genotyping using polymerase chain reaction:** The existence of insertions, deletions, or outside sequences introduced during homology-directed repair have the potential to be confirmed by PCR amplicons obtained from genomic DNA around the suspected cut site. Genewiz was used to Sanger sequence the purified amplicons, or BamHI or PacI were used as templates for restriction digests.
- **Statistics:** In terms of statistics, the data was summarized and shown using the matplotlib and seaborn tools in Python, respectively, boxplots and means with 95% confidence intervals are generated.
- **Stable allele genotyping using fluorescence:** The microscopic examination of immobilized larvae or pupae was carried out using a dissecting microscope (SMZ1500, Nikon) that was outfitted with a fluorescent light source, ECFP, and dsRed filter sets.

4. Results

4.1 Transgenic lines were created for underdominance

Figure 1 shows the results of the first method, which included administering the underdominance prototype strains tetO-LexA-NIPP1-AeHex1g-tTAV2-tetO-Gal4Groucho and tetO-UAS-NIPP1-AeHex1g-tTAV2-tetO-LexAGroucho. Both structures were introduced into the egg hatching process when the parents were given tetracycline. The development of the larvae that made it through these injections was also aided by tetracycline. This was vital to ensure that the transgenic insertions remained stable and that the potentially harmful constitutive production of NIPP1 was prevented. Oxitec provided the constructs necessary for genomic integration, which relied on the piggyBac transposition mechanism. A rate of 6.7% from embryonic to adulthood and a rate of 4.5% from adulthood was recorded. From the 170 G0s and 10 G0s that were crossed individually, no transgenic adults were found in the progeny. The embryo-to-adult survival rate following microinjection in D. melanogaster usually ranges from 12.5% to 37.5%, according to O'Connor and Chia (2002). Low survival rates and a lack of transgenic adults need a fresh approach. Our best guess is that the low survival rate was due to either inexperience with injections or temporary expression toxicity caused by the design, or perhaps both.

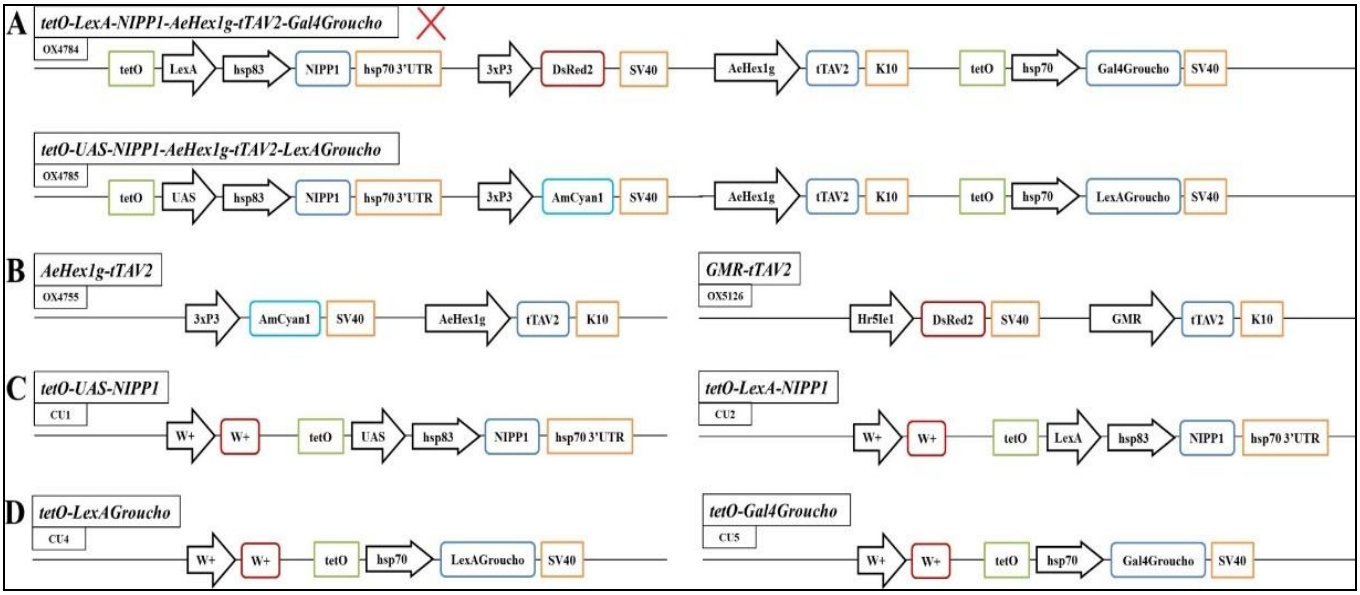

Fig 1: Cloned components, both alone and in combination, were used to create injection models of an underdominance system

4.2 tTAV2 expression is detected

Following the protocol outlined in Section 2.2.10, the function of the components was monitored by assessing their mRNA expression levels using RT-qPCR. To begin with, we wanted to know whether the tTAV2 expression level was sufficient to upregulate NIPP1. Consequently, would the anticipated impact of cell death be brought about by elevated NIPP1 levels.

Due of the lack of site-directed construct integration in the genome, we made sure to assess the expression levels of each component before doing any functional analysis or crosses. A number and a letter were used to identify transgenic lines that were created from offspring that tested positive for the G1 marker. The numbers correlate to the G0 adult number, while the letters indicate that the lines were developed from the same G0 parent. The eight transgenic lines chosen for RT-qPCR analysis display varying degrees of tTAV2 expression for both AeHex1g-tTAV2 and GMR-tTAV2 (Figure 2). The AeHex1g promoter's spatial expression in D. melanogaster has not been previously studied. Specifically expressed in female mosquitoes' fat bodies, this promoter was created and tested in the Aedes aegypti mosquito. Both sexes exhibit expression, according to the findings of the head, thorax, and abdomen fractions. Unlike Aedes aegypti, it does not have a fat body limit and does not have the expected female-specific expression.

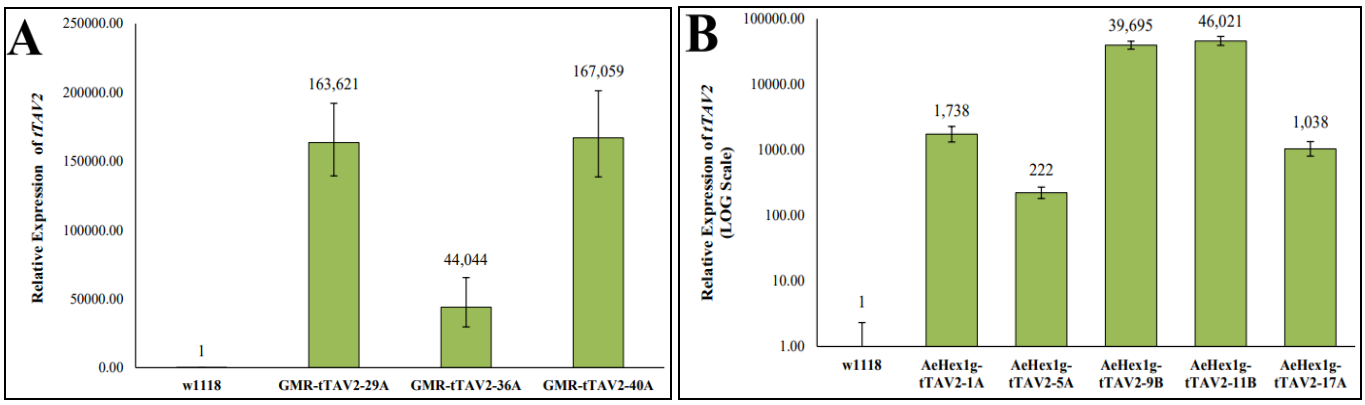

Fig 2*:* Both the GMR-tTAV2 and the AeHex1g-tTAV2 driver lines exhibit variation in tTAV2 expression across insertions

The x-axis shows different driver line insertions, while the y-axis shows the relative expression of tTAV2 with respect to w1118. W1118 is used as a control as it does not express tTAV2. What really important is the relative expression of tTAV2 over all of the lines, not the high relative expression figures that are essentially artifacts showing fold differences compared to 0. A control amplification using RNA polymerase II is used to standardize the relative expression levels of the cDNA input. With the help of the error bars, we can see the standard deviation of the triple technical replicates. The GMR-tTAV2 lines are these (A). For every cDNA sample, two biological replicates-that is, two adult female heads of D. melanogaster-were used. Lines 29A and 40A seem to have a higher level of tTAV2 expression. As for (B), these lines stand for AeHex1g-tTAV2. The other insertions may be more easily compared to the highly expressive lines 9B and 11B since the y-axis is on a logarithmic scale. Two mature female dolphins (D. melanogaster), or replicates, were used to produce each cDNA sample. It seems that lines 9B and 11B have much greater tTAV2 expression.

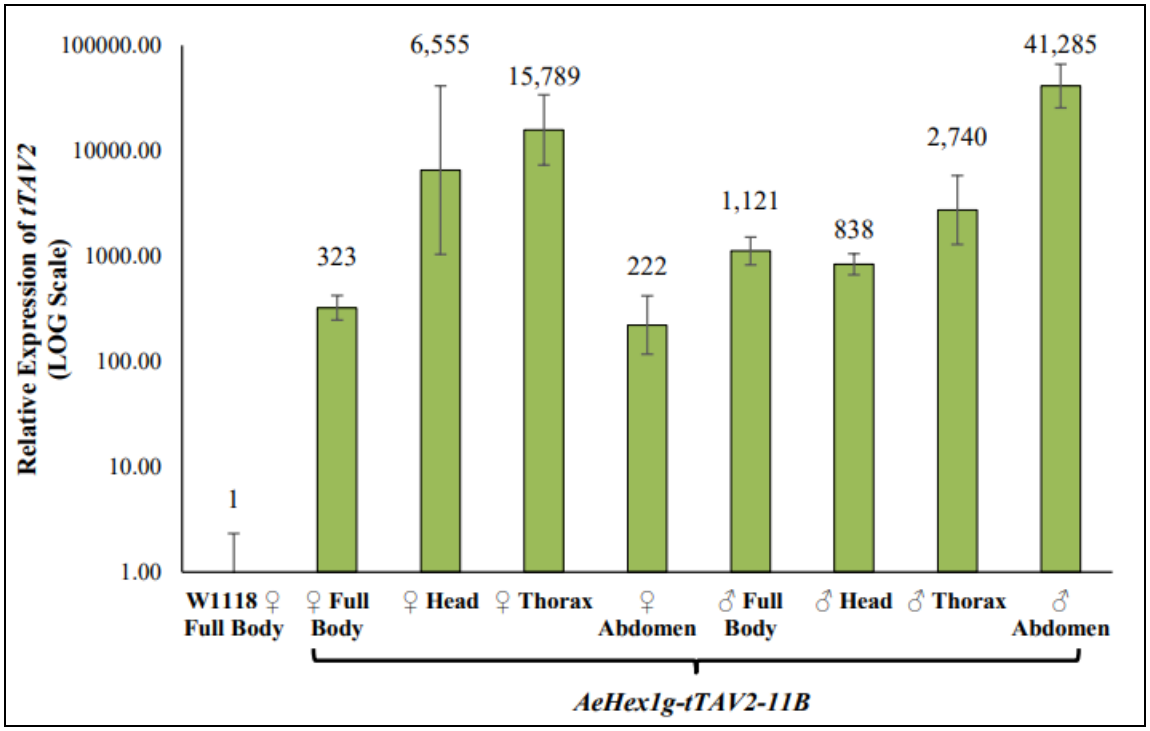

Fig 3: Transcriptional activation of tTAV2 by AeHex1g does not mimic the promoter's expression pattern in Aedes aegypti

For various cDNA samples, On the y-axis, a logarithmic scale displays the relative tTAV2 expression compared to w1118. By comparing the expression level data to the RNA polymerase II control processes, the amount of cDNA input is regulated for. As you can see from the error bars, there is a standard deviation for the three technical replicates. Expression fold differences are shown in comparison to the w1118 control, in which tTAV2 is set to 1. Over the error bars, you can see the expression values. Each cDNA sample is the biological equivalent of one biological replication, and it was created using one adult D. melanogaster (whole or split). The most prominent feature of the male AeHex1g-tTAV2-11B abdomen seems to be tTAV2 expression, whereas the expression varies throughout the rest of the body.

4.3 NIPP1 expression detected

Now that we know tTAV2 expression is present, we need to know whether the UAS-NIPP1 lines express NIPP1 when GAL4 is not present (basal expression). While tTAV2 expression varied between the lines tested, NIPP1 expression levels were very consistent across all three RT-qPCR stock lines. The three lines show that NIPP1 expression is basal or leaky in comparison to w^{1118} endogenous expression. Primers used to amplify endogenous and transgenic NIPP1 sequences were used. The presence of 5xUAS sequences and limited promoter sequences might be the reason for this.

Although UAS sequences should need Gal4 for upregulation, the observed basal expression might be caused by hsp83 sequences or UAS. Given the paucity of compelling evidence for UAS-only basal expression (reportedly very low in the absence of Gal4) and the frequent presence of Hsp83 near minimal promoters in Drosophila, it seems probable that Hsp83, the minimum promoter in this system, is driving the basal expression of NIPP1.

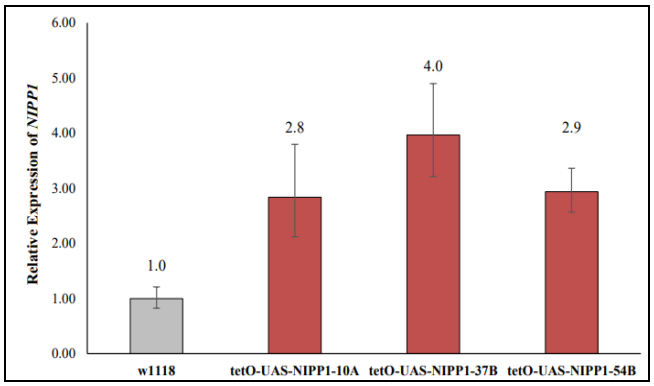

Fig 4: None of the tetO-UAS-NIPP1 lines differ in their basal NIPP1 expression.

The x-axis shows different killer line insertions, while the y-axis shows the relative expression of NIPP1 compared to w1118. We standardize the amount of cDNA input by comparing the expression level results to the control reactions of RNA polymerase II. As you can see from the error bars, there is a standard deviation for the three technical replicates. In the w1118 control, the endogenous NIPP1 level is fixed at 1, and changes in expression fold are shown relative to this. The values of the expressions are shown above the bounds of the mistake. Each cDNA sample consisted of two biological replicates, or two adult female heads of D. melanogaster. We only used heads because we expected NIPP1 overexpression in the eyes after crossing to GMR-tTAV2. This RT-qPCR technique detects endogenous and transgenic NIPP1 sequences, however NIPP1 baseline expression in UAS-NIPP1 lines increases the amount of NIPP1 expressed by an additional 3-to 4-fold. Thermo Fisher Scientific's TURBO DNase was used to digest the gDNA.

4.4 Transgenic lines with tetO, UAS, NIPP1, Ae, Hex1g, tTAV2, and Lech Groucho do not exhibit any longevity. Post-Tet rearing

When fed on an Off-Tet diet for an extended period of time, adults of the prototypical No viable offspring are generated by the tetO-UAS-NIPP1-AeHex1g-tTAV2-tetO-LexAGroucho strain. offspring. People who were raised or given a diet without tetracycline will be called Off-Tet, while people who are given a diet with tetracycline will be called On-Tet. Upon transitioning to an Off-Tet diet, the majority of transgenic lines that were homozygous for independent insertions failed to produce transgenic adults. All offspring should carry the gene for transgenes as this is an inbreeding homozygous transgenic line. The results showed that just one of the six insertion lines could successfully create adults that could not carry the Off-Tet diet on to the next generation. The other five lines failed to produce any offspring that made it beyond the late pupal stages. Some of the fatalities that occurred during the pupal stage were linked to specific anomalies seen in the pupal cases. Furthermore, there was a decrease in the quantity of larvae reaching the third instar, indicating that death had far-reaching consequences. to the pupal stage. Only NIPP1 is expected to be a tetracycline repressible killer in this build. Nevertheless, I had previously shown that the individual component analysis reveals very little NIPP1 upregulation. For this reason, I set out to find more explanations for the context's lethality.

After being reared on Tet, the majority of the transgenic lines-5 out of 6-were unable to produce viable adults when given an Off-Tet diet. An adult-bearing first-generation offspring was successfully generated from insertion line 21B. Despite keeping the adults off-tet and crossing them, no viable larvae were produced.

Table 1: When Off-Tet, no healthy progeny are produced by tetO-UAS-NIPP1-AeHex1g-tTAV2-tetO-LexAGroucho adults suitable for stocking stables

tetO-UAS-NIPP1-AeHexlg-tTAV2-tetO-LexAGroucho lines	Transgenic larvae amongst the first generation Off-Tet	Transgenic adults amongst the first generation Off-Tet	Transgenic larvae amongst the second generation Off-Tet
9C	Yes	No	-
9D	Yes	No	-
12A	Yes	No	-
15A	Yes	No	-
15E	Yes	No	-
21B	Yes	Yes	No

4.5 TetO sequences have the ability to boost expression upstream by a minimum of 1900 bp.

Other components than NIPP1 could have caused the fatality in transgenic lines that lack tetracycline, such as tetO-UAS-NIPP1-AeHex1g-tTAV2-tetO-LexAGroucho, since the cause of the mortality phenotype is yet unknown. Furthermore, we examined the early pupae cDNA samples for the expression levels of tTAV2, a virus that is known to be toxic to insects when present in sufficient amounts. The expression levels of tTAV2 are significantly elevated in early pupae that are reared off-the-grid. Positive reinforcement of tTAV2 expression seems to be occurring via tetO sequences. The enhancing interaction may occur at least as long as the distance between the tetO operator and responsive promoter is at least as much as these tetO sequences, which are the closest to tTAV2 1900 bp. In the finished design, the tetO downstream is the greatest choice for the feedback loop as it is the enhancer that is closest to tTAV2.

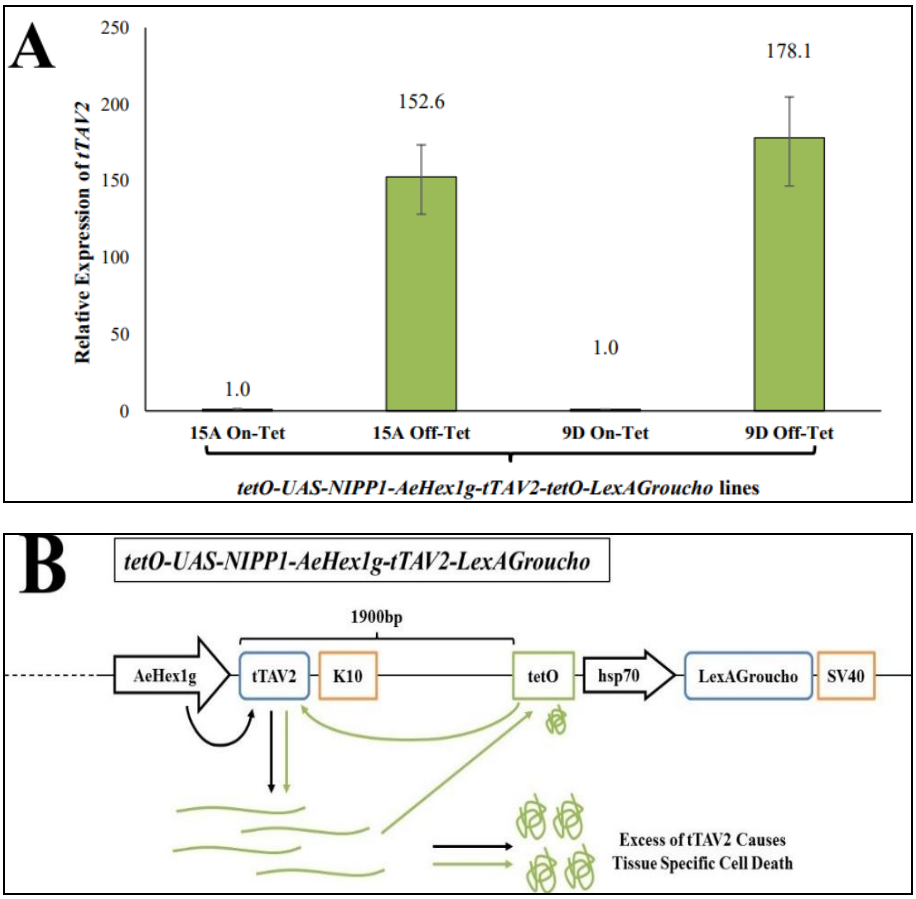

Fig 5: Off-Tet, tetO-UAS-NIPP1-AeHex1g-tTAV2-tetO-LexAGroucho lines show a 150-fold increase in tTAV2 upregulation.

As the killer line insertions are presented on the x-axis, the tTAV2 relative expression is shown on the y-axis. We standardize the amount of cDNA input by comparing the expression level results to the control reactions of RNA polymerase II. As you can see from the error bars, there is a standard deviation for the three technical replicates. Lines of tetO-UAS-NIPP1-AeHex1g-tTAV2-tetO-LexAGroucho that received tetracycline medication have their expression fold changes displayed relative to the level of tTAV2, which is set to 1. The values of the expressions are shown above the bounds of the mistake. Each cDNA sample consisted of two biological replicates, or two D. melanogaster early pupae. Both lines showed a 152- and 178-fold increase in tTAV2 expression, respectively

5. Conclusion

In the presence of It seems that either teto or the contacts between these components were disrupted since there was very little upregulation of NIPP1 by ttav2, and no UAS or lexa between teto and hsp83. A further context-dependent feature is the robust positive feedback loop between ttav2 and teto. Despite their 1900 bp distance. One possible solution to this issue is to use enhancer-blocking insulators like dctcf, which is an orthologue of the well-studied CTCF insulator in mammals found in the faraway fruit fly. In conclusion, contrary to other reports, the teto-ttav2 system seems to be able to render Gal4Groucho and lexagroucho fusion proteins in D. Melanogaster deadly. While there are few instances of modularity in biology (e.g., 3xP3 promoter sequences consistently trigger the downstream expression of any gene with tissue specific messenger RNA), most biological processes rely on their surroundings. Variability and noise are ever-present in biological systems, even in the best-case scenarios.

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