ROLE OF GALECTIN IN THE REGULATION OF NEURAL EXCITABILITY BY *Drosophila* SIALYLTRANSFERASE

A Senior Scholar Thesis

by

NIRAJ KC

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biochemistry
Genetics

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Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Role of Galectin in the Regulation of Neural Excitability by *Drosophila* Sialyltransferase. (May 2012)

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Galectins belong to a family of endogenous lectins with affinity for β-galactosidase-containing oligosaccharides. They are differentially expressed by several types of cells of the immune system in vertebrate organisms. Through lectin-carbohydrate interactions, they can interact with cell-surface and extracellular matrix glycoconjugates like glycoproteins and glycolipids and can promote cell growth, affect cell survival, modulate cell adhesions and induce cell migration. Role of galectins has been mostly investigated in the immune system and homeostatis. It is believed that galectins may be involved in other crucial functions, such as regulation of neural functions via collaboration with the pathways of neural sialylation. However, the relationship between galectin and sialyltransferase functions in the nervous system has never been established. I hypothesize that galectins work together with sialyltransferase to regulate neural

excitability. Using *Drosophila* as a model system, I focus on testing this hypothesis. In my project, I will generate galectin mutants and carry out the functional analysis of these mutants. I will investigate if galectin function contributes to neurological phenotypes of Drosophila Sialyltransferase (DSIAT) mutants. If the hypothesis is correct, I anticipate detecting genetic interactions between galectin and DSIAT mutant alleles. Depending on the mode of the interactions, I can expect that the mutant phenotype will be enhanced or ameliorated. These results will shed light on the molecular and genetic mechanisms of galectin function in the nervous system.

DEDICATION

This is dedicated to my lovely parents Mr. Shambhu Kumar KC and Mrs. Chandra Kumari KC.

ACKNOWLEDGMENTS

I would like to extend my deepest gratitude to all the individuals who inspired and helped me to carry out this research. I want to thank the Department of Biochemistry and Biophysics for giving me an opportunity to carry out the necessary research work and to use the departmental resources. I am deeply indebted to my supervisor, Dr. Vladislav Panin, from Texas A&M University whose help, stimulating suggestions and encouragement helped me during the time of research and writing of this thesis. I am grateful to have mentors and lab colleagues like Dr. Dmitry Lyalin, Courtney Caster, Hillary Witzenman, and Morgan Ritz who provided immense support during my time in the lab. Especially, I would like to give my special thanks to my dear friends Dheeraj Pandey and Manoj Rajaure for their encouragement and support that enabled me to complete this work.

NOMENCLATURE

Neu5Ac	N-acetylneuraminic Acid
Neu5Gc	N-glyconulneuraminic Acid
DSIAT	Drosophila Sialyltransferase
UDP	Uridine Diphosphate
GlcNac	N-acetylglucasamine
N	Amino Group
C	Carboxyl Group

TABLE OF CONTENTS

Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Glycoconjugates are the products of an important biochemical reaction called glycosylation in which sugars are added to protein and lipid molecules. Glycoconjugates are also called “third language of life” following nucleic acids and proteins (Schauer, 2004). A specific type of glycosylation, catalyzed by sialyltransferase, in which sialic acid is added to the protein or lipid is called Sialylation. Sialic acids (Figure 1) belong to a family of 9-carbon-alpha-keto acids (Angata and Varki, 2002) and are composed of nine-carbon sugar backbone unlike most other sugars of vertebrate-glycoconjugates which have five or six. Sialic acids are crucial complex carbohydrates and are located at non-reducing end of glycans attached to glycoconjugates (Figure 2). They are negatively charged and appear largely on cell surface in mammals and other vertebrates. They play an important role in mediating cell to cell recognition and cell adhesion. There is evidence that sialic acids are prominently involved in cell-signaling mechanism (Schauer, 2004). Other important functions include regulation and turnover of glycoprotein, pathogen-host recognition, immune system functioning, and nervous system development.

This thesis follows the style of The Journal of Neuroscience.

Studying biochemical pathways of sialylation in fruit fly *Drosophila melangoster* has many advantages. For instance, powerful molecular genetics, low genetic redundancy, thoroughly-studied development, various possible physiological and behavioral assays are some of them. Previous studies have indicated that the genome of *Drosophila* lacks some essential genes encoding important enzymes necessary for the synthesis of sialic acid, such as the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNac kinase that is necessary for the conversion of UDP-GlcNAc to ManNac-6-phosphate (precursor for Neu5Ac) (Angata and Varki, 2002) (Figure 3). However, *Drosophila* does contain several other orthologous genes which are believed to contribute in the sialylation pathway (Angata and Varki, 2002). Moreover, *Drosophila* possesses unique functional α-2, 6 sialyltransferase (DSIAT) that has significant homology to mammalian ST6Gal sialyltransferase (Repnikova et al., 2010). Recent experiments indicated that DSIAT is involved in the regulation of neural excitability and development in fruit flies. However, the molecular mechanism of these functions remains to be elucidated. By analogy with vertebrate systems, we hypothesized that sialylation could be involved in "masking" of galactose residues, which suggested a possibility of interplay between sialylation pathway and interactions between terminal galactose residues and lectins, such as galectins.

Figure 1. Sialic acids are composed of 9-carbon-alpha-keto acids.

Galectins belong to a family of endogenous lectins with affinity for β-galactosidase-containing oligosaccharides (Rabinovich et al., 2007) (Figure 4). They are differentially expressed by several types of cells of the immune system in vertebrate organisms (Liu, 2005; Rabinovich et al., 2007). Through lectin-carbohydrate interactions, they can interact with cell-surface and extracellular matrix glycoconjugates like glycoproteins and glycolipids and can promote cell growth, affect cell survival, modulate cell adhesions and induce cell migration. Current research and accumulating evidence indicate that galectins play important roles in the immune cell response and homeostasis. On the other hand, the role of galectins in the nervous system remains largely unknown. My project is focused on the elucidation of this role using Drosophila as a model system. Employing two different methods which are described in Chapter II below, I will generate a novel galectin mutant fly stock. Then I will combine this mutant with DSIAT mutant to form a double mutant and carry out various functional analyses on this mutant. Comparing the results from the double mutant with DSIAT mutant and the wild type flies will provide deeper insights on the role of galectin in the neural excitability by Drosophila sialyltransferase and hence in the nervous system.

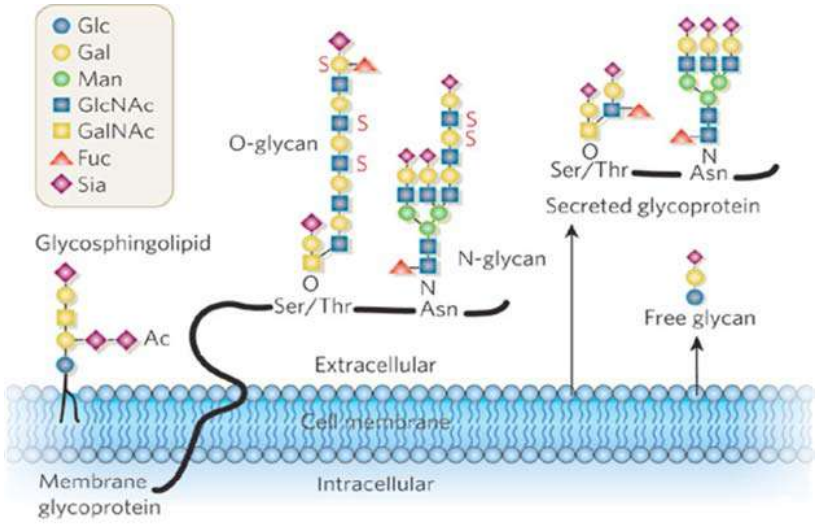

Figure 2. Sialic acids are located at non-reducing end of glycans. They are attached to glycoconjugates and appear largely on cell surface of mammals and other vertebrates.

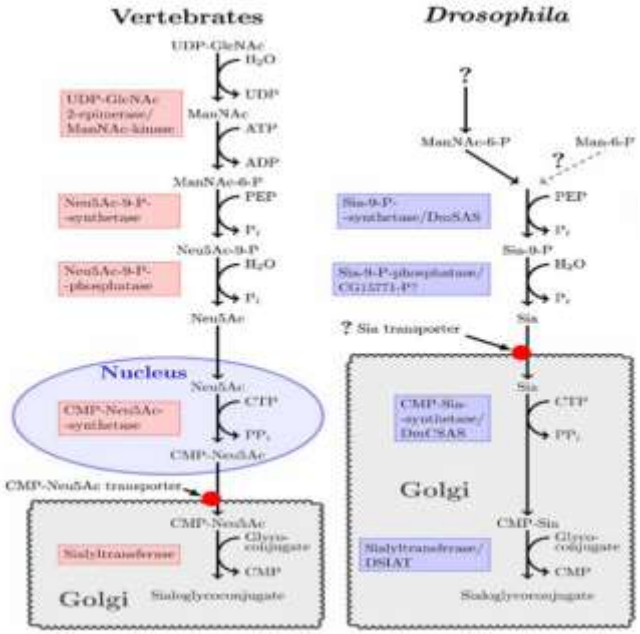

Figure 3. Sialylation pathway. *D. melanogaster* carries most of the enzymes necessary for sialylation except the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNac kinase that is necessary for the conversion of UDP-GlcNAc to ManNac-6-phosphate(precursor for Neu5Ac). Adapted from Glycoconj J DOI 10.1007/s10719-008-9154-4.

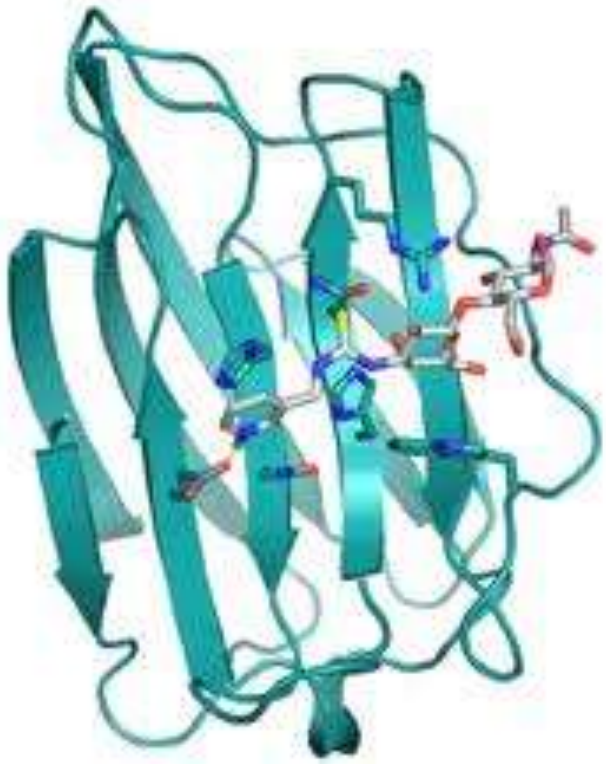

Figure 4. Galectins bind to β-galactosidase-containing oligosaccharides. Courtesy: www.organic.lu.se

CHAPTER II

METHODS

Creating a novel galectin mutant stock is the key to my research. I employed various biochemical tools and genetic assessment throughout my research. To generate the deletion of *Drosophila* galectin gene, two strategies will be approached.

FRT-mediated deletion

The first strategy involves the use of FLP recombinase and the FRT-bearing transgenic stocks which will facilitate *trans*-recombination events to create the deletion in one chromosome and duplication in another (Parks et al., 2004). This part of research was carried out in collaboration with Morgan Ritz who is another undergraduate research worker in Dr. Panin's lab. To start with, I created a stable stock containing fØ7544 (frt1) and an observable marker bristle (Bl) on the same chromosome. This process required several generations of fly-crossings which is summarized below:

Cross 1: female ywf; f Ø7544 × male yw/Y ; Bl/Cyo

Cross 2: female yw; Bl/Cyo × male ywf/Y ; f Ø7544

Progenies from crosses 1 and 2 were crossed as below:

Female yw/ywf ; f Ø7544/Bl × Male yw/Y ; Bl/Cyo

The progeny from the above cross were scored for red eye, bristle and curly wings with genotype (yw or ywf); (f Ø7544, Bl)/Cyo and crossed with balancer to produce a balanced stock as below:

Step 1 Male (yw or ywf)/Y; (f Ø7544, Bl)/Cyo × Female yw; Bl/Cyo

Step 2 Male (yw or ywf)/Y ; (f Ø7544, Bl)/Cyo × Female (yw or ywf)/yw; (f Ø7544, Bl)/Cyo

The progenies from step 1 were scored for red eyes, bristle marker and curly wings and self crossed as in step 2 to maintain a stable stock.

This stock of flies generated above was crossed with another stock containing other transposable element (frt2) which we obtained from *Drosophila* stock collection. Crosses place two FRT-bearing transposon insertions in *trans* and the heat shock-driven FLP recombinase. Once the FLP recombinase is activated, it results in the recombination between the two FRT elements and results in the generation of deletions. The goal was to generate the deletion between two transposable elements which will create the novel galectin mutant and the deletion can be detected by PCR. Following the bristle marker, the progenies will be selected and their genomes will be analyzed by PCR and gel-electrophoresis. The positive candidates will be verified by DNA sequencing service provided by Plant Technologies in Borlaug Center of Texas A&M.

Hobo hopping deletion

The strategy involves the use of the hobo P{wHy} hybrid transposable element to create the deletion. This hobo hopping deletion method can be used to generate sets of precisely mapped deletions in a given region of *Drosophila* genome (Huet et al., 2002) (Figure 5). Once activated by hobo transposase, the hybrid transposable element hops randomly to the either direction and copies itself to the new location. Local hops are favored more than the hops covering the longer distance across the genome. The recombination between the newly copied element and the original element results in the deletion. Hence, this strategy exploits the local transpositional and recombinational properties of the hobo element (Huet et al., 2002).

Stocks containing hobo in galectin P{wHy} and hobo transposase In(2LR)/ cyo,P{hs-H\T} were ordered from Bloomington Fly Stock Center, Indiana. These stocks were used as parents to create several generations of flies to give the final galectin mutant. The reciprocal cross was:

21281 yw; P{wHy} × 6602 yw; $\frac{\text{In(2LR)}}{\text{cyo,P\{hs-H\T\}}}$

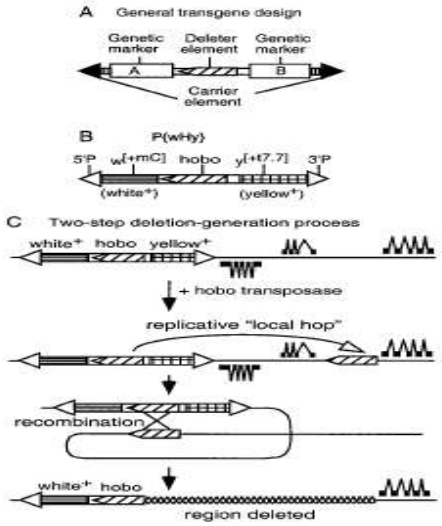

Figure 5. Basic Strategy of Hobo Local Hopping mediated deletion. (Huet et al., 2002)

Elimination of hobo confined deletion

Genomic DNA of the candidate flies was extracted using REDExtract-N-Amp Tissue PCR Kit from Sigma-Aldrich. A PCR test was used to identify deletion events confined within the hobo element. Some local hops are short and cover only small region away from the original position. In some cases, they cover far enough to destroy the marker on the right which in our case is yellow body gene but do not pass the 3' P element (Figure 5). Hence, the flies with these events will lose the yellow marker but will not have the deletion we seek. To eliminate these events, a PCR test based on the retention of both P element ends [3] was done. The expected amplified product is about 360 base pairs in length and should be observed in non-candidates only. The primers used were pendout2 (5'-CGACGGGACCACCTTATGTT-3') and wHy_genomic_primer1 (5'-

TTCCATCCACCACCTCGATG-3'). The products of the PCR reactions were run on 1.2% agarose gel along with the controls.

Deletion mapping using the inverse PCR

The inverse PCR protocol of J. Rehm available at www.fruitfly.org/about/methods/inverse.pcr.html was used with few exceptions. The restriction enzyme used was AluI, and the primers were Ph-EA1 5'-GGGCATAATCTATTTCGCTTTCT-3' and Ph3-2 5'-CGAGTATTTTGTGTGCCGCAAGT-3'. All the flies which failed to amplify a PCR product during the hobo confined deletion test were tested using this method. Since the flies did not give any amplification products in the previous test, they are not the hobo confined deletion and hence, using this method, the region covered by the deletion was determined. This was done using PCR, gel-electrophoresis, and DNA sequencing at the end.

Once the galectin mutant has been created, it will be combined with DSIAT mutant to create a double mutant genotype by several generations of fly-crossing. This double mutant will be missing activities of both the galectin and DSIAT genes. These flies will be then tested for mutant phenotypes like neural excitability and life span and compared to both the wild-type flies and DSIAT mutant flies. This comparison will shed more light in the role of galectin in the sialylation pathway.

One possible problem with the proposed method is that deletion of the galectin gene may turn out to be lethal and the further analysis may be impossible. Since galectin has other crucial functions in the cell like their roles in the immune cell response and homeostasis, the deletion of the gene may be lethal and the fly may not survive. If this happens, then I will employ new methods to create tissue-specific deletion of galectin gene. The tissues of target will be central nervous system (neurons and glial cells) where sialyltransferase is exclusively expressed (Repnikova et al., 2010).

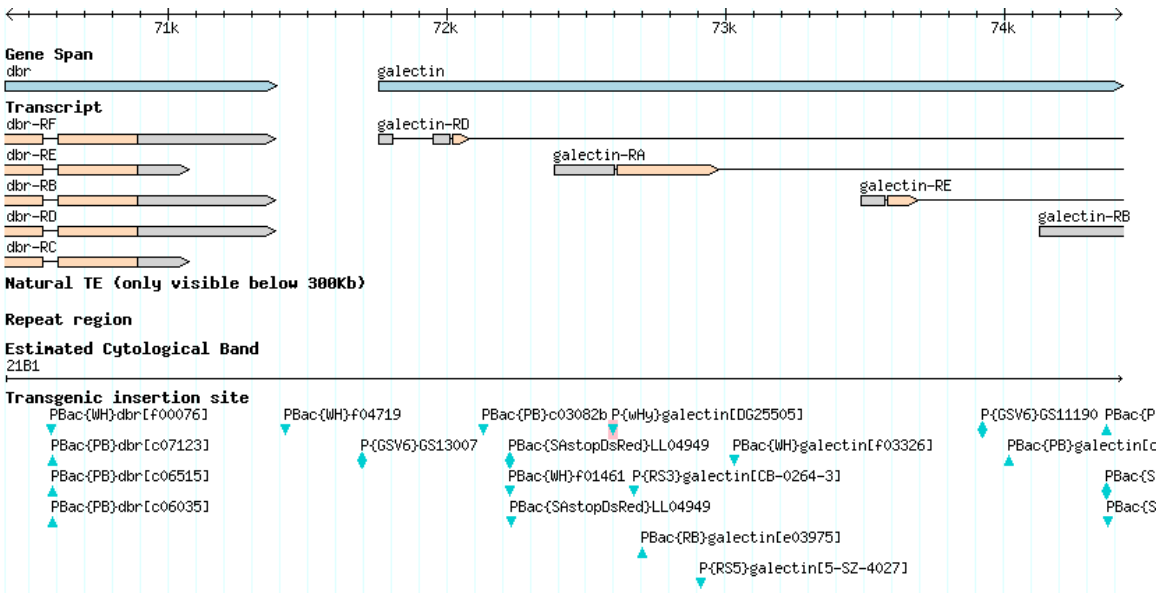

Figure 6. Genomic map of *Drosophila* Genome. It shows the position of hobo element and the galectin gene. (http://flybase.org/cgi-bin/gbrowse/dmelstocks/?ref=2L;start=70421;stop=74421;nav4=1;plugin=)

CHAPTER III

RESULTS

Hobo hopping deletion

The flies crossed following the hobo hopping deletion protocol (Huet et al., 2002) were scored for yellow body, red eyes and either of bristle or curly wings but not both. The flies with these phenotypes represent the jumping of the hobo transposable element towards the right direction where the galectin gene is located in the chromosome (Figure 6). However, for the first trial started in July 2011, the efficiency was very low (Figure 7). Out of 2015 flies examined after setting up the cross, only 3 had the phenotypes sought and the overall efficiency was 0.15%. Out of 3 candidate flies, only two flies successfully produced progeny which was then checked using multiple PCR to ensure the deletion if any (Chapter II, methods).

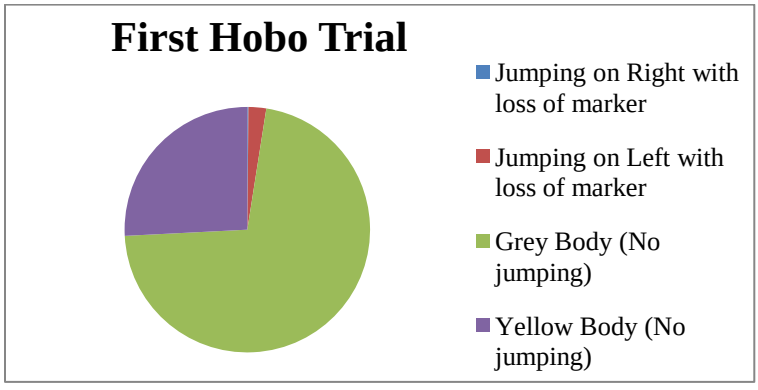

Figure 7. Summary of the first round of hobo hopping trial.

Since the right deletion is a scarce event even after the hobo hopping, the original protocol of hobo hopping method was modified to include more frequent and prolonged heat shock. The flies were subjected to heat shock for one hour every day until they hatched instead of 30 minutes every other day, as it was suggested in the original protocol. This modification further increased the expression of the hobo transposase and hence, the local hopping was more likely to take place. The efficiency was improved to 1.05% and 40 flies with the sought phenotypes were obtained out of 3,815 flies scored (Figure 8).

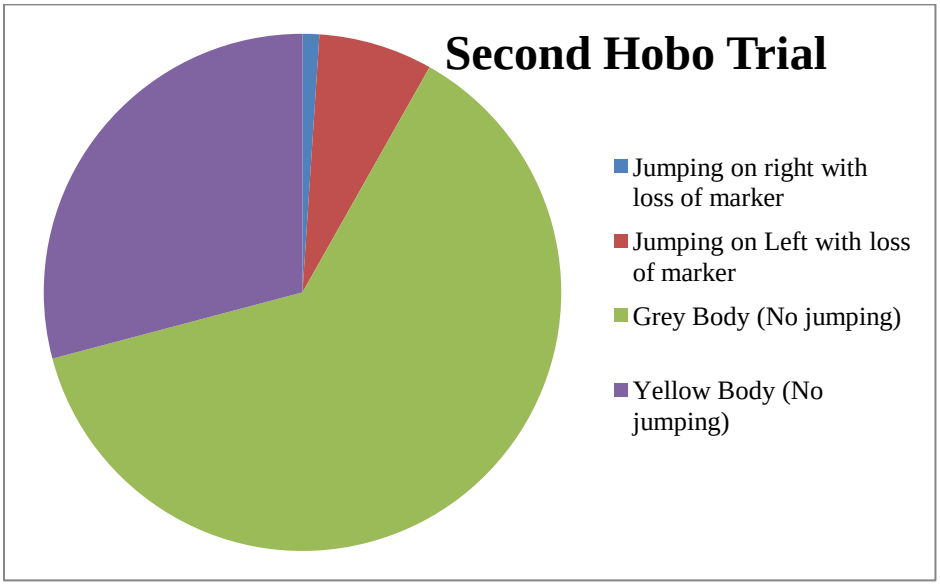

Figure 8. Summary of the second round of hobo hopping trial.

Out of the 40 candidate flies obtained, 5 flies did not produce any progeny and hence, could not be further tested. The remaining 35 flies were used to generate stable lines that were checked using multiple PCR reactions to ensure the deletion if any. For generating these stable lines, the candidate flies were crossed with yw; Bl/Cyo flies individually.

The progeny from the first cross were scored for curly wings, red eyes and yellow body and were then self-crossed to create stable mutant fly lines corresponding to each unique recombination event.

Elimination of hobo confined deletion

Out of the 37 fly strains tested (2 from first hobo cross and 35 from second), 4 strains showed an amplified bands of about 360 base pairs and 33 did not (Figure 9). Those which amplified were eliminated while the fly strains which failed to amplify were retained for further analysis by inverse PCR. The candidates which were eliminated using the method were nkc7, nkc25, nkc4 and nkc5. The PCR amplification to give a DNA product of 360 base pair represents an event confined to the P{wHy} transgene: a small deletion extending from one end of the hobo element and removing part of the yellow transgene, thereby inactivating it. Hence, the fly with this event lose the yellow marker but does not represent the deletion far enough to cover the galectin gene on the right hand side of the hobo transposable element.

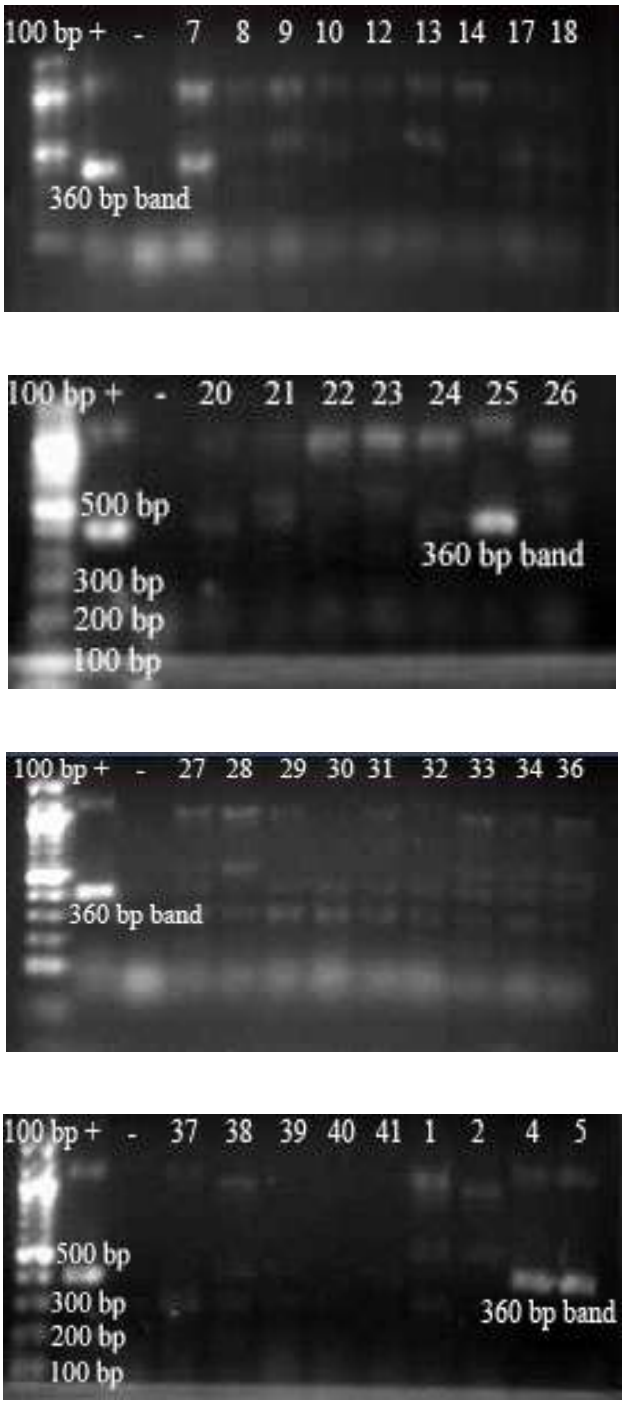

Figure 9. Agarose gel-electrophoresis of the PCR products (nkc7-nkc5) to eliminate hobo confined deletion events. Gel was loaded in the order, left to the right, 100 bp molecular marker, positive control (yw; P{wHy}), negative control (wild type), and the mutant candidates. From top to bottom: Amplification product observed for nkc7, nkc25, nkc4 and nkc5.These candidates were eliminated. No amplification product was observed for the third row.

The positive control used during this test was the original hobo in galectin (yw; P{wHy}) stock which contains the original hobo P{wHy} hybrid transposable element. Hence, the positive control retains both the P element ends. Therefore, it gives the idea what band to expect in the flies with the hobo confined deletion events. The negative control used was a wild type fly that lacks the hobo transposable element. Hence, no band was observed in the negative control.

Deletion mapping using inverse PCR

Due to the time constraints, not all the mutants were tested using the inverse PCR. Out of the two mutants tested (nkc1 and nkc2), nkc1 was homozygous lethal. Hence, all the flies had curly wings due to the presence of the balancer chromosome with Cyo marker as the homozygous Drosophila die during the developmental stage (no flies with straight wings present). On the other hand, nkc2 was not homozygous lethal and contained flies with both curly and straight wings. It is expected that nkc1 which is homozygous lethal is likely to contain the deletion covering the galectin gene more than nkc2 which is not homozygous lethal. This is because of potentially important roles of galectin for fly viability (possible involvement in the immune response, homeostasis and other crucial processes).

Genomic DNA was extracted as described in Chapter II (methods) followed by the enzymatic digestion with AluI. RNase was used to get rid of RNA contamination during

the DNA prep. The digested DNA was ligated and PCR amplified. The gel pictures, showing the progression of this process, are shown in Figure 10. A 300 bp DNA band is observed in both the mutant nkc1 and nkc2 at the end. However, some extra DNA band is observed in nkc2. PCR conditions will be adjusted to improve the resolution of the DNA bands. Then, PCR amplified DNA will be extracted from the gel and sent for sequencing from which the region covered by the deletion will be determined.

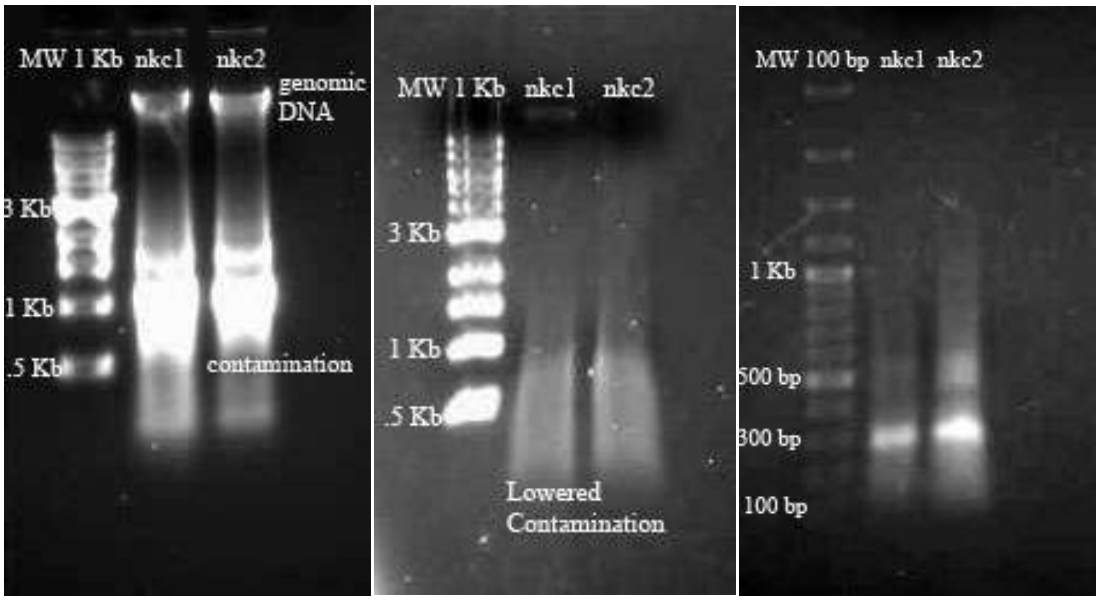

Figure 10. Inverse PCR to map the deletion events. From left to right: a. Genomic DNA extraction from mutants nkc1 and nkc 2. b. Digestion of genomic DNA with AluI and RNase. Note the RNA contamination is reduced significantly and genomic DNA has been digested. c. Ligation and PCR amplification of digested DNA. A 300 bp band is observed in both the mutants. Note the difference between the products in nkc1 and nkc2.

CHAPTER IV

SUMMARY AND CONCLUSION

In this project, I have generated thirty three potential mutants that may include the deletion of the galectin gene. Employing various genetic and molecular tools, I have eliminated the non-candidates from the total mutant pool. Over the research period, I employed two different strategies to generate the novel galectin mutant. One involved use of FLP recombinase and the FRT-bearing transgenic stocks which facilitated *trans*-recombination events to create the deletion in one chromosome and duplication in another. The other method used the hobo P{wHy} hybrid transposable element to generate sets of precisely mapped deletions in a given region of Drosophila genome, which in our case is the region containing galectin gene. The region contains two galectin genes in a row.

For the remaining of the research period, I will be carrying out inverse PCR for other candidate lines and verifying the deleted region. Depending upon how far the hobo transposable element has hopped to the right (Figure 5), I might get the deletion of one galectin gene or both the galectin genes or none. Some may be useful while others may not. The one with one galecting gene deleted is the one useful in my project and I will be combining this single galectin mutant with DSIAT mutant to make a double mutant. This double mutant lacks the activities of both DSIAT and galectin and will be subjected to different functional analysis. The relationship between galectin and sialyltransferase

functions has never been established. My hypothesis is that the galectins work together with sialyltransferase to regulate neural excitability, which suggests that galectin function contributes to neurological phenotypes of DSIAT mutants. By creating a novel galectin mutant and combining with DSIAT mutant, it is possible to reveal the relationship between galectin in the sialyltransferase functions. I anticipate to detect genetic interactions between galectin and DSIAT mutant alleles. Depending on the mode of the interactions, it can be expected that the mutant phenotype will be enhanced or ameliorated. These results will shed light on the molecular and genetic mechanisms of galectin function in the nervous system.

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