Development of sex-specific differences in *Drosophila melanogaster* neurons

by
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Huong T. Duong, Author

AN ABSTRACT OF A THESIS OF

Huong T. Duong, for the degree of the Honors Baccalaureate of Science in General Science (pre-Pharmacy) presented on May 30 2013.

Title: The development of sex-specific differences in *Drosophila melanogaster* neurons

Abstract approved:

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Dr. Barbara Taylor

Many diseases cause a heavier burden on women than on men; however, treatment guidelines are based largely on data on men (NCBI, 2012). It becomes obvious that certain differences in genetics between men and women account for specific sex-related diseases, but there is too spare research focus on that issue. Consequently, there is a rising concern about identifying biological and physiological differences between men and women to understand the significance of the difference for diagnosis and treatment. One way to approach this problem is to study divergent gene expression between males and females in the development of the brain. The project "The development of sex-specific differences in *Drosophila melanogaster* neurons" was conducted to find how sexual differences in gene expression between males and females affect the development of a small cohort of neurons, using *D. melanogaster* as a model. Two genes *dsx* and *Dl* were investigated in this study because it was previously shown that these genes participated in sex determination and/or neural production (Baker et al., 1988; Burtis et al., 1989; Artavanis - Tsakonas et al., 1999). The time course of expression used in the lab provided testable models for how *dsx* and *Dl* expression might be regulated. Using immunohistochemical (IHC) techniques to visualize gene expression, as well as an EdU labeling method to detect cell division, sex-specific distinction in *dsx* and *Dl* expression in second and third larvae was found between male and female in four abdominal neuroblast lineages. It was discovered that the expression of *dsx* and *Dl* persistent in males and transient in females. The result obtained from this project is critical to explore a potential dynamic relationship between *dsx* and *Dl* that has not yet been discovered, which in turn can be used to acquire understanding how sex regulation genes work inside a brain to create the differences in male and female.

Key words: *dsx*, *Dl*, knock-out gene, gene expression, gene cascade, immunohistochemistry, EdU labeling.

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I/ INTRODUCTION

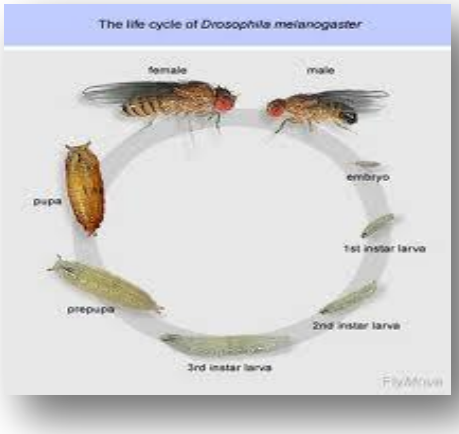
The analysis of FDA and pharmaceutical-industry practices has found that pharmaceutical data were collected without accounting for sex differences. According to the survey conducted by FDA, most of the drug dosage and their side effects reflect testing primarily in men, not in women. The consequence of limited comprehensive knowledge of sex differences can directly influenced medication effect (GAO, 1992). This raised attention to the necessity to consider sex differences in human biomedical research. It has been suggested that funding agencies require researchers to justify sex inequalities in grant proposals and should favor proposals that include both sexes. FDA also recommends that physicians and the public should be aware of sex differences in drug reactions and dosages; and that medical schools should train physicians in how diseases, symptoms, and drug responses can differ by sex (Nature, 2010).

However, the conduct of biomedical research involving the participation of human beings is associated with a variety of ethical concerns pertaining to such values as dignity, bodily integrity, autonomy, and privacy. When a person has tissue removed (with their consent) as part of a treatment intervention and is asked for permission to allow a piece of that tissue to be used in a related ongoing genetic study, it is required that a research project has to be described to an experimental subject with enough precision to allow for meaningful, valid informed consent. By contrast, it is difficult, if not impossible, for a participant in a research protocol to give meaningful prospective consent to the use of tissue in a possible future research protocol that cannot currently be described (Kapp MB, 2006).

In order to approach the goal of understanding the sex differences, the basic understanding about how genes work inside the cell should be considered carefully. Since

conducting human research is limited by the aforementioned barriers, *Drosophila melanogaster* was used as an ideal model in this project because of several reasons. First of all, *Drosophila* has a short life cycle; flies grow quickly in a laboratory environment. At room temperature (25^{o}C), it takes *Drosophila* about 10 days to complete the whole life cycle, from an embryo to the first, second, third larvae and finally to a mature adult. Second, *Drosophila* possesses well-defined genetics which is easy to manipulate and study. More important, *Drosophila* genome was sequenced in 2000 and turned out to be surprisingly similar to human (Adams et al., 2000). In fact, research proposed that several genes involved in sex differentiation; for example *dsx*, were conserved in animal kingdom. The previous finding suggested the evolution of these gene's products have similar molecular functions between flies and humans (Dauwalder et al., 1996). It is now clear that we human use *Dmrt* gene, which is congruent with *dsx* gene in *Drosophila,* to regulate our sexual dimorphisms (Held, 2009). Because homologous of *dsx* genes is found in humans, the results of this study can be used in further research of sex-related diseases in mammals and humans.

Figure 1.1: Drosophila's life cycle (FlyMore): it only takes 10 days for *Drosophila* to complete a life cycle at 25^{o}C, from an embryo to the first, second, third larvae and finally to a mature adult.



In *Drosophila*, *doublesex* (*dsx*) gene plays an essential role in sex differentiation in a central nervous system (CNS) (Baker et al., 1980). In order for *dsx* to do this function, *dsx* must interact and/or regulate other genes that also participate in the neural development process. Specifically, *dsx* products have to regulate a number of downstream cascade genes that in turn participate in further sex-specific differentiation. One of these genes that have been proposed to be involved in this process is *Delta* (*Dl)*. *Dl* is the gene that regulates the cell fate decision in neural development (Cornbrooks et al, 2006). Even though it is clear that the products of both *dsx* and *Dl* are important for the normal neural maturation, how those genes connect together is poorly understood. In this project, we focus on study the relationship between *dsx* and *Dl* to exploit how these two genes interact in the differentiation of sex-specific development in a central nervous system (CNS). By doing that, the project aims to contribute to the understanding of how basic programs control the process of sex-specific divergence.

1.1 The neuroblast (NB) division pattern in the central nervous system

Understanding the division pattern for the development of neurons inside a brain is crucial for this study. In *Drosophila*, the central nervous system (CNS) is created by the embryonic and

postembryonic divisions of neuronal stem cells, called neuroblasts (NBs) (Doe et al., 1985). NBs are organized in a way where each of them has a unique identity as determined by position and gene expression (Truman et al., 1988).

During the embryonic development period, NBs in the ventral nervous system segregate from an undifferentiated sheet of neuroectodermal cells before generating neurons for the larval stage. NBs divide asymmetrically as stem cells to produce series of ganglion mother cells (GMC). Each of GMC then divides once, equally to generate two neurons. The neurons arising from each NB are highly diverse but unique to NBs (Doe et al., 1985). Group of cells that includes NB and all of its progeny is commonly known as a NB lineage.

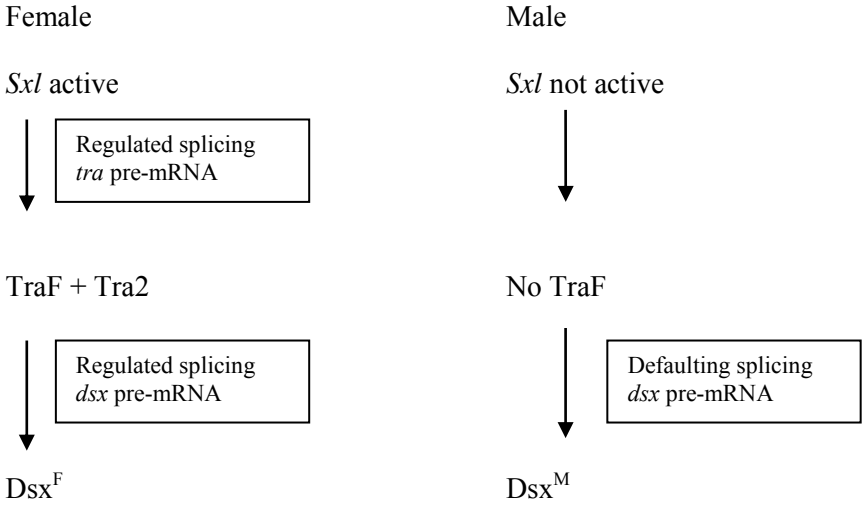
After the embryonic divisions, NBs in *Drosophila* become dormant, and then re-activate during the larval period to launch a series of postembryonic stem cell divisions which create adult-specific neurons (Truman et al., 1988). These immature adult-specific neurons arrest their development until the onset of metamorphosis at pupa stage. A set of four male-specific abdominal NBs were observed were observed by Truman and Bate (1988). By following the development of these NBs and lineages, this system is ideal to investigate the mechanism that direct the wiring of sex-specific adult neural networks.

In addition to a detailed understanding of NB division patterns, previous studies have also determined what genes are needed for Drosophila sex determination and neural development. An important sex-determination gene is *doublesex* (*dsx*), which gives rise to different proteins in males, Dsx^M, compared to females, Dsx^F. Another gene, *Delta* (*Dl*), which encodes a protein involved in neural development, is expressed in one of these male-specific lineages at the same period of time. Characteristics of these genes will be presented in the next two sections.

1.2 *dsx* in the sex determination hierarchy

dsx is found in the bottom of the sex determination hierarchy (Baker et al., 1987). *dsx* expression is differentiated in *Drosophila* via an alternative splicing intron mechanism which was controlled by the combination actions of *transformer (tr*a), and *transformer 2 (tra 2)* gene products (Baker et al., 1987, Baker et al., 1988, Slee et al., 1990). *tra*, and *tra 2* are in turned regulated by the *Sex-lethal* (*Sxl*) gene, the gene at the top of the sex-determination pathway (Nagoshi et al., 1988). The *Sxl* gene is only active in females; its product regulates the splicing of *tra* pre-mRNA to produce TraF protein. Together with Tra2, TraF regulated the splicing of the *dsx* pre-mRNA, which is translated into Dsx^F protein. It was proved that the absent of the TraF products in mutant females flies, dsx pre-mRNA is spliced into male-specific dsx mRNA (Nagoshi et al., 1988). In males, due to the absence of Sxl protein, *traF* transcript is not made and *dsx* primary transcripts are processed by default into the male-specific dsx^M transcript. As a result, the production of sexually dimorphic proteins is created: Dsx^F in females and protein Dsx^M in males.

Figure 1.2: The way Dsx^F and Dsx^M are made in *Drosophila melanogaster*



In the absence of any functional *dsx* gene product, as in flies homozygous for *dsx* loss of function mutations *Df(3R) dsxMR+15*, both male and female flies develop as intersexes (Hildreth, 1965; Baker et al., 1980). The sex-specific terminal NBs in the mutations showed the lack of postembryonic division in either male or female larvae in nervous systems. Study of intersexes suggested *dsx* influenced the formation, function of neural tissues important for reproductive behaviors (Taylor et al., 1992). In addition, Dsx^M has been proved to play an essential role in larval development for two male-specific neuronal lineages produced by NBs in the terminal abdominal region of the CNS at late 3rd instar (Taylor et al., 1992). From all of the previous research work, it is clear that the product of *dsx* is critical for female sexual behavior and required for the development of male-specific NBs, which suggest an involved in neuronal framework necessary for a male sexual behavior.

Although a number of studies investigated the role of *dsx* in the development formation and function of CNS in pupae and adult Drosophila (Taylor et al., 1992; Lee G et al., 2002), the knowledge of *dsx* expression pattern in earlier larvae stages is still limited. In this research, we focus on finding the expression of this gene in 2nd and 3rd instar in the interaction with the *Dl* gene.

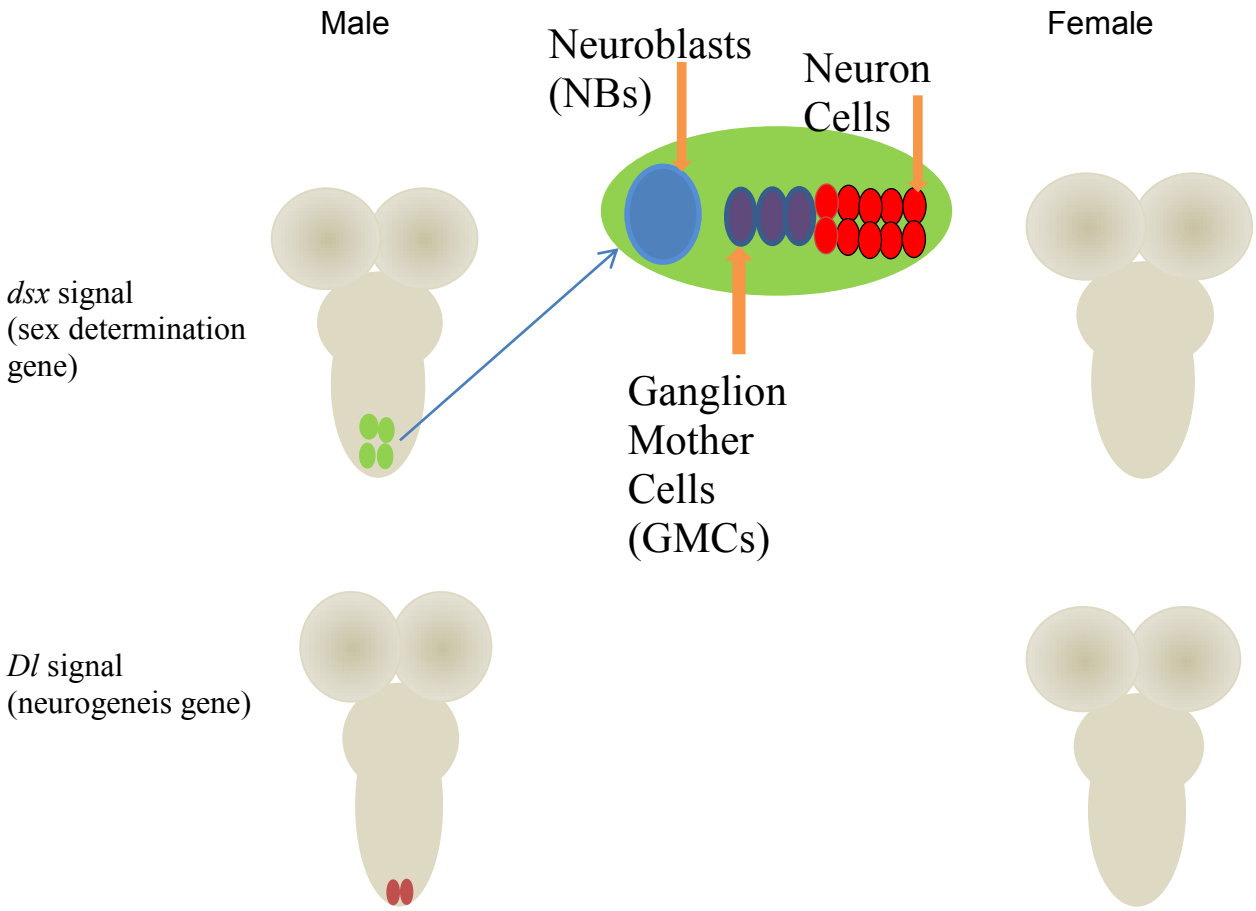
1.3 *Dl* and its role in neural development

Dl is a gene encoding for the protein Dl that functions as a signaling for Notch receptor. Dl- Notch signaling model is known as fundamental mechanism for mediating cell fate decisions during CNS development (Artavanis-Tsakonas et al., 1999; Whitford et al., 2002). Whenever Dl binds to Notch receptors on neighboring cells, a series of down-stream transcriptional events are

invoked that restrict differentiation of the Notch-expressing cell. Thus in CNS, the Notch-expressing cell is prevented from adopting certain non-neuronal fates by default (Artavanis-Tsakonas et al., 1991; Doe et al., 1998)

Dl expression is an indicative of an intrinsic developmental program of identity in adult lineage neurons. A study Dl in post-embryonic development of the *Drosophila* CNS revealed two Dl-expressing lineages in the terminal abdominal ganglion only present in male. Specifically, one of these abdominal Delta lineages produces Dl positive neuritis. It is one of two lineages in the ganglion which likely arise from the male-specific abdominal NBs that continuing to proliferate in the late larval and pupal stages (Truman and Bate., 1988; Taylor and Truman, 1992). Dl is highly enriched in the terminal neurites of these neurons, but do not form initial contacts with other Dl neurites, suggesting that they contribute to development of male-specific neuronal networks (Cornbrooks et al, 2006).

Figure 1.3: NB division and Dl staining pattern. Both male and female Ab-NBs begin dividing in the second instar stage. Female Ab-NBs stop dividing in the third instar stage but male Ab-NBs continue thoughout the larval period.



1.4. Hypothesis

The discovery of atypical expression pattern of *dsx* and *Dl* in *Drosophila* might be the key to encode many problems in modern neuroscience. Thus the ultimate goal of this research is to examine the relationship between Dsx and Dl, for the purpose of finding the mechanism involving in the development of sex-specific difference in CNS. To approach this goal, we examined the expression pattern of these two genes specifically in the abdominal region of the *Drosophila* CNS in various larvae stages, ranging from 2nd to late 3rd larvae.

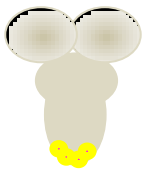
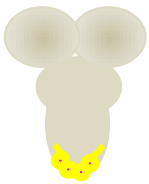
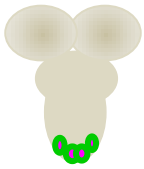
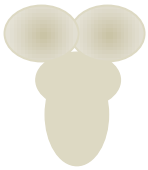
As mentioned earlier, Dsx is a transcription factor for many downstream genes, and my working hypothesis was that *dl* would be one of those genes controlled by Dsx. From the types

of proteins involved, I hypothesized that in the postembryonic NBs Dsx^M would be a transcription activator for *Dl*, while Dsx^F would be a transcription inhibitor for *Dl*. It was predicted that Dsx^M would express through the entire time period of 2nd to 3rd larvae to promote Dl expression in males in all 4 NBs in the abdominal region. In contrast, Dsx^F would express through the time period of 2rd to 3rd larvae and prevent Dl expression in females. Dsx^F then would turn off completely due to cessation of cell division in 4 NBs before the larvae entered pupal stage. For a control experiment, *Dsx* loss-of-function mutation would also be used, in which it was predicted to show neither *dsx* nor *Dl expression*.

Figure 1.4: Hypotheses

Image code: *dsxGal4; UAS GFP* expression = Green Dl expression = Red Dsx + Dl = Yellow

Edu (dividing cells) = Pink nuclei

	2nd instar	3rd instar
Male		
Female		

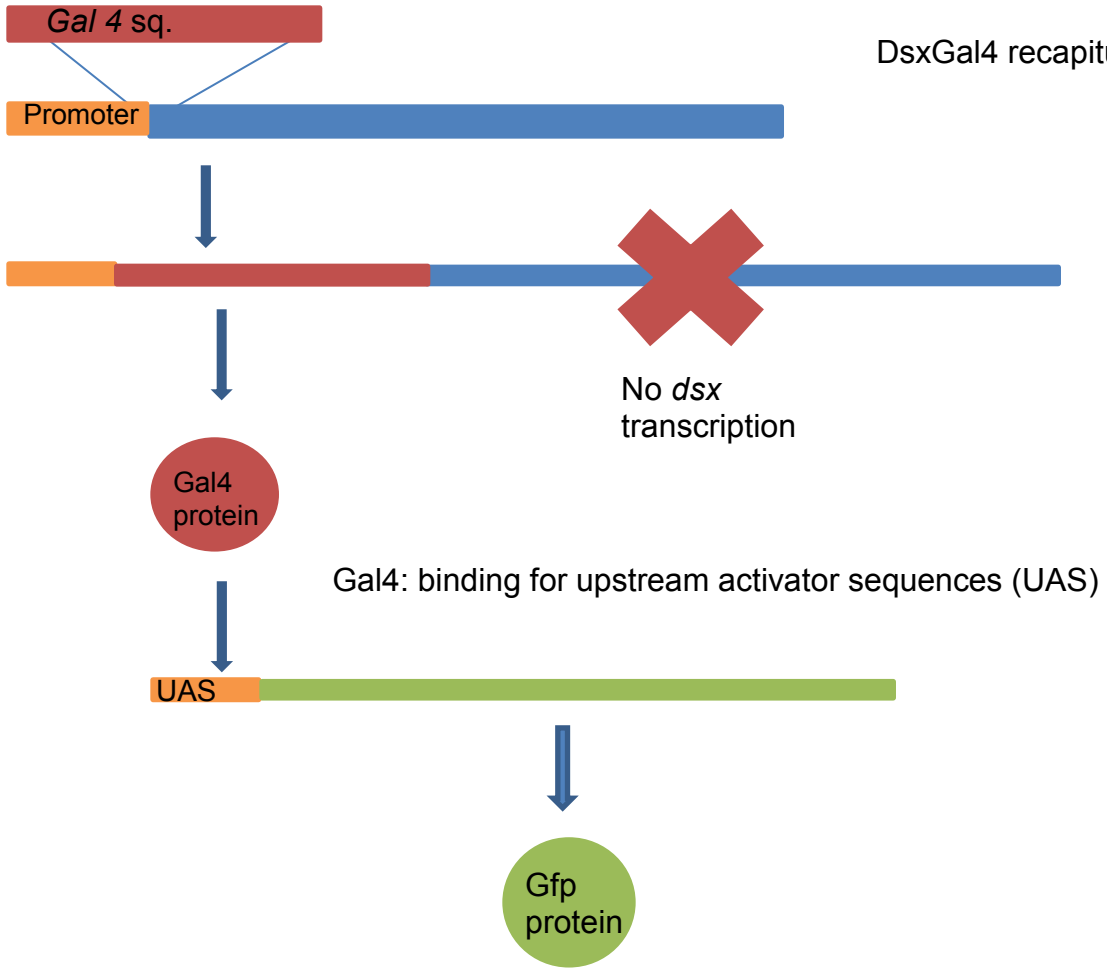
II/ MATERIALS AND METHODS

Various techniques were applied to conduct experiments for this research, including knock-out gene method to generate the fly strains, immunohistochemistry for protein staining, EdU labeling for tracing cell division pattern, and confocal microscope for image processing.

2.1 Targeted insertion of GAL4 into *dsx* locus/ Knock - out gene

For the purpose of visualizing *dsx* expression in the cell, the GAL4 coding sequence was inserted into the first, non - sex - specific, coding exon of *dsx*. The resulting allele, *dsxGAL4*, was produced. GAL4 protein is a yeast transcription activator that binds to upstream activating sequences (UAS) and can initiate transcription of downstream genes. In my experiments, several different UAS-green fluorescent protein (UAS-*GFP*) reporter genes were used. The presence of GAL4 protein in a cell thus promotes the Green fluorescent gene to be transcribed, which in turn produces GFP. When mating the male with the *dsxgal4* gene and the female with UAS-*GFP* gene, some of their progeny will contain both essential chromosomes to produce GFP in the dsx expression pattern. In other word their genotype will be *dsxGal4; UAS GFP*, which can be detected either by its native color or using antibody staining. A similar cross of *dsxGal4; UAS GFP* was proved to be a sensitive, specific marker for Dsx-expressing cells (Rideout et al., 2010).

Figure 2.1: Knock out gene method



2.2 Strains of *D. melanogaster* and Crosses

Table 2.1: Parents and progeny used in this research

Female	Male	Progeny of interest
w;dsxGal4/TM3	*y;;UASmcd8GFP;dsx(M+R15)/TM3*	*UAS-GFP* *mcd8;dsxGal4/Tm3*
w;dsxGal4/TM6B	*w;BsY.UASmcd8GFP;dsx(M+R15)/TM6B*	*UAS-GFP* *mcd8;dsxGal4/TM6B*

w;dsxGal4/TM6B	*y;;UAS-GFPnls;dsx(M+R15)/TM6B*	*UAS-GFP nls;dsxGal4/TM6B*
w;dsxGal4/TM6B	*y;;UASmcd8GFP;dsx(M+R15)/Tm6B*	*Df(3R)dsx(M+R15)/TM6B*

Strains of *D. melanogaster* used in the lab were from Flybase. The dsxGal4 inserted line was the gift from Dr. Michael McKeown, Brown University.

Two types of balancers (in third chromosome) were used in this study to identify larval and adult fly genotypes. The first balancer, *TM3*, has a dominant mutation for Stubble of fly. The second balancer, *TM6B*, has a dominant mutation for Tubby. Because the homozygous balancer was lethal, no progeny could survive if they carried both balancers. Thus those balancers would help to determine exact genotyped of interest.

Df(3R)dsx(M+R15) is a complete loss-of-function allele of *dsx* so no Dsx protein was created. The cross with *Df(3R)dsx(M+R15)* was generated as a control experiment.

All virgin female and male were collected and incubated in 25^{o}C for 3 days before collecting eggs. After 3 days, the crosses were transferred to apple agar plates for egg collection. The crosses were continually transferred to new plates every two hours of approximately four times during the collection day, so that various larvae stages could be obtained. The egg collection process usually occurred in 5 days, and the overnight eggs from crosses were not used in this experiment to ensure the time of each image.

Time course experiment was used in this study, in which CNSs of larvae were collected every two hour for approximately 4 times per day in one week. By doing that, the precise age of larvae could be determined. CNSs were harvested from larvae with age range from 48 hour (0h 2nd instar) to hour (wandering stage). Only larvae that had fluorescent signal or did not carry the

TM6B or Tb balancer were chosen to dissect under the microscope. Larvae sex was later sorted by its color, in which brown larvae were male, and black larvae were female. In most case, larvae were fed with dried yeast before dissecting. In some other cases, larvae also were fed with yeast containing EdU for cell division labeling. Detail of the EdU labeling method will be mentioned in the next sections.

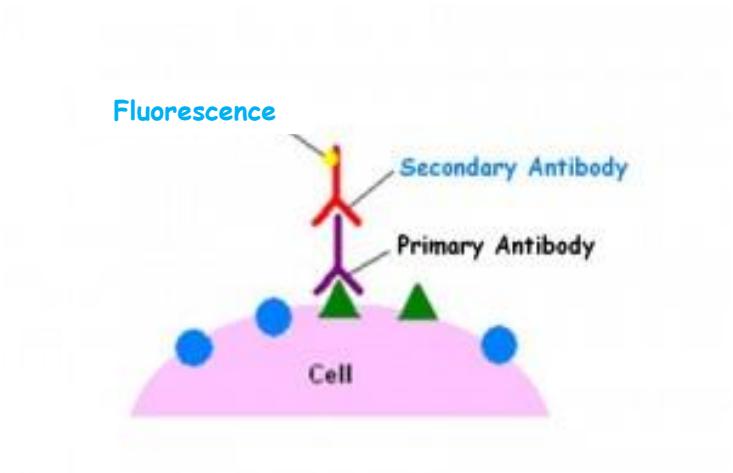
2.3 Immunohistochemistry

After sorting *Drosophila* larvae into males and females, CNSs were dissected separately in phosphate buffered saline (PBS) before being fixed in PBS-TX for 15 min. The CNSs were then transferred into the eppendorf tube and put in the primary antibody solutions containing PBS-TX, NGS 1:10, NaAzide 1%, anti -GFP 1:200, anti - Delta 1:20 for 1 day at 4ºC. After that, the CNSs were washed by PBS-TX at least 3 times, before applying the second antibody solution containing PBS-TX, NGS 1:10, anti-mouse Alexa 555 (2^{nd} antibody of Dl) 1:300, anti-rabbit Alexa 488 (2^{nd} antibody of DsxGal4) 1:300 for 3 hours. In next step, CNSs were taken out of second antibody solution and washed 3 times by PBS-TX, before incubating with DAPI 1% solution in 15 min at room temperature. The CNSs were then washed again and mounted in prolong reagent. For other labeling experiments, anti-Notch (1:20) was used to identify cell neighbors of Dl. Since Notch signal only appeared in the presence of Dl protein, this staining was used as substitution for Dl signal detection.

The anti-Notch bTAN20 and anti-Delta C594.9B developed by Dr. Artavanis-Tsakonas were obtained from the Developmental Studies Hybridoma Bank (DSHB) developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The AlexaFluor 488 goat anti rabbit IgG (H+L): used to detect Gfp signal and

the AlexaFluor 555 donkey anti mouse IgG (H+L) was used to detect Dl signal (Invitrogen Molecular Probes company, 4849 Pitchford Ave, Eugene, OR, 97402-9165).

Figure 2.2: The model of Immunohistochemistry (Ramos-Vara, 2005): Signal of interest (eg: *Dl*, *dsxGal4; UAS GFP*) is recognized by primary antibody, which in turn binds specifically to secondary antibody. Since secondary antibody has an attached fluorescent tag, one can determine whether signal presents in the cell by detecting a fluorescent tag.



2.4 Labeling of dividing cells with the EdU click - it reaction.

The Click- iT EdU assay is a novel method that is used to detect DNA synthesis, the most accurate way to measure a cell's ability to proliferate. The compound 5'-ethynyl-2'-deoxyuridine (EdU) is a nucleoside analog of thymidine and is corporated into DNA during active DNA synthesis (Anna E. Kliszczak et al., 2011). It contains an alkyne and the AlexaFluor dye contains the Biotin TEG azide. Detection is based on a click-it reaction, a copper-catalyzed reaction between the azide and the alkyne. The advantage of using Click-it EdU labeling is the small size of the dye azide, which allows for efficient detection of the incorporated EdU using mild conditions (Kliszczack et al., 2011). Standard paraformaldehyde-based fixation and detergent

permeabilization are sufficient for the Click-iT detection reagent to gain access to the DNA. The EdU assay is fully compatible with other antibody staining protocols and direct DNA staining, such as with DAPI, including dyes. It can also be multiplexed with surface and intracellular marker detection using antibodies. In addition, Click-iT EdU uses bio-orthogonal moieties, producing low background and high detection sensitivities (Anna E. Kliszczak et al., 2011).

If EdU labeling method was used, larvae was either fed with yeast containing EdU or the CNSs were dissected and put in EdU 1% solution before fixing. In either way no Azide was added in the primary antibody solution to prevent the interference with EdU-click it reaction.

Figure 2.3: The mechanism of EdU labeling (Anna E. Kliszczak et al., 2011) one can detect whether cell is under division by speculating EdU positive signal due to "Click-iT" reaction.

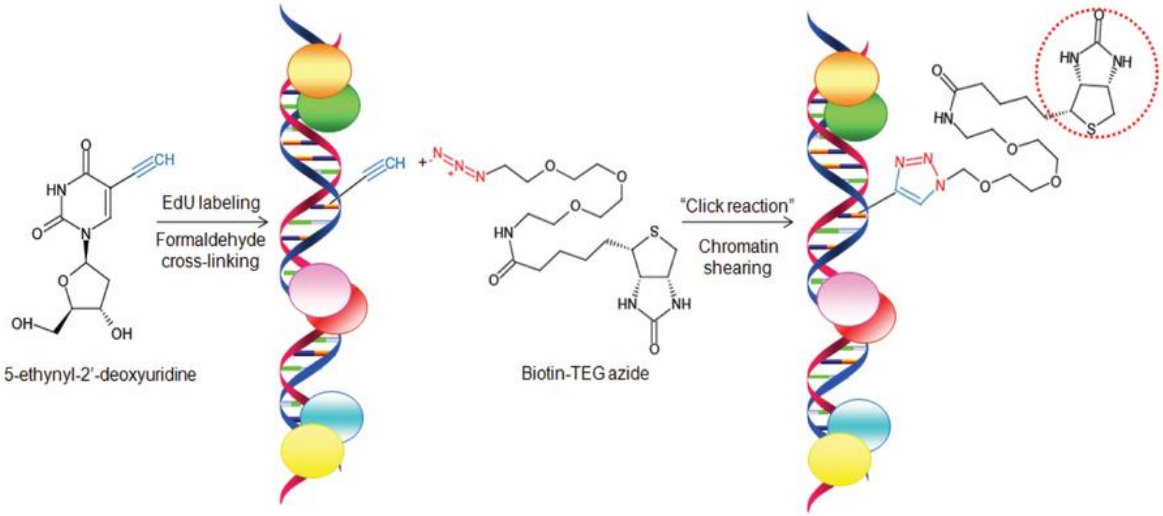
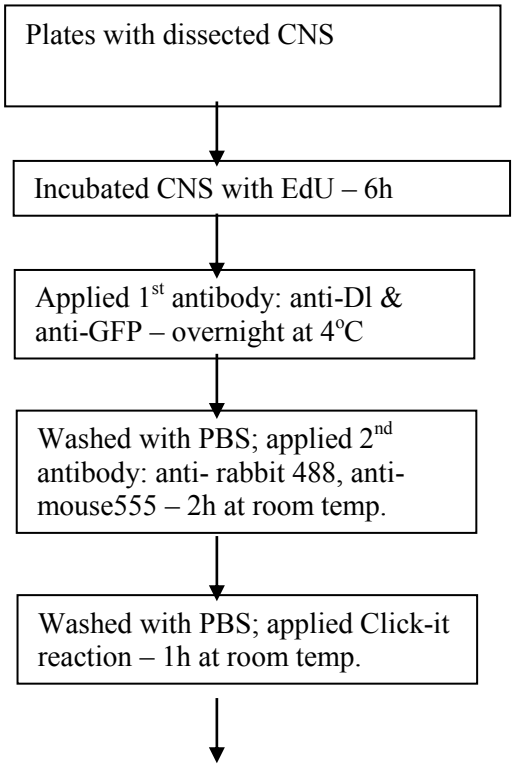


Table 2.2: Click-iT reaction cocktail

Reaction components	Number of Eppendorf tubes				
	1	2	4	5	10
1x Click-iT reaction buffer	430µL	860µL	1.8mL	2.2mL	4.3mL
CuSO4	20µL	40µL	80µL	100µL	200µL
Alexa Fluor azide	1.2µL	2.5µL	5µL	6µL	12.5µL
Reaction buffer additive (1:10)	50µL	100µL	200µL	250µL	500µL
Total Volume	500µL	1mL	5mL	12.5mL	25mL

Figure 2.3: Summary procedure



Washed with PBS, stained with DAPI 0.1%, mounted with prolong reagent

2.4 Microscopy and image processing

All of the slides were visualized by using the Zeiss LSM 510 Meta Confocal Microscope with Axiovert 200 motorized microscope and version 3.2 LSM software. Oil immersion objectives was used in this project, Plan-NEOFLUAR 40x/1.3 oil DIC (1056-602(1083-997) and Plan-APOCHROMAT 63x/1.4 oil DIC (1113-108). Four laser lines were used to detect signals: 488 nm to detect GFP, 555 nm to detect Dl, 647 nm to detect EdU and 405 nm to detect DAPI.

2.5 Analysis of the Data

Images were captured on the confocal and all of the data about observation of sample were saved in T-drive before importing in Microsoft Excel. A total 126 samples were analyzed in this study. All images were further processed using PTS program to minimize the background affect.

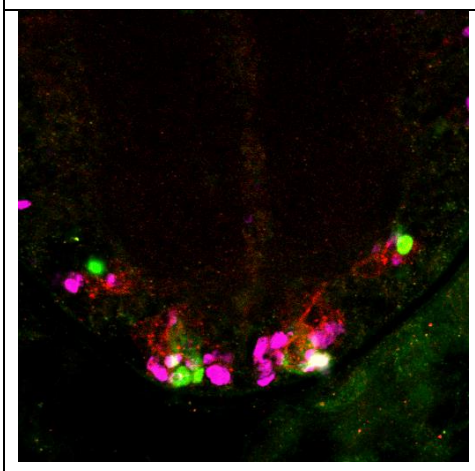
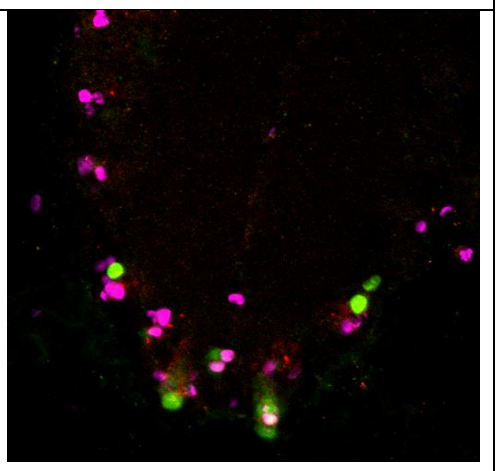
III/ RESULT

3.1 At 68h after egg lay (AEL) (2nd instar larvae): both male and female showed *dsxGal4; UAS GFP* **and** *Dl* **expression**

My hypothesis was that the four sex-specific NBs in the abdominal ganglia of males and females would express *dsx*. The analysis of CNSs from larvae in the second stage, specifically around 68h after hatching, showed that there were clear signals of *dsxGAL4; UAS-GFP* and Dl in the terminal abdominal ganglia of both male and female. All male CNSs at this stage were found

to have *dsxGAL4; UAS-GFP*- and Dl-positive label in four NBs in an abdominal region while 70% female images were found express both proteins. As it can be seen in Fig. 3.1a, which showed male *Drosophila* at 68h, both *dsxGAL4; UAS-GFP* (green) and Dl (red) signal were observed in four NBs of the abdominal region in CNSs. Two out of those four NBs were also detected to have EdU signal. Compare the result to Fig. 3.1b which showed female *Drosophila* at 68h. Dl signal was fainter than that found in the male CNSs. Like males of the same age, EdU signal was detected in two out of four NBs in female CNSs at 68h.

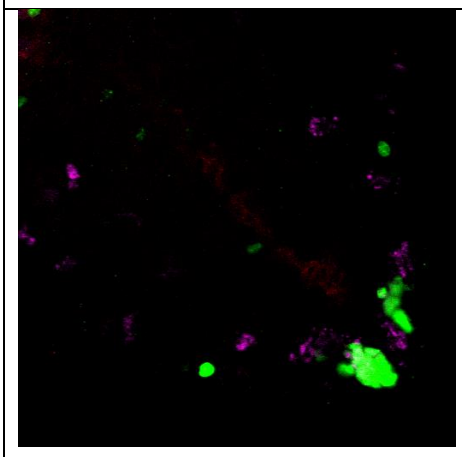
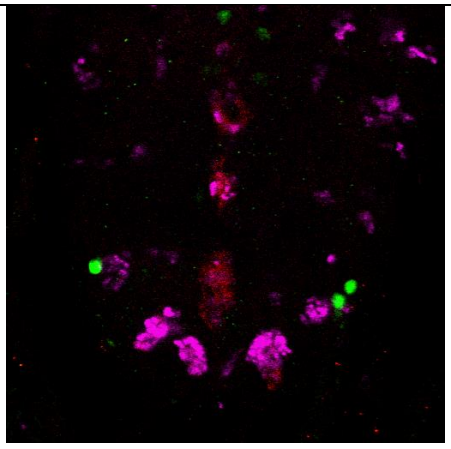
Figure 3.1: Larvae at 68h AEL: The green signal indicated the presence of Dsx driven GFP signal, the red signal indicated the presence of Dl, and the pink signal indicated the presence of EdU signal, which stained for the cell division pattern. Cells which have all three signals appear white.

Fig 3.1a: Male *Drosophila* at 68h after egg lay	Fig. 3.1b: Female *Drosophila* at 68h after egg lay (AEL).
	

3.2 At 70h AEL (2rd instar): male larval CNSs express *dsxGal4; UAS GFP* and *Dl*, female CNSs infrequently express *dsxGal4; UAS GFP* or Dl

Larvae at the early third instar (70-72 hrs) started showing the difference in expression of Dsx and Dl between male and female. Specifically, in most case, *dsxGal4; UAS GFP* signal was not observed in all four of NBs in female, although one or two GFP-positive NBs were detected in many CNSs and occasionally a Dl-positive NB was also observed. As it can be seen in Fig.3.2b, no *dsxGal4; UAS GFP*-positive NBs were found in this female CNS although faint Dl signal was detected. In contrast, clear Dl and *dsxGAL4; UAS-GFPnls* signal were found in male CNS. Signal of EdU (purple) was clearly seen in all four NBs in the abdominal region of both male and female CNSs.

Figure 3.2: Larvae at 70 AEL: both Dl and *dsxGAL4; UAS GFPnls* signal were detected in male. EdU positive neurons were found in the posterior abdominal region of the CNS, no *dsxGAL4*. In female*:UAS GFPnls* NBs were detected although with less quantity in regard to male.

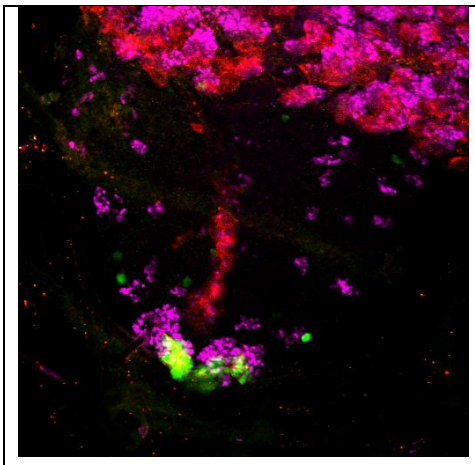
Fig. 3.2a: Male at 70h AEL	Fig. 3.2b: Female at 70h AEL
	

3.3 At mid-3rd instar larvae: male expressed both *dsxGal4; UAS GFP* and *Dl*; neither *dsxGal4; UAS GFP* nor *Dl* expression was found in female

As Fig.3.3a illustrates, in male 78h CNSs, GFP signal was found in all four NB lineages where as strong neurite Dl signal was only found in bilateral NB lineages of abdominal region. This pattern was also observed in male 79h, 93h and 96h. The image of male at 96h (Fig. 3.3c) showed the projections were newly emerged from NBs. Compared to males, no *dsxGAL4; UAS-GFP* or Dl signal was clearly detected in later third instar female CNSs (Fig. 3.3b. 3.3d). The analysis of EdU signal of larvae at the age from 78h to 96h showed that all four *dsxGAL4; UAS-GFP* NBs in males were EdU-positive and similarly positioned Edu-positive neuroblasts in females. However, in 96h female CNSs, no EdU labeling was detected although four *dsxGAL4; UAS-GFP* and Dl-positive NBs were also EdU-positive in male CNSs (Fig. 3.3c, d).

Figure 3.3: Larvae at mid-3rd instar stage: the expression of both *dsxGAL4; UAS GFPnls* and *Dl* were seen in male; neither GFP nor Dl was seen in female. At 96h AEL, no EdU signal was found in female.

Figure 3.3a: male at 78h AEL	Figure 3.3b: female at 78h AEL



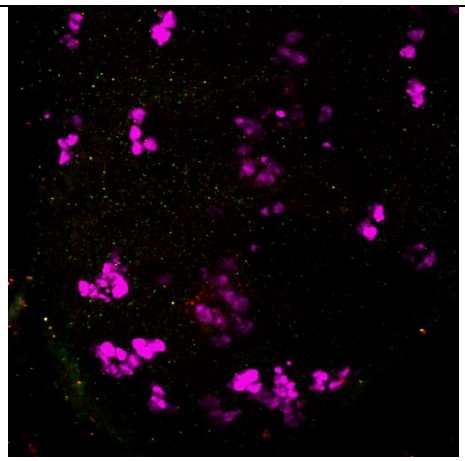
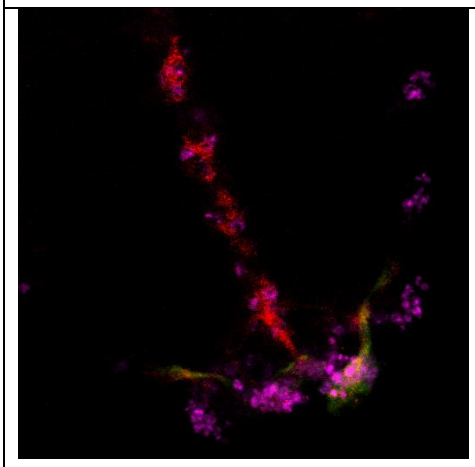
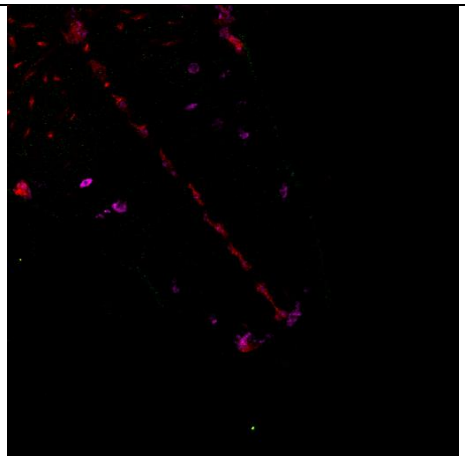


Figure3.3c: male at 96h AEL

Figure 3.3d: female at 96h AEL



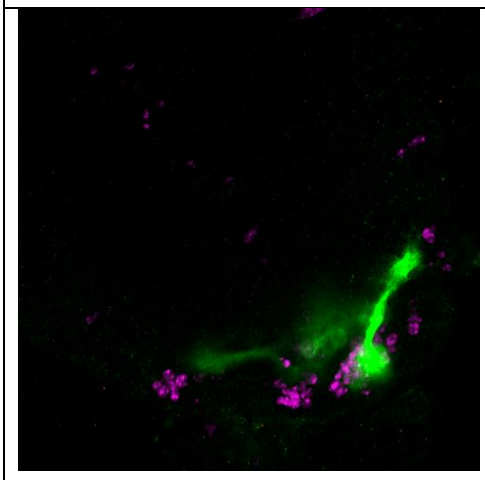
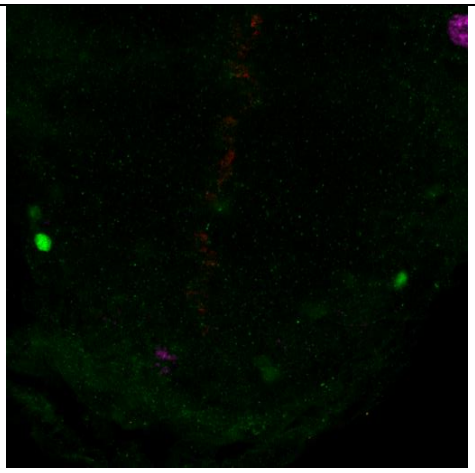


3.4 At late third larvae (102h) to pupae: male expressed *dsxGal4; UAS GFP* **and** *Dl***; neither** *dsxGal4; UAS GFP* **nor** *Dl* **expression was found in female**

The significant difference in expression of GFP and Dl signal between male and female was reported in larvae stage of 102h to pupae. Here *dsxGal4; UAS GFP* signal was strongly

expressed in male (Fig. 3.4a) in all four NB lineages. Dl/Notch signal was also found in this stage. Four NBs showed positive to EdU signal. In contrast with female none of Dl, GFP, EdU signals were found in the NBs of abdominal region. There were several GFP signals were reported outside NBs, which indicated that there were no issue concerned for antibody reliable (Fig. 3.4b).

Figure 3.4: Larvae at 102h AEL: *dsxGal4; UAS GFP* and *Dl* continued expressing in male; neither *dsxGal4; UAS GFP* nor *Dl* expression was found in female.

Fig.3.4a: Male at 102h AEL	Fig.3.4b: Female at 102h AEL
	

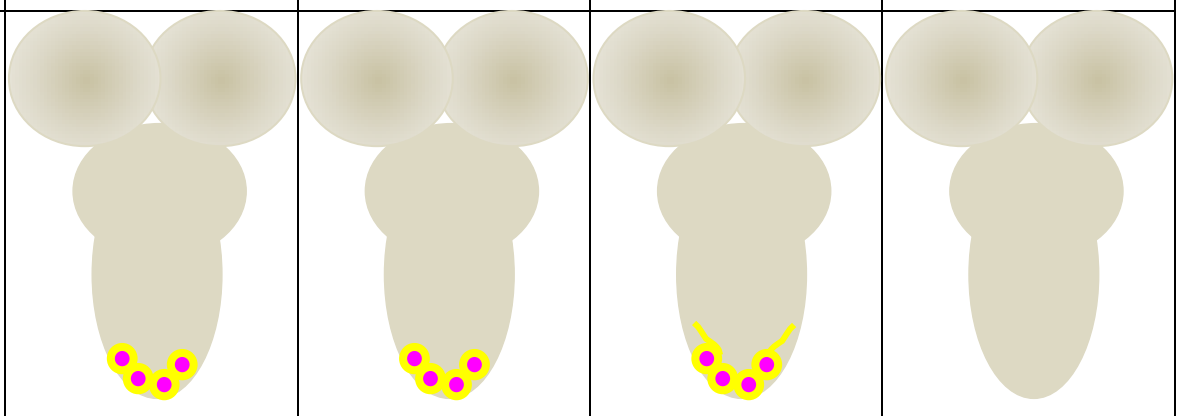
3.5 CNS from *dsx* loss of function mutations showed no Dl signal

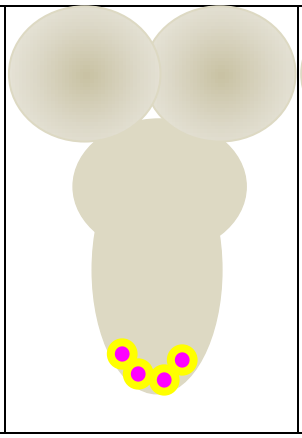
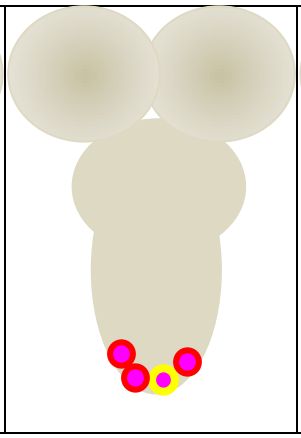
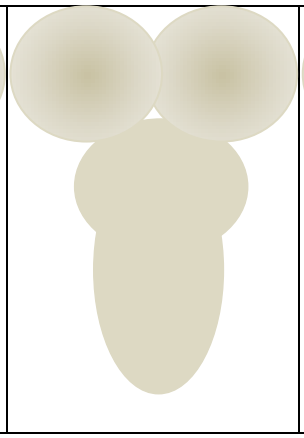
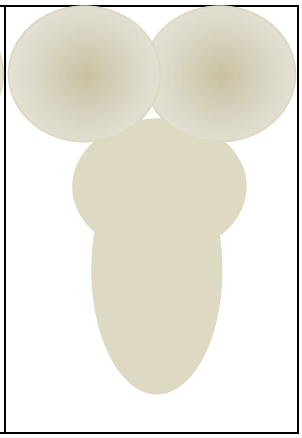
dsx loss of function mutation line - *dsx(M+R15)/dsxGal4* - created neither GFP nor Dl positive signal (image not shown). This pattern was observed between late second to third larvae stage.

Table 3.1: Overall result: it was observed persistent co - expression of *dsx* and Dl in all 4 NB lineages from 2nd to 3rd instar. In constrast, there was transient expression of dsx and Dl from 2nd to 3rd instar in femal. Dsx^F turned off before NBs ceased to divide.

Image code*: dsxGal4; UAS GFP* expression = Green, Dl expression = red Dsx + Dl = yellow

Edu (dividing cells) = pink nuclei

	Late 2nd instar	Early 3rd instar	Late 3rd instar	*dsx(M+R15)/dsxGal4* (control)
Male larvae				

Female larvae				

IV/ DISCUSSION

dsx is known to be a transcription factor for many downstream genes involved in the differentiation of sex features; while *Dl* is known to be a gene that determine cell fate decisions in neural development. Based on the nature of these genes, it is possible that *Dl* is one of those genes that are controlled by *dsx*. I hypothesized that Dsx^M would be the transcription activator for Dl while Dsx^F would be a transcription inhibitor for Dl. It was predicted that in males, Dsx^M would be expressed in the terminal NBS through the entire time period of 2^{nd} through the 3^{rd} larval stage to promote Dl expression, while in females, Dsx^F would express in the homologous NBs from early 2^{nd} to 3^{rd} larvae, to suppress Dl expression and then would turn off later.

In my results, it was found that in *dsxGal4; UAS GFP* males four NBs in the abdominal CNS expressed both GFP and Dl from the late second instar through the third larval instar stage. In *dsxGal4; UAS GFP* females, four NBs also expressed GFP and Dl in the late second instar stage. In the early third instar, GFP expression and Dl expression were both lost from the four neuroblast lineages. During the period of loss, a few EdU-positive NBs and progeny were seen and those were occasionally only Dl-positive or only GFP-positive, which made it difficult to conclude that Dsx^F was responsible for the loss of Dl expression. In addition, other experiments

ongoing in the laboratory found that in early second instar *dsxGal4; UAS GFP* CNSs, when the abdominal NBs first begin to divide, terminal NBs are EdU-positive but neither Dl- or GFP-positive. In males and females, the earliest signal appears to be Dl and only in the last part of the second instar is the *dsxGal4* driven GFP signal detectable. Therefore, data obtained from the lab did not fully support the hypothesis proposed. The results suggest that the interrelationship between *dsx* and *Dl* molecular activities might be far more complex than hitherto image.

Even though current finding might propose a potential dynamic relationship between *dsx* and *Dl*, other possibility should not be eliminated. The alternative explanation would also be considered, in which two possible explanations can be ruled out.

4.1 CNS from *dsx* loss of function mutations showed no Dl signal

When CNSs from *dsx(M+R15)/dsxGal4* third instar larvae were labeled for GFP and Dl expression, no GFP or Dl expression was detected. At this time of development, only males would have been expected to have NBs expressing GFP and Dl signal. The absence of any signal suggests that none of the four sex-specific NBs were present.

4.2 The role of Dsx^M in Dl expression in male

The co-expression of Dsx^M and Dl in *Drosophila* without interruption from late 2^{nd} to late 3^{rd} instar confirmed the hypothesis that Dsx^M was a transcription activator for *Dl* gene. Unlike previous study that showed Dl expression in only two male-specific NBs (Truman et al., 1993), the results from this research found Dl/Notch signal was observed in all four NBs in abdominal region of *Drosophila*'s CNS.

4.3 The role of Dsx^F in Dl expression in female

The data collected in the lab did not support for the hypothesis that Dsx^F was a transcription inhibitor for Dl. The co-expression of *dsxGal4;UAS GFP – Dl* found at 2nd instar (68h AEL) illustated that Dsx^F protein did not inhibit the Dl expression. One might think that Dl protein had high stability, in which the *Dl* gene was shut off by Dsx^F but its product still presented in the cell. Nevertheless, if it was the case, then when *dsx* totally turn off in later stage (late 3rd instar) Dl signal would be reappear in the cell. In fact, both *dsxGal4; UAS GFP* and *Dl* signal were not detected at late 3rd instar. Furthermore, at 70-72h AEL, it was occasionally observed the expression of only Dl-positive or only GFP-positive. Explanation about the existence of Dl-DsxF interaction was thus rejected. All of those information suggested that there was no direct connection between Dsx^F and Dl in CNSs of *Drosophila*'s larvae.

It is possible that while Dsx^M plays a role in Dl expression, Dsx^F is not involved in any activities of Dl regulation at any level.

4.4 Ongoing research

Advancing study conducted in Taylor's lab revealed that *Dl* gene was expressed prior to *dsx*. Specifically, Dl signal was detected in both sexes at 56h larvae (8h 2nd instar). No *dsxGal4; UAS GFP* signal was detected in either male or female at that time. When tracing down to even ealier stage, 48h (0h 2nd instar), it was found that all of four NBs in Abdominal region showed no *dsxGal4; UAS GFP* and *Dl* signal in both male and female. The only detected signal was EdU, which indicated that lineages started dividing at this time.

4.5 New model for *dsx – Dl* and future research

The new finding suggested that there would be a dynamic relationship between *dsx* and *Dl*. Based on observation from the empirical data collected in the lab, I would like to propose the new hypotheses for *dsx-Dl* relationship:

Dl is the upstream gene for *dsx* when NBs start dividing: The finding that *Dl* expression happens prior to *dsx* may be a clue that it is Dl that regulates the expression of dsx. The production of Dl would activate the transcription of *dsx*, followed by the alternative in mRNA splicing at the 2nd instar to create different Dsx proteins in male - Dsx^M and female - Dsx^F.

Dsx^M in turn determines the further expression of Dl: Dsx^M may have a positive feedback for Dl expression from late 2nd to late 3rd instar. Dsx^M would be crucial for Dl to continue express in male, which explains a lack of Dsx^M larvae leads to the absent of Dl protein in intersexes *dsx(M+R15)* and female larvae.

Dsx^F creates no effect on Dl expression. The cessation of Dl expression in female in 3rd instar, as proposed, is due to the lack of Dsx^M.

To test the hypotheses, I will use the line that have Dl mutation in which Dl cannot function appropriately and find out whether I can see *dsx* signal at any level. If no *dsx* expression can be found, the new hypotheses would be supported. I will also generate *dsxGal4;UAS Dl-RNAi* line to eliminate Dl expression under *dsx* control. Last but not least, I will repeatly conduct experiment in the early third instar for female to find the pattern of DsxF – Dl expression to confirm the result.

The alternative hypotheses for the new model is that both Dsx^M and Dsx^F activate Dl expression in the NBs. In the early 3rd instar of female larvae, there are other sex (female) specific factor(s), besides Dsx, which can compensate for absence of Dsx and can upregulate Dl expression in absence Dsx. In the absence of this sex specific factor(s) and Dsx in the late

3rd instar, there is no expression of Dl in female larvae.there would be no correlation between *dsx* and *Dl*. *dsx* is perhaps necessary for the maintenance or survival of the NBs but plays no direct transcriptional role in activating Dl. In another word, both genes may independently determine sex-specific characteristics but have no effect on other.

4.6 Significance of the Result

The detection of both spatial and temporal of *dsx* and *Dl* in early neural development as reported in the result would be considered as a significance result. The different between *dsx* and *dl* expression occurring in the very early period of larvae in Drosophila suggests that the neural development of male and female start diverging very soon after zygote is produced. Since a paralogs of *dsx* genes is found in humans, this finding would be a key factor to decode biologic and physiologic differences between men and women.

Application derived from this project's finding would be benefit for diagnosis and medication therapy in human diseases. Because men and women are genetically different, several diseases arise more in women than in men and vice versa. For example, motor neuron diseases (MNDs), a group of progressive neurological disorders that destroy motor neurons, the cells that control essential voluntary muscle activity, occur more commonly in men than in women (NINDS, 2012). The current lack of investigation in sex-related factors contributes for an inappropriate treatment, creating unnecessary expense and side effect for patient. Sex has been shown to be a more important determinant of variability in drug disposition than age, and it is prudent that future protocols for pharmacokinetic studies should regard age, sex, the menstrual cycle and oral contraceptive steroids as potential sources of variability (Wilson K., 1984).

To treat those diseases effectively, it is important that the difference in genetic factors need to be carefully considered (Mittelstrass et al., 2011). Study of sex-specific differences inside a brain helps to define the most appropriate population for treatment and to determine whether drug benefits or harms differ by sex. Thus, finding the pattern of *dsx-Dl* expression in male and female can be applied for future pharmacokinetics to develop medicines that specifically target on sex-specific genes causing diseases.

V/ REFERENCES

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richard S, Ashbutner M, Henderson FM. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185-95.

Artavanis-Tsakonas S, Delidakis C, Fehon RG. (1991). The Notch locus and the cell biology of neuroblast segregation. *Annu Rev Cell Biol* **7**,427-452.

Artavanis-Tsakonas S, Rand MD, Lake RJ. (1999). Notch signaling: Cell fate control and signal integration in development. *Science* **284**,770-776.

Baker BS, Nagoshi RN, Burtis KC. (1987). Molecular genetic aspects of sex determination in *Drosophila*. *BioEssay* **6**, 66-77.

Baker BS, Ridge KA. (1980). Sex and the single cell. I. On the action of major loci affecting sex determination in *Drosophila melanogaster*. *Genetics* **94**, 383-423.

Baker BS, Wolfner MF. (1988). A molecular analysis of *doublesex,* a bifunctional gene that controls both male and female sexual differentiation in *Drosophila Melanogaster*. *Genes Dev*. **2**, 477-489.

Burtis KC, Baker BC. (1989). Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs coding related sex-specific polypeptides. *Cell* **56**, 997-1010.

Cornbrooks C, Bland C, Williams DW, Truman JW, Rand MD. (2006). Delta expression in post-mitotic neurons identifies distinct subsets of adult-specific lineages in *Drosophila*.*JNeurobiol* **67**, 23-38.

Christian P, Carney G E, Taylor BJ, White K. (2002). Reaper is required for neuroblast apoptosis during Drosophila development. *Development* **129,** 1467-76.

Dauwalder B, Amaya-manzanares F, Matrox W. 2. (1996). A human homologue of the Drosophila sex determination factor *transformer-2* has conserved splicing regulatory functions. *Nat*. **115**, 24-32.

Doe CQ, Goodman CS. (1985). Early events in insect neurogenesis I: Development and segmental differences in the pattern of neuronal precursor cell. *Dev*. *Biol*. **111**,193-205.

Putting gender on the agenda. *Nat*. **299**, 465-665.

GAO (U.S. General Accounting Office). 1992. Women's Health: FDA Needs to Ensure More Study of Gender Differences in Prescription Drug Testing.

Helf, Lewis. (2009). Quirks of Human Anatomy. *Cambridege University press* **111**, 79-88.

Hildreth PE. (1965). *Doublesex*, a recessive gene that transforms both males and females of *Drosophila* into intersexes. *Genetics* **51**, 659-678.

Kapp MB. (2006). Ethical and legal issues in research involving human subjects: do you want a piece of me?. *J Clin Pathol*. **59**, 335-339.

Kliszczak A, Rainey M, Harhen B, Boisvert F, Santocanale C. (2011). DNA mediated chromatin pull-down for the study of chromatin replication. *Scientific Report* **95,** 122-126.

Lee G, Hall JC, Park JH. (2002). Doublesex gene expression in the central nervous system of *Drosophila melanogaster*. *J. Neurogenetics* **16**, 229-248.

Institute of Medicine (US) Board on Population Health and Public Health Practice. Sex-Specific Reporting of Scientific Research: A Workshop Summary. Washington (DC): National Academies Press (US). 2012. Why sex-specific reporting is important.

Mittelstrass K, Ried JS, Yu Z, Krumsiek J, Gieger C, Prehn C, Roemisch-Margl W, Polonikov A, Peters A, Theis FJ, Meitinger T, Kronenberg F, Weidinger S, Wichmann HE, Suhre K, Wang-Sattler R, Adamski J, Illig T.(2001). Discovery of sexual dimorphisms in metabolic and genetic biomarkers. *PLoS Genet* **2011**, 7-21.

Nagoshi RN, McKeow M, Burtis KC, Belote JM, Baker BS. (1988). The control of alternative splicing at genes regulating sexual differentiation in *D. melanogaster*. *Cell*. **53**, 229-236.

NINDS. 2002. Motor Neuron Diseases Fact Sheet.

Rideout EJ, Dornan AJ, Neville MC, Eadie S, Goodwin SF. (2010). Control of sexual differentiation and behavior by the *doublesex* gene in *Drosophila melanogaster*. *Nat Neurosci*. **13**, 458-467.

Slee R, Bownes M. (1990). Sex determination in *Drosophila melanogaster*. Quart Rev *Biol*. **65**, 175-204.

Taylor B J, Truman JW. (1992). Commitment of abdominal neuroblasts in *Drosophila* to a male or female fate is dependent on genes of the sex-determining hierarchy. *Development*. **114**, 625-642.

Truman JW, Taylor BJ, Awad TA. (1993). Formation of the adult nervous system. *Plainview*. **1**, 1245-1275.

Whitford KL, Dijikhizen P, Polleux, Ghosh A. (2002). Molecular control of cortical dendrite development. *Annu Rev Neurosci*. **25**, 127-149.

Wilson K. (1984). Sex-related differences in drug disposition in man. *Clinical Pharmacokinetics* **3**, 198-202.

Rideout E, Dornan A, Neville M, Eadie S, Goodwin S. (2011). Control of Sexual Differentiation and Behavior by the *doublesex* gene in *Drosophila melanogaster*. *Nat Neurosci*. **4**, 458-466.

Ramos-Vara. (2005). Technical Aspects of Immunohistochemistry. *Vet Pathol* **42**, 405-426.