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Characterization of Histidine Decarboxylase in Drosophila Using an Internal FLAG Epitope

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Characterization of Histidine Decarboxylase in *Drosophila* Using an Internal FLAG Epitope

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Abstract

Histamine is a neurotransmitter in arthropods and is responsible for synaptic transmission in vision, mechanosensation, temperature sensing and sleep cycle in *Drosophila*. Histamine is synthesized by the enzyme histidine decarboxylase (HDC). While histamine is detectable within tissues using current immunofluorescent labeling techniques, immunological approaches have not been successful for HDC itself, with both direct antibodies and terminal epitope tags determined to be ineffective. In order to avoid loss of the epitope tag through putative N- and C-terminal proteolytic cleavage, known to occur for HDC in other organisms, an internal epitope tag that does not disrupt enzyme function was utilized. A 6xHIS internal epitope tag had been previously employed and though it was not successful as a method to detect the HDC protein, it did demonstrate that an internal *SacI* restriction site could be used without disrupting the protein's function and a FLAG epitope tag was employed instead. We found that an internally located HDC-FLAG tag is an effective method for determining the location of HDC in a variety of tissues. In the brain of *Drosophila*, HDC is found to co-localize with histamine in histaminergic cells and structures. In larvae, HDC was found to localize to the same 10 pairs of cells in the ventral nerve cord found to be histaminergic. In adults, HDC was detected in central brain neuropil region where synaptic terminals are located, cells in the central brain, and photoreceptors as well. Photoreceptor localization was not as strong as expected, displaying a punctate staining pattern in the photoreceptors. Embryo analysis showed very restricted HDC localization, except for a novel pattern on

their lateral edges, postulated to be the chordotonal organs. While the digestive tract displayed immunofluorescence in adult tissue sections, whole mount gut immunofluorescence experiments stained negative for FLAG. In summary, the internal HDC-FLAG epitope tag has been shown to be an effective, useful tool for HDC detection in tissue and has the capacity for enabling new discoveries in histamine biology in *Drosophila*.

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Background

Histamine has been identified as a neurotransmitter the photoreceptors of the widely used model organism, *Drosophila melanogaster* (Sarthy, 1991; Burg et al., 1993). Among the most important functions of histamine in flies are vision (Burg et al., 1993; Melzig et al., 1996) and mechanosensation (Melzig et al., 1996), with other functions such as temperature sensing (Hong et al., 2007), the regulation of the sleep-wake cycle (Oh et al., 2013) and mediating mating behavior (Ingles et al., 2013) having been reported. Histidine decarboxylase (HDC) is the enzyme that is responsible for synthesizing histamine in *Drosophila.* The objective of this thesis is to demonstrate the effectiveness of a FLAG-labeled HDC protein as a tool for detecting HDC protein in the tissue of *Drosophila,* which can be used to determine whether HDC is present in the same locations as histamine, or if the localizations are different.

Histamine was first discovered to be a neurotransmitter in the eyes of *Drosophila melanogaster* (Hardie, 1989, Sarthy et al., 1991). It is produced by HDC from the amino acid histidine in a one-step, pyridoxal phosphate-dependent reaction (Epps, 1945). The *Hdc* gene has been mapped out, sequenced and several mutant stains have been identified in *Drosophila* (Burg et al., 1993). The nonlethal mutant allele Hdc^{JK910} has a premature stop codon, which leads to a greatly reduced amount of the enzyme (Birdsey, 2007). This mutation leads to near-undetectable levels of histamine (Burg et al., 1993). HDC is well conserved, having orthologs in many species, including zebrafish (Puttonen et al., 2013), chickens, cows (Zimin et al., 2009), rats (Krusong et al., 2011),

mice (Krusong et al., 2011) and humans (Zenthow et al., 1991). Histamine-deficient flies have been shown to be functionally blind and also have mechanosensory defects (as demonstrated by failure to properly perform grooming motions) (Melzig et al., 1996).

A critical part of understanding the cellular and molecular biology of histamine revolves around knowing how histidine decarboxylase is localized and regulated- in particular where HDC is localized in the nervous system and if cells that contain histamine do so because of their ability to synthesize histamine. There is a clear need for an effective method to detect HDC to both identify it in tissues that are known to be histaminergic, as well as potentially discover new tissues that have not been identified as histaminergic using histamine immunochemistry. Fluorescent microscopy has been used for the detection of HDC activity in tissue, which has its limitations. An effective detection method could provide insight into the subcellular localization of HDC, especially with confocal microscopy. Intracellular differences between histamine and HDC localization are expected. Western blot techniques could be utilized with an immunological detection tool available. The size of the HDC protein could be determined, analysis of any proteolytic processing it may undergo *in vivo* and even identify the process required for this proteolytic cleavage.

Molecular Regulation of HDC

There is a 60% similarity in amino acid sequence between *Drosophila* HDC and mammalian HDCs, such as those found in mice, rats and humans (Burg et al., 1993, Fig. 1). This similarity can allow us to make some limited inferences about *Drosophila* HDC

based on mammalian orthologs. The mouse HDC has been shown to undergo post-translational, PEST-mediated cleavage from an inactive 74 kDa form to a significantly smaller, active 55 kDa form (Yamamoto et al., 1993). This cleavage is facilitated by caspase-9 and mediated by tandem aspartate residues at 517-518 and 550-551 (Furuta et al., 2007.) This cleavage removes the N- and C-terminal ends, which has been determined, in rats, to contain PEST (proline, glutamate, serine, and threonine) domains, which have been linked with degradation signaling (Rogers et al., 1986; Dartsch et al. 1998.) Multiple isoforms can be processed from the initial protein product, possibly resulting in differentially regulated versions depending on the location in the body (Fleming and Wang, 2000).

Neurotransmitter Action of Histamine

After the release of histamine as a neurotransmitter from the photoreceptor cells into the synaptic cleft and binding onto the HclA channel on the L1 and L2 interneurons (Hardie, 1989), after which the surrounding glial cells have been shown to take up the released histamine. Histamine is conjugated to β-alanine through the action of the protein Ebony, yielding carcinine (Edwards and Meinertzhagen, 2010). The protein encoded by the gene Inebriated (ine) is responsible for transporting carcinine back into photoreceptors (Gavin et al., 2007). Carcinine is then shuttled back to the photoreceptor cell using ine, where the enzyme Tan deconjugates the histamine and β-alanine (Edwards and Meinertzhagen, 2010). This system can be demonstrated by feeding histamine to histamine-deficient HdcJK910 flies. Supplementing the nutrition of these mutant flies with exogenous histamine will rescue the various Hdc^{JK910}phenotypes,

restoring vision and mechanosensation. This rescue lasts for about a week before histamine degrades. Histamine can be observed in the photoreceptors and mechanoreceptors, but notably not in the central brain neurons in the uptake experiments (Melzig et al., 1998). What role that the histamine recycling system has in relation to HDC is not well understood.

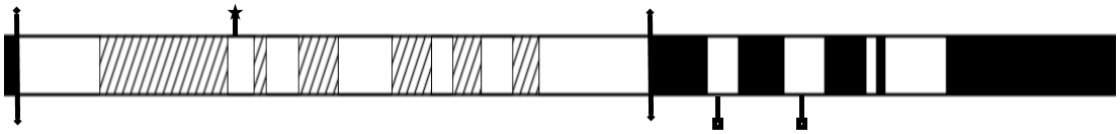

Figure 1: Diagrammatic representation of a multiple sequence alignment of HDC protein. *Rattus norvegicus, Mus musculus, Homo sapiens,* and *Drosophila melanogaster* HDC, plus *Drosophila melanogaster* dopa decarboxylase amino acid sequences were aligned. Strongly conserved and/or identical regions are indicated by hatched boxes, moderately to poorly conserved regions are indicated by white boxes and very poorly conserved regions are indicated with black boxes. The point marked with the star is the location of the *SacI* site where the FLAG epitope tag was inserted for this research. The points marked with boxes represent the tandem aspartates required for PEST-mediated cleavage of HDC in mice (at amino acid residues 530-531 and 580-581 in the alignment). The lines cutting through the protein with diamond marks represent the PEST cleavage sites for Rat HDC, with the length of protein outside the lines being cleaved off. The very large poorly conserved region on the C-terminus is due to *Drosophila* HDC, which has many more amino acids than that of other species in this region of the protein (See Appendix III for the full alignment).

Expression Data of HDC

A useful approach for assisting in the detection of the HDC protein during development and in various tissues is determining the stages that the *Hdc* mRNA is being expressed, to assist in detection. Northern blots and tissue *in situ* hybridizations have previously identified tissue-based expression of HDC in adults (Burg et al., 1993). For other developmental stages, the employed method was RNAseq, enabling for comprehensive snapshots of all coding RNA expressed at a specific moment in time (Chu & Corey, 2012), although tissue specificity is not possible. *Hdc* is shown to be expressed at about 12 hours into development (stage 15), until the third instar of larval development (Graveley et al., 2010). Expression is also detected in early pupae, as well as after 24 hours until emergence as an adult (Graveley et al., 2010). Adult males and females have the same amount of *Hdc* expression, which for females decreases after one day of adulthood to about one half to one third the expression seen in day one (Graveley et al., 2010). Males maintain this level of expression until day 30 (Graveley et al., 2010). In the adult, most *Hdc* expression is located in the head, with the eyes and central brain having strong expression (Burg et al., 1993). This data can assist in determining which locations and developmental stages to examine for the HDC protein, rather than observing at random points in development. Detection of histamine has served as a method of *Hdc* expression detection, through the identification of the enzymatic product. While this technique may be useful for identifying gene fragments

that regulate expression of *Hdc*, it does not allow for the detection of the protein itself- which could undergo additional regulation.

Epitope Tagging of HDC

Histamine can be detected with an antibody, by first fixing tissue with 1-ethyl-3(3-diamethylaminopropyl)-carbodiimide ("carbodiimide") to make cross-linked histamine conjugates (Panula et al., 1988). The localization of histamine and HDC is predicted to be equivalent, but it is possible that either may be present in a location where the other isn't. Experiments in mice show slight differences in localization of histamine and HDC in mast cells (Wagner et al., 2003). It is also possible that histamine may escape from structures during dissection, eliminating the ability to detect histaminergic structures. For these reasons, there have been attempts to generate methods for the direct detection of HDC in *Drosophila,* using immunofluorescence techniques. However, these methods had been generally unsuccessful, as initial attempts to make an antibody directly against the HDC protein did not result in acceptable levels of specificity (personal communication with Dr. M. Burg). This is likely due to the fact that the amino acid sequence of HDC is very similar to that of dopa decarboxylase (DDC). Certain isoforms of the DDC protein have 50% sequence identity to HDC based on local sequence alignment (Wada et al., 1987). An antisera specific to HDC in humans, rats and mice was later developed, though this antibody has no evidence of working in flies (Francis et al., 2012). Fusion protein epitope tagging was also attempted but this method likewise resulted in no detection. N- and C-terminal

tagging was not seen as an option, due to the post-translational, PEST-mediated cleavage on both ends of the protein that likely takes place. This protein processing of HDC is seen in other organisms, such as rats (Fleming and Wang, 2000) and mice (Yamamoto et al., 1993). This proteolytic processing of HDC is also predicted to occur in flies, so for this reason, internal epitope tag insertion was pursued instead.

The Hdc^{JK910} mutant histamine-deficient phenotype has been rescued using transgenic forms of *Hdc*, suggesting that all regulatory information of the *Hdc* gene is contained within the gene itself (Burg & Pak, 1994; Burg & Pak, 1995; Burg & Pak, 1995). This demonstrates that rescue with altered transgenic forms of *Hdc* is possible and expression should be very similar to wild-type. This is important to clearly demonstrate before attempting transgenic experiments.

Analyzing predicted amino acid sequence to identify and avoid hydrophobic regions, conserved regions, or anything resembling an active or regulatory site is necessary for the determination of adequate internal insertion sites for epitope tags (Jarvik & Telmer, 1998). This was done by examining the 3D crystal structure of DDC as a base (Fair, 2012) and determining where a site may exist that will allow the insertion of an epitope tag far from the catalytic site of HDC.

For *Hdc* in *Drosophila*, two different candidate restriction sites were identified for internal, PCR-based epitope tagging of *Hdc*- a *SacI* site and *SalI* site, located in the second exon of the *Hdc* gene (Payne 2006). The location of the epitope insert was selected based on the structure of DDC, placing it in a location that is likely not a

catalytic or regulatory region, as well as being faced outward on the exterior of the protein. Tagged genes were inserted into a P-element containing pCasPeR-3 plasmid vector and used in germline transformation experiments. A 6xHIS tag (consisting of 6 histidine residues) was utilized initially. Insertion into the *SacI* site produced an epitope-labeled gene that rescued the Hdc^{JK910} histamine deficiency phenotype, but the *SalI* insertion yielded histamine-deficient flies (Fair et al., 2012; Hage et al., 2012). The *SacI*-6xHIS construct rescued the histamine deficient phenotype, but was not effective for detection of HDC in tissue or with western blots. A *SacI*-FLAG construct was also made as an alternate, should the *SacI*-6xHIS experiment fail (Payne, 2006). This project details the use of *SacI*-FLAG as a candidate for detection of HDC.

The FLAG epitope is an 8-residue long sequence consisting of (AspTyrLysAspAspAspAspLys) (Einhauer & Jungbauer, 2001.) The amino acid composition and sequence were chosen very carefully in order to maximize the antigenicity of the tag, with the aromatic tyrosine residue, supported by the first asparagines. The lysine plus asparagine repeats make the tag hydrophilic, which also increases antigenicity and pushes the epitope out in the 3D structure of a protein (Einhauer & Jungbauer, 2001). The M2 antibody is the most versatile of the FLAG antibodies available, not requiring calcium and functioning well on the C-terminus or at an internal site, in addition to on the N-terminus (Einhauer & Jungbauer, 2001). This makes the FLAG tag and the M2 antibody useful for our needs. It was purchased for our experiments through Sigma-Aldrich, St. Louis, MO (Cat. #: F1804 Sigma).**Methods**

Germline Transformation with the *Hdc*-FLAG Gene

The *Hdc*-FLAG construct was made by previous members of the Burg lab and placed into the pCasPeR-3 vector (Payne et al., 2006). The *p{Casper3-gHdc-SacIFLAG, w^+}* plasmid, with a HDC-FLAG insert and *mini-white* (colored eye) gene marker, was injected into the germline cells of white-eyed fly embryos by technicians at Genetic Services Inc. (Fig. 2). About 130 injected flies produced were then mated to white-eyed flies, from which colored-eye progeny were screened for and collected. Each individual fly collected was designated as a new line. Two such transformation injections were performed in total. These flies were then crossed to *+;Sco/CyO;Sb/TM3,Ubx* flies and chromosomal linkage was determined (see Figure 4). 52 lines were generated from the second transformation. Only first or third chromosome inserts were retained, as the Hdc^{JK910} allele is present on the second chromosome. Some of the X and third chromosome insert lines were bred into a w^-;Hdc^{JK910} mutant background to demonstrate that the HDC-FLAG protein was functional.

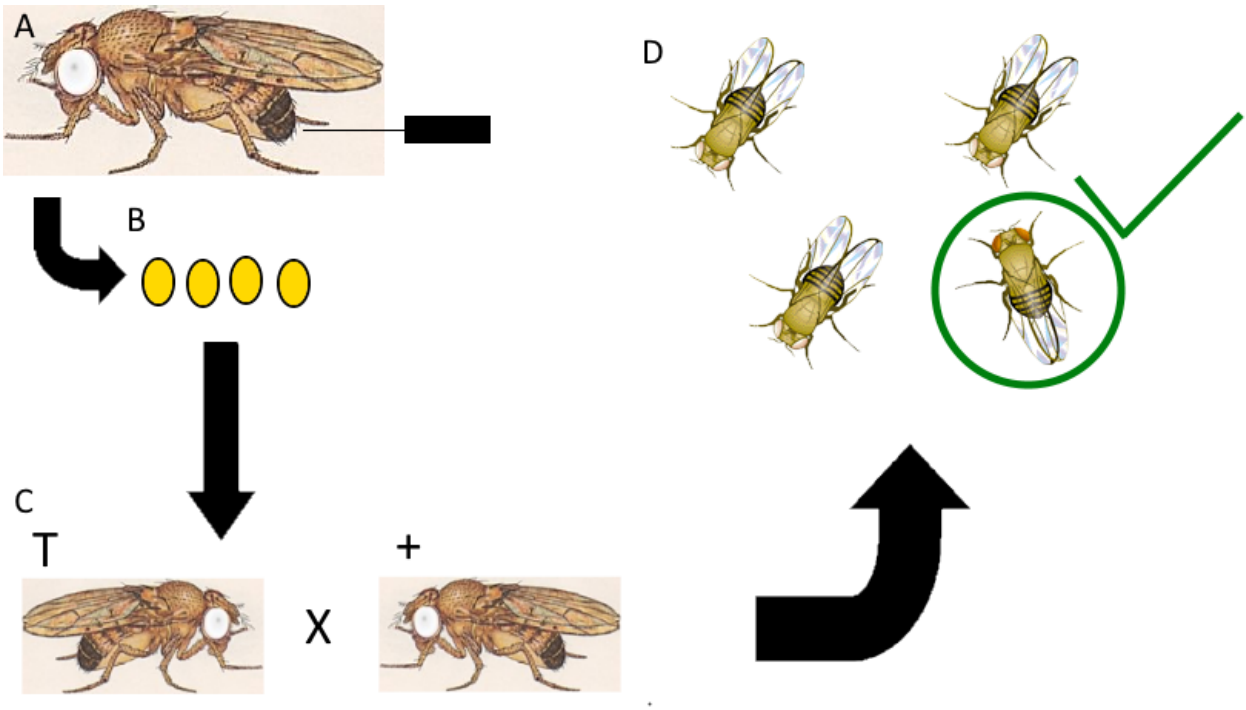

Figure 2: Diagrammatic summary of germline transformation of *Drosophila*. A) A gravid female, white-eyed fly has the plasmid injected the germline cells of the eggs inside of it. B) The eggs are laid and flies are collected. C) Emerging flies are mated to white-eyed, wild-type flies. D) The offspring of the flies from the cross in C are collected. Transformants have the *mini-white* gene as a marker, indicating that any fly with orange eyes contain the transgene. These flies are then crossed to a marker strain to determine linkage. *Image credit:* http://www.indiana.edu/~oso/lessons/Genetics/bw_st.html for side view flies *and* http://www.exploratorium.edu/exhibits/mutant_flies/mutant_flies.html for top view flies.Histamine Immunofluorescent Labeling

Immunofluorescent labeling for histamine was performed on wild-type, *HdcJ*^{K910} and transgenic experimental flies with inserts on the first and third chromosomes, as per Melzig et al., 1996. Third instar flies were initially the subject to most of these experiments, with second instar later being preferred. A 4% buffered carbodiimide solution in PBS was used as a fixative (see Appendix II). Brain tissue was dissected in the cold fixative and allowed to fix for 2.5-3 hours on ice. Afterwards, the tissue was washed with *Drosophila* Ringer's solution and blocked with 5% normal goat serum in TBS plus 0.3% Triton X-100. Initially, rabbit histamine polyclonal antibody (Incstar, Inc.), diluted 1:1000 in 1% normal goat serum (NGS) in Tris-buffered saline (TBS) plus 0.3% Triton X-100 was used. 1:2000 and 1:5000 dilutions were later determined to be sufficient for detection. The tissue was incubated in the primary antibody solution at 4° C overnight (approximately 14-16 hours). After incubation, the tissue was equilibrated to room temperature for 30 minutes. Brains were washed in 1% NGS, TBS 0.3% Triton X-100 solution and treated with 1:1000 anti-rabbit Alexa-Fluor 555 nm antibody (red) diluted in 1% NGS, TBS 0.3% Triton X-100 for 1.5 hours, shielded from light. The tissue was then washed for 20 minutes, twice with 1% NGS in TBS with 0.3% Triton X-100 afterwards, then washed in PBS for 10 minutes, mounted on slides and visualized with a TRITC filter.

FLAG Immunofluorescent Labeling

FLAG Immunostaining was performed in a similar manner as above, but with the following alterations: Brains were dissected in ice-chilled 4% paraformaldehyde fixative, buffered to about 7.0 pH (see Appendix II), and incubated for 15-30 minutes. The dilution of the primary antibody- M2 anti-FLAG (Sigma) was 1:500 for all experiments. The secondary used was goat anti-mouse, Alexa-Fluor 488 nm (green) with a 1:1000 dilution. Fluorescent microscopy utilized the FITC filter.

Histamine and FLAG Double Immunofluorescent Labeling

Double labeling using histamine and the FLAG epitope antibody to detect HDC-FLAG and histamine simultaneously was performed to determine colocalization of the two and whether or not there were any intracellular localization differences. For this procedure, second and third instar larval brains were dissected in 4% buffered carbodiimide and allowed to fix for no more than 2.5 hours. They were washed twice with *Drosophila* Ringer's solution, then allowed 15 minutes to fix with the 4% buffered paraformaldehyde on ice and then washed again with Ringer's several times. 5% NGS in TBS with 0.3% Triton X-100 was used to block for one hour. Both primary antibodies were diluted in 1% NGS in TBS 0.3% with Triton X-100, in the same vessel. Rabbit histamine antibody was diluted 1:5000 and the M2 mouse anti-FLAG was diluted 1:500. The primary antibody solution enabled both antibodies to incubate with the brains simultaneously, at 4° C, overnight. The tissue was treated in the same fashion as stated above, but used a mixed antibody solution containing a 1:1000 dilution of both goat

anti-mouse Alexa-Fluor 488 nm and goat anti-rabbit Alexa-Fluor 555 nm simultaneously. Images were taken with both TRITC and FITC filters, taking the image at the same focal plane with each filter separately. On some samples an aqueous mountant with fluorescent decay-inhibitors was used before visualization. Signal intensity of the FLAG epitope appears to be reduced with this procedure when compared to the FLAG immunofluorescence alone, so exposure time needed was increased with some images in order to visualize the FLAG signal.

FLAG Immunofluorescent Labeling of Adult Tissue Sections

Sectioned tissue from adult heads and bodies of both transformant and wild-type flies were collected and stained for the FLAG epitope. Adult flies were placed into Tissue-Tek™ CRYO-OCT compound (Fisher Scientific, Inc.) and covered with a second layer of the solution and frozenusing conductive metal stubs and dry ice. A cryostat (Lieca, inc.) was used to slice sections at approximately 20 µm thickness, which were placed on a slide and kept at -20° C until use. Two methods were used to fix tissue samples: a pre-fixation and post-fixation method. Post-fixation methods entailed incubating the slides in 4% buffered paraformaldehyde after the frozen sections were acquired. Different fixation times were carried out to obtain the clearest visualization. The pre-fixation procedure involved a partial dissection of specimens, allowing the paraformaldehyde fixative to perfuse through the intact tissues of the fly, before proceeding with the cryosectioning procedure. As with the post-fixation, the fly was frozen using dry ice, a conductive stub and Tissue-Tek™ CRYO-OCT compound.

Specimens were transported to the cryostat apparatus and allowed to equilibrate to the cabinet temperature, then sections were cut at an ambient temperature of -10° C, as indicated by the cryostat. Fixation times using this method ranged between 1 hour and 2 hours. Prefixation for one hour without heat treatment (for flattening sections) produced the best resolution and tissue morphology. Sections could be stored for up to one week in a -80 °C freezer.

After samples were sectioned, they were circled with a PAP pen (EMS, Inc.), then washed with *Drosophila* Ringer's solution twice. Slides with sections were kept in a humidity chamber during all incubations. They were blocked with 5% NGS in TBS with 0.3% Triton X-100 for one hour. M2 mouse FLAG antibody was diluted 1:500 in 1% NGS in TBS with 0.3% Triton X-100 and incubated on the slides overnight at 4° C. Slides were then washed with 1% NGS in TBS with 0.3% Triton X-100 and incubated 1.5 hours, with goat anti-mouse Alexa-Fluor 488 nm diluted 1:1000 in 1% NGS in TBS with 0.3% Triton X-100 (being shielded from light). Slides were washed again with 1% NGS in TBS with 0.3% Triton X-100, washed with PBS, sealed with cover glass and Fluoro-Gel mountant (EMS, Inc), then visualized with fluorescent microscopy.

Embryo and First Instar Collection and Immunofluorescent Labeling

HDC-FLAG transformant lines and wild type embryos were analyzed with FLAG immunostaining. An egg collection system was used to obtain stage 12 to 17 embryos. Egg laying plates were made from agar, sugar and grape juice (Featherstone et al., 2009). Each of the transformant and control lines had a one hour initial egg lay, followed

by a two-hour long egg laying period, then a second 2-hour egg laying period. The egg plates were incubated at 21° C overnight, resulting in embryos whose ages varied between 24 and 19 hours old. Embryos between stages 12 and 17 were selected, as their nervous systems were at a more advanced stage of development. It was determined through examining RNAseq data that HDC expression began one or two hours prior to the larvae hatching from the egg (Graveley et al., 2010). To get embryos that were very close to hatching, a the same as above was employed, but a 25° C incubation was used to induce more rapid development of embryos. This resulted in some of the embryos having developed fully into first instar larvae, but a number were still around stage 15-17.

Collected embryos were dechorionated using a 50/50 mixture of household bleach and distilled water. They were not allowed to incubate in the bleach for longer than 3 minutes. The embryos were washed with the embryo wash solution and/or distilled water to remove excess bleach. Then, they were transferred to a tube with heptane and an equal amount of 4% paraformaldehyde was added. The tube was shaken vigorously for about 20 seconds, then rocked at room temperature for 20 minutes. Afterwards, the aqueous paraformaldehyde layer was removed and an equal volume methanol was added. The tube was shaken and vortexed for 20-30 seconds. The heptanes layer was then removed and the tube was stored at 4° C until ready (typically no more than 2 hours.) The embryos were rehydrated gradually, as follows: the methanol was removed and an 80/20 mixture of methanol/PBS was added and the tube rocked for 5 minutes. This was repeated with a 60/40, 20/80 and 0/100 mixture of

methanol/PBS as well. The embryos were then blocked with a mixture of PBTA for 30 minutes and 5% Normal Goat Serum for 30 minutes (this was later changed to an hour of blocking with 5% NGS in TBS with 0.3% Triton X-100 for 1 hour.)

The M2 FLAG antibody was used as the primary antibody, at a 1:500 concentration diluted in 1% NGS in TBS with 0.3% Triton X-100. The embryos were incubated at 4° C overnight, with rocking. Afterwards, the antibody solution was removed and embryos were washed twice, 10 minutes each with 1% NGS in TBS with 0.3% Triton X-100. Then the secondary was applied: 1:1000 anti-mouse Alexa Fluor antibody, 488 nm diluted in 1% NGS in TBS with 0.3% Triton X-100. Incubated, shielded from light, with rocking, for 1.5 hours. Then the secondary antibody solution was removed and the embryos were washed twice, for 20 minutes each time, with 1% NGS in TBS with 0.3% Triton X-100, then for another 10 minutes with PBS. The embryos were transferred to a slide and mounted with Fluoro-Gel (EMS, Inc).

Imaging proceeded in a manner similar to that of the brains. Exposures varied from 100 to 500 ms, in order to best visualize structures. Oil immersion was required for 100x magnification.

For first instar larvae, the same method as above was employed and newly hatched larvae (within _ hours of hatching) were picked out from the plate, no more than 1-2 hours old. These larvae were dissected with the brains still attached to the mouth hooks. They were processed for immunostaining in the same manner as later larval stages (see above).

Image Acquisition and Processing for all Microscopy

Images were acquired with an Olympus BX51 microscope and the software used was MicroSuite Basic Edition version 2.7. Images were subjected to minor image editing for presentation. Some images had their saturation increased to better visualize structures, though most images were not adjusted. Merges between two different fluorescent images entailed pasting the respective red and green channels from the two images into the same image document.

Being limited to epifluorescent microscopy, it was not possible to capture all HDC-FLAG-containing cells in a single image without manipulating the images. For data presentation purposes, a focal plane series of images were taken the image data program ImageJ was used to generate Z-stacks, allowing all relevant details to be presented into one image (Fig. 3).

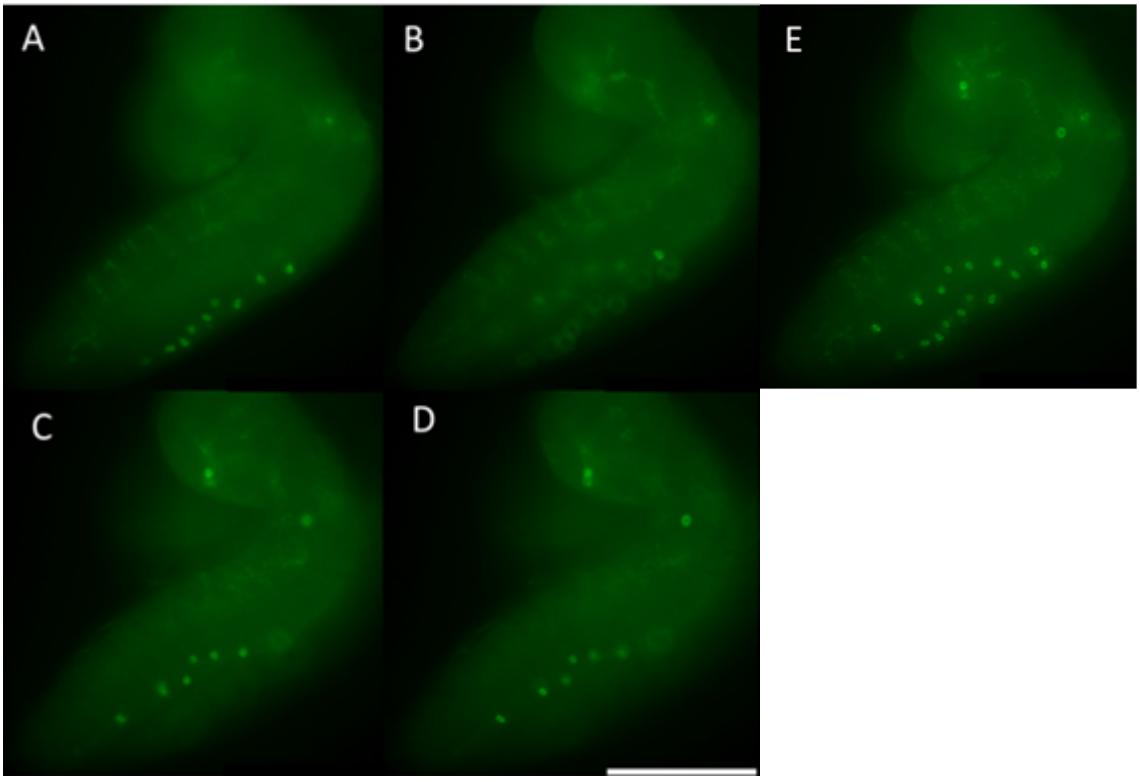

Figure 3: Assembling a Z-stack. Focal plane image series of w^{-}; Hdc^{+}; *gHdc-SacIFLAG*$^{78\text{-}5}$, second instar brain. (A)-(D) are four images taken in a focal plane Z-series. (E) Is the Z-stack of the four previous images, allowing all the details to be observed in one image. Throughout the results section, the image that best displays and demonstrates the most accurate representation of the data will be shown. Scale bar = 100 µm.

Results

Characterization of Transformant Lines: Larval CNS

The primary goal of this work was to determine the localization of HDC-FLAG in the fly larval brain using immunocytochemistry. The second instar brain was used as a consistent stage for these experiments. Several lines were analyzed to determine whether a consistent pattern could be detected be characterized.

There were two major controls utilized in these experiments: wild type (Oregon-R strain, with either wild-type or w^- eyes) and w^-;Hdc^{JK910}. Wild-type functioned as both a positive and negative control to compare results of transformant line results for histamine and FLAG immunoflurorescent detection. The w^- Hdc^{JK910} mutant also served as a negative control for non-specific immunofluorescence, as this fly should have no histamine or FLAG in its nervous system. The typical wild-type pattern of immunofluorescence features 10 pairs of cells in the ventral nerve cord of the larval brain (Fig. 4.; see also Python and Stocker, 2002). One pair of histaminergic cells are present per segment, except for one (segment A7), which lacks histamine. Another pair can be seen between the ventral ganglia and the lobes of the central brain, as well as two pairs of cells in each of the lobes and staining in the nerve terminals from these cells. No cells or structures stained for FLAG in wild-type brains (Fig. 5). Hdc^{JK910} displayed no immunostaining of cells for either histamine or FLAG (Fig. 6). Comparing wild-type FLAG immunolabeling to transgenic lines stained in the same manner should show that any FLAG immunofluorescence seen in experimental transformant lines is not due to endogenous proteins that could bind the FLAG antibody.

To determine whether the *gHdc-SacIFLAG* transgene in some transformants was present and resulted in a functional enzyme, histamine immunocytochemistry was performed on second and third instar larval brains of *gHdc-SacIFLAG* lines (see Appendix I for line summary). Most of the analyzed transgenic lines placed into Hdc^{JK910} background displayed cellular histamine localization nearly identical to wild type (Fig. 7).

Analysis of the transformant lines was performed using immunocytochemistry with antibodies against the FLAG epitope. The objective of this analysis was to determine whether the internal FLAG epitope was effective at detecting HDC in tissue, as well as confirming that the signal observed is actually HDC. Previous research has identified the localization of histamine in the larval brain, as well as the expression of HDC using a GFP reporter with an HDC promoter (Python and Stocker, 2002; M. Burg, pers. Communication). If the signal from the FLAG fluorescent immunochemistry of an anti-FLAG stain is similar to or the same as the expected histamine patterns, it was inferred that it is most likely HDC-FLAG that this signal represents.

The typical pattern seen in the larval brain for HDC-FLAG featured the following: 10 pairs of histaminergic cells on the ventral ganglion (Fig. 4). An additional pair is located between the central brain and ventral nerve cord (Fig. 4). There are two cells present in each of the two lobes of the central brain as well as extending neuropil in both the ventral ganglia and lobes (Fig. 4). Processes projecting from cell bodies can also be seen (Fig. 4). This pattern closely matches the staining pattern seen with histamine immunofluorescence, as seen in both wild-type and transgenic lines.

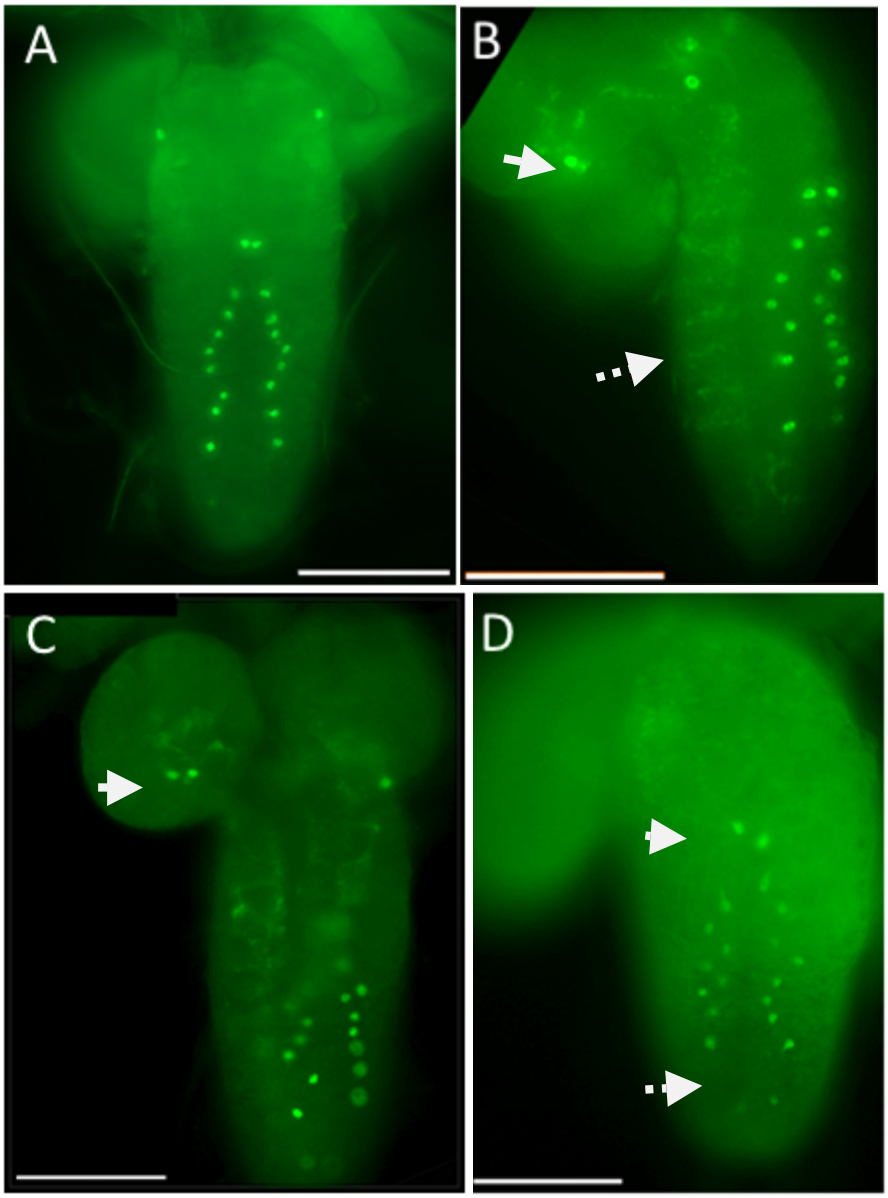

Figure 4: Typical HDC-FLAG immunofluorescence staining pattern. A) *gHdc-SacIFLAG*$^{55\text{-}4}$ larval brain preparation displays all 10 ventral nerve cord cell pairs, plus an additional pair near the central brain (Z-stack of 4 images). B) *gHdc-SacIFLAG*$^{78\text{-}5}$ (Z-stack of 4 images) and C) *gHdc-SacIFLAG*$^{55\text{-}4}$ show the central brain cells (arrows). (B) also demonstrates the nerve terminal staining (dashed arrow). D) *gHdc-SacIFLAG*$^{135\text{-}1}$ demonstrates neurite projection staining (arrow). Note that a cell pair is missing from a segment (dashed arrow). Scale bar = 100 μm.

To confirm that the neurons and neuropil that stained with the FLAG antibody are the same cells that contain histamine, double labeling of histamine and FLAG with immunofluorescence was also utilized. Merging of color channels of histamine immunostain images with the corresponding FLAG images shows that the two colocalize in nearly all cells and structures (Fig. 7, 9). This confirmed observations made with the FLAG-only stain and strongly suggests that the immunofluorescence seen is HDC and not another protein. In addition, the capability of the transgenic HDC-FLAG to produce histamine is demonstrated, with a transgenic line in an Hdc^{JK910} background showing signal for both histamine and FLAG (Fig. 7). The intensity of the histamine immunofluorescence also appears on par with wild type, suggesting no significant reductions in enzymatic activity (Fig. 7). While all cell bodies demonstrate histamine and HDC-FLAG colocalization, the other cellular structures display unequal localization. Nerve roots are found to contain histamine evenly, while HDC-FLAG appears to demonstrate more punctate staining. When co-localized in a cell, histamine also appears to stain slightly stronger when compared to HDC-FLAG (Fig. 9).

Double labeling experiments were also used to determine subcellular localization differences between histamine and HDC in the cell body of neurons in HDC-FLAG transformant flies. FLAG immunofluorescence was primarily gathered around the edges of the cell body, suggesting HDC localization there. This was different than the localization of histamine, which appeared to be evenly distributed in the cell body (Fig. 8).

In some of the transformant lines, ectopically localized histamine was found in structures where ectopic HDC-FLAG expression was also localized. This results lends evidence to that what is being detected actually is the HDC-FLAG protein and is not a result of any FLAG cross-reacting protein, as HDC is synthesizing histamine in those ectopic regions that typically are not histaminergic (Fig. 10).

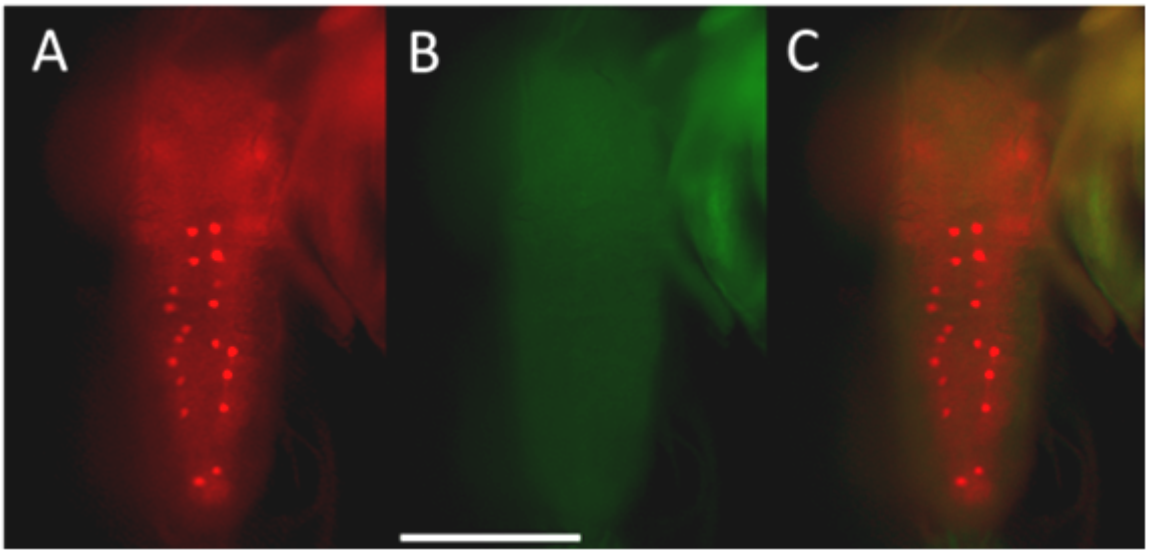

Figure 5: Wild-type brains show no FLAG Immunofluorescence. Histamine and FLAG double immunofluorescent labeling of wild-type second instar brain. Brains were stained with both histamine and FLAG antibodies on the same sample. A) Histamine immunofluorescence stain. B) FLAG immunofluorescence stain. C) Merge of the previous two images. There is no signal in the FLAG immunostain (B), demonstrating that any signal seen in experimental HDC-FLAG samples is due to the presence of the transgene. The typical pattern of histaminergic cells is seen here, as well, displaying 9 of the 10 typical histaminergic cell pairs. Scale bar = 100 µm.

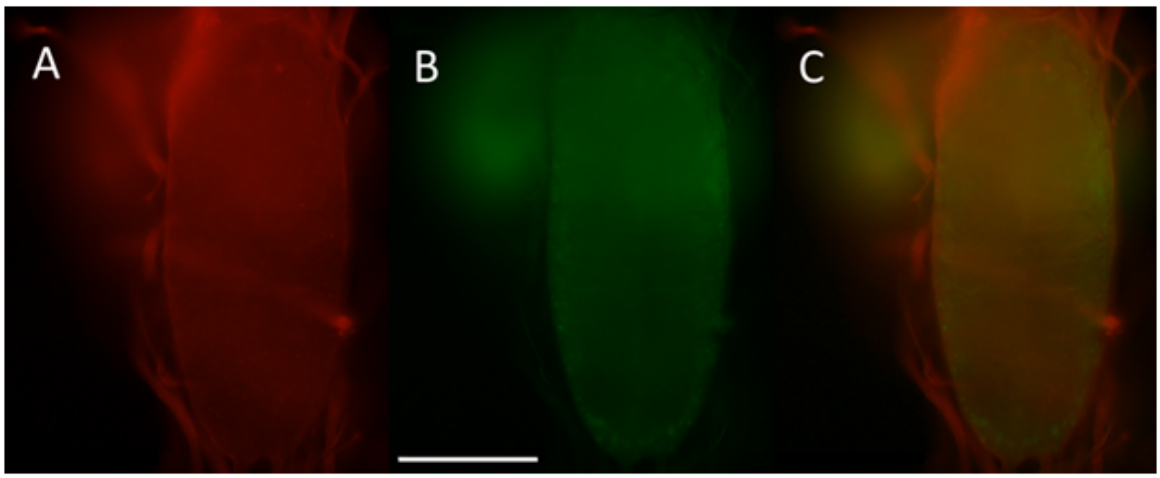

Figure 6: *Hdc*^{JK910} brains show no histamine or FLAG immunofluorescence. Histamine and FLAG double immunofluorescent labeling of *Hdc*^{JK910} second instar brain. A) Histamine immunostain. B) FLAG immunostain. C) Merge of the two. The *Hdc*^{JK910} has no detectable histamine and the cells seen in wild-type preparations are not visible here. The histaminergic cells do not have any signal for FLAG, either. Scale bar = 100 µm.

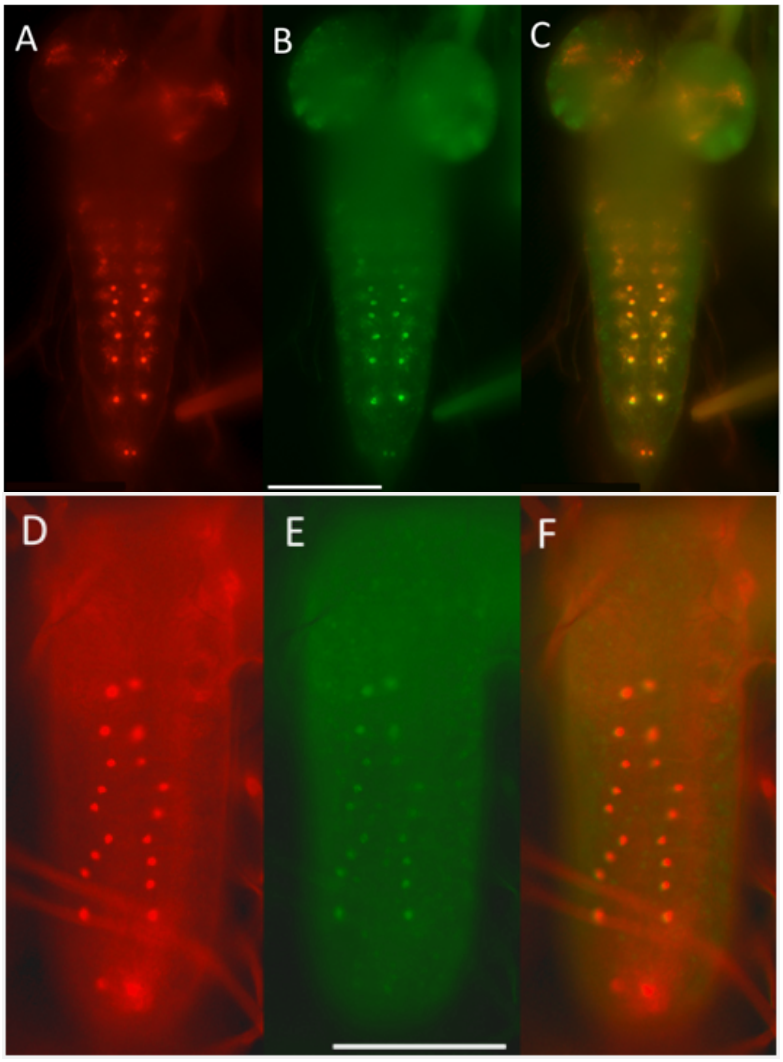

Figure 7: HDC-FLAG and histamine colocalize in the cells in the ventral nerve cord.

A-C) Histamine and FLAG double immunofluorescent labeling of second instar *gHdc-SacIFLAG*$^{78\text{-}5}$ brain, Z-stack of 4 images. D- F) Histamine and FLAG double immuno-fluorescent labeling of second instar *gHdc-SacIFLAG*$^{135\text{-}1}$ brain, in an *Hdc*^{JK910} background. A, D) Histamine immunofluorescence stain. All 10 pairs of histaminergic cells in the ventral ganglia are visible here. B, E) Anti-FLAG immunofluorescence stain. C, F) Merge of the respect sets of two images. All cells in the merge colocalize, demonstrating that HDC is present in the same locations as histamine, although the terminal pair has weaker FLAG signal. Scale bar = 100.

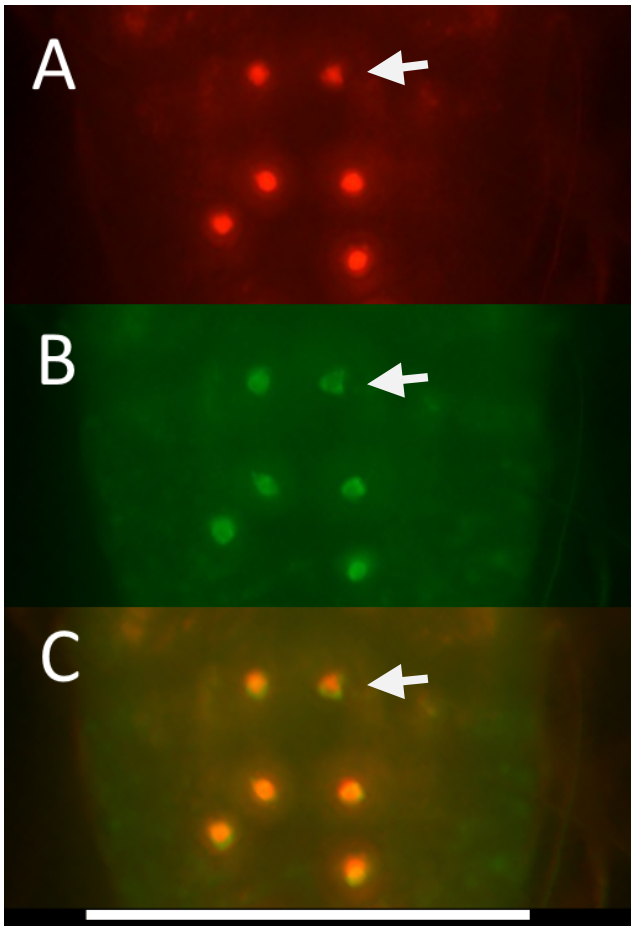

Figure 8: HDC-FLAG appears to localize more on the edges of the cells, while histamine localization is centered in the cell. FLAG and histamine double immunofluorescent labeling in a second instar *gHdc-SacIFLAG*$^{78\text{-}5}$ brain. This image takes a selection of cells from Figure 7D-F. A) Histamine immunostain, B) HDC-FLAG immunostain and C) a merge of (A) and (B). The arrows point towards a cell that has noticeably different localization. HDC-FLAG appears to localize more on the edges of the cells, while histamine is more centered. Scale bar = 100 µm.

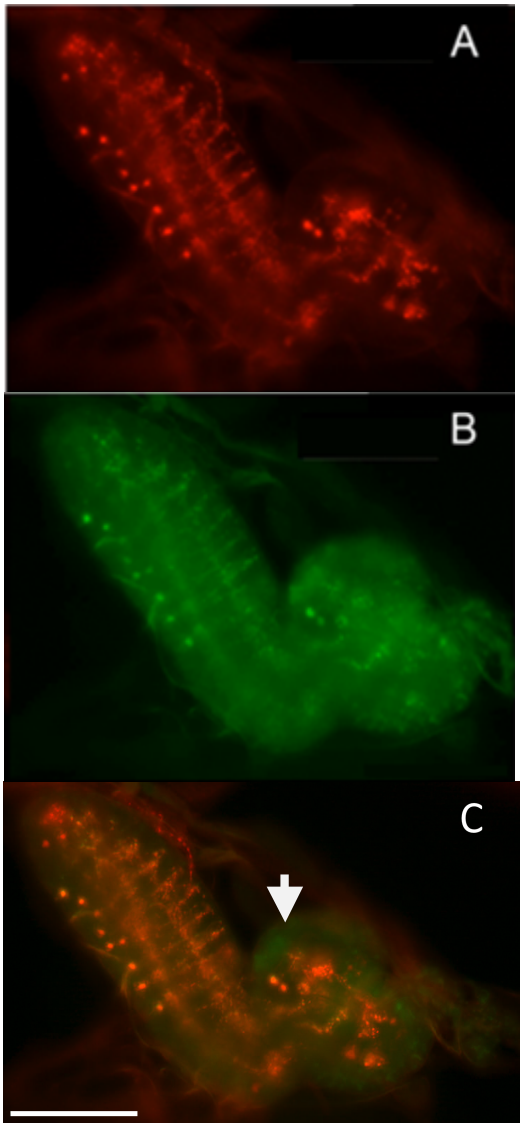

Figure 9: Anywhere that there is histamine, HDC appears to be present. Histamine and FLAG double immunofluorescent labeling of a second instar *gHdc-SacIFLAG*$^{78\text{-}5}$ brain. A) Histamine immunostain. B) FLAG immunostain. C) Merge of two previous images. These images show that the central brain cells also have histamine and HDC-FLAG colocalizing (arrow.) Note also the nerve terminals for the histaminergic cells and neuropil in the ventral ganglia and central brain have both histamine and HDC-FLAG colocalizing. Scale bar = 100 μm.

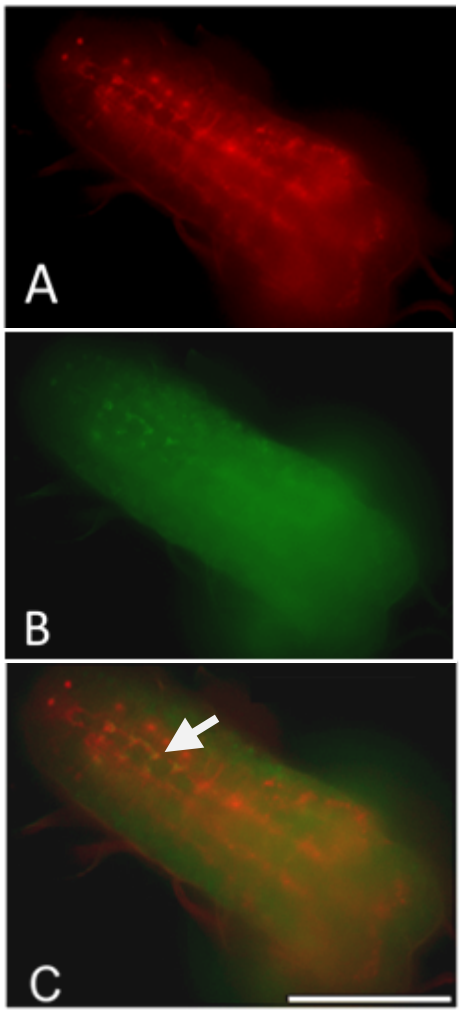

Figure 10: Ectopic expression of HDC also leads to ectopic localization of histamine. Histamine and FLAG double immunofluorescent labeling of second instar brain of gHdc-*SacIFLAG*$^{6\text{-}1}$. A) Histamine immunostain. B) FLAG immunostain. C) Merge of the previous two images. Much of the neuropil has colocalization of histamine and HDC-FLAG. A particular part of the segmented neuropil (arrow) has noticeably more FLAG signal than histamine. Scale bar = 100 µm.

HDC-FLAG in Adults

Larval brains have been the standard preparation for the majority of the project as the immunostaining preparation is simple. The consistent, easily observable stereotyped pattern of the 10 histaminergic cell pairs in the ventral ganglia has been used to gauge the effectiveness of the FLAG tag as a method of HDC detection and has been used to characterize transformant lines that bear the *gHdc-SacIFLAG* transgene. Deviations from the typical pattern are easily detectable and are detailed in a later section (see page 51). However, most interest in the *Drosophila* nervous system is focused on the adult brain and development occurring in the embryo. While the data we have acquired regarding the adult brain is less in volume than of the larval brains, we have still obtained evidence that establishes the FLAG-tagged HDC as being an effective tool for detection of HDC in adult tissue, enabling some novel discoveries.

Histamine immunostaining has been performed on adult brains in the past, showing histamine localization in the photoreceptors, lamina and medulla, as well as central brain neuropil and cortex (Pollack and Hofbauer, 1991; also see Fig. 11). FLAG-HDC also strongly stains in the adult brain with the FLAG antibody, most notably having strong neuropil staining and matching the cellular locations that have been detected with histamine previously (Figs. 11 and 12). However, the lamina and medulla layers stain weakly for the FLAG epitope in HDC-FLAG transformant flies compared to the histamine immunostaining and the FLAG signal in the central brain neuropil. The photoreceptor FLAG staining for HDC-FLAG is also unlike what is seen in the histamine

immunostaining (Fig. 13). While the histamine staining appears mostly even over the length of the photoreceptors (Fig. 11), FLAG staining appears mostly localized to punctate regions in the photoreceptor cell body (Fig. 12). These areas of staining appear to be located on the edges of the eye in cryosectioning experiments. To better visualize this unexpected result, whole eye FLAG immunostains were performed. The punctated staining pattern appears to be more numerous than in the tissue sections, seemingly being present in a more proximal region of the photoreceptor (Fig. 13). With current microscopy capabilities, it was not possible to determine precisely what localization these packets have within the photoreceptors.

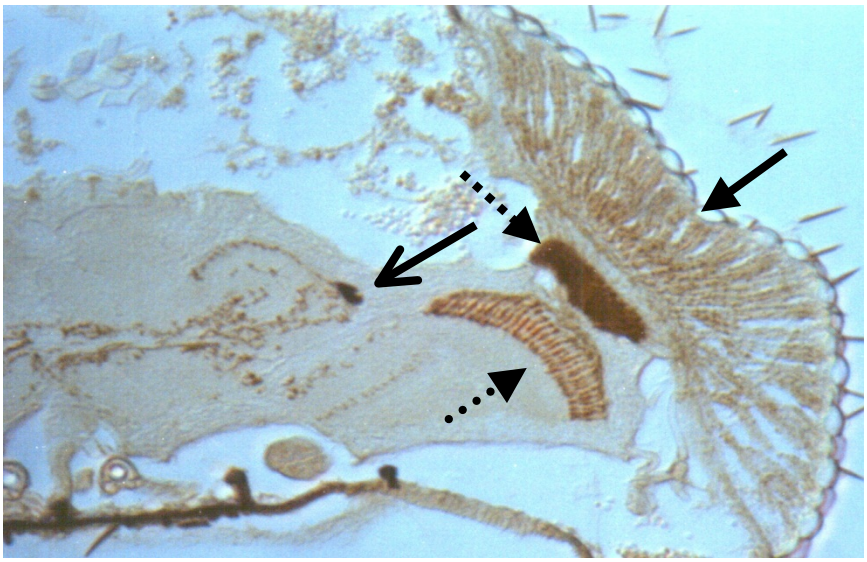

Figure 11: Histamine immunostaining of wild-type adult head cryosection shows histamine in visual system and the central brain. The brown stain indicates the presence of histamine, generated from horseradish peroxidase (HRP) conjugated to the secondary antibody. Main areas of histamine localization include photoreceptors (solid arrow), lamina (dashed arrow), medulla (dot-dashed arrow) and a histaminergic cell and neuropil (open-tipped arrow). (image supplied by M. Burg, unpublished.)

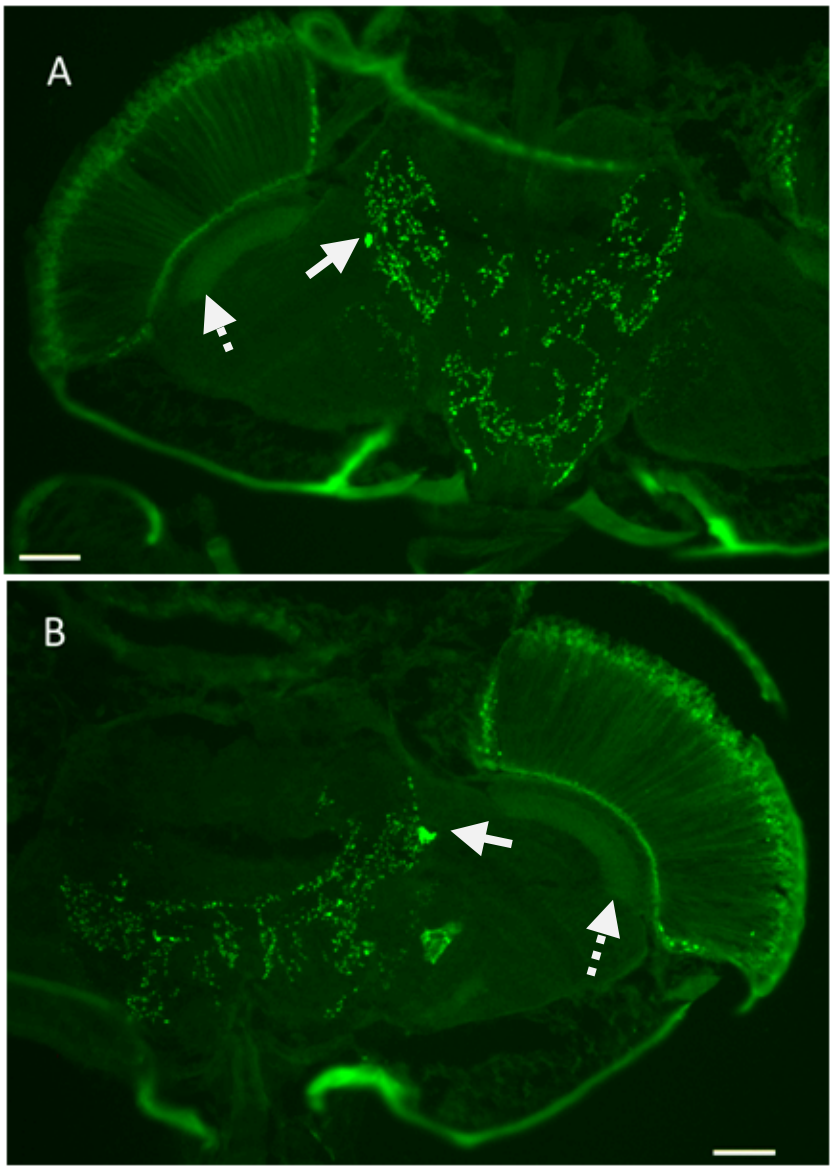

Figure 12: FLAG immunofluorescent labeling shows HDC-FLAG localization very similar to that of histamine. Anti-FLAG immunostain of adult head tissue section of gHdc-*SacIFLAG*$^{135\text{-}1}$. A) and B) are tissue sections from different planes of depth. Extensive neuropil staining is observed in the central brain complex. Single cells (solid arrows) match the relative locations on the histamine stain. The lamina (dashed arrows) appears to stain, but is not as strong as that seen with histamine. The medulla is visible, but does not appear to have any significant level of FLAG staining at all. Scale bar = 100 µm.

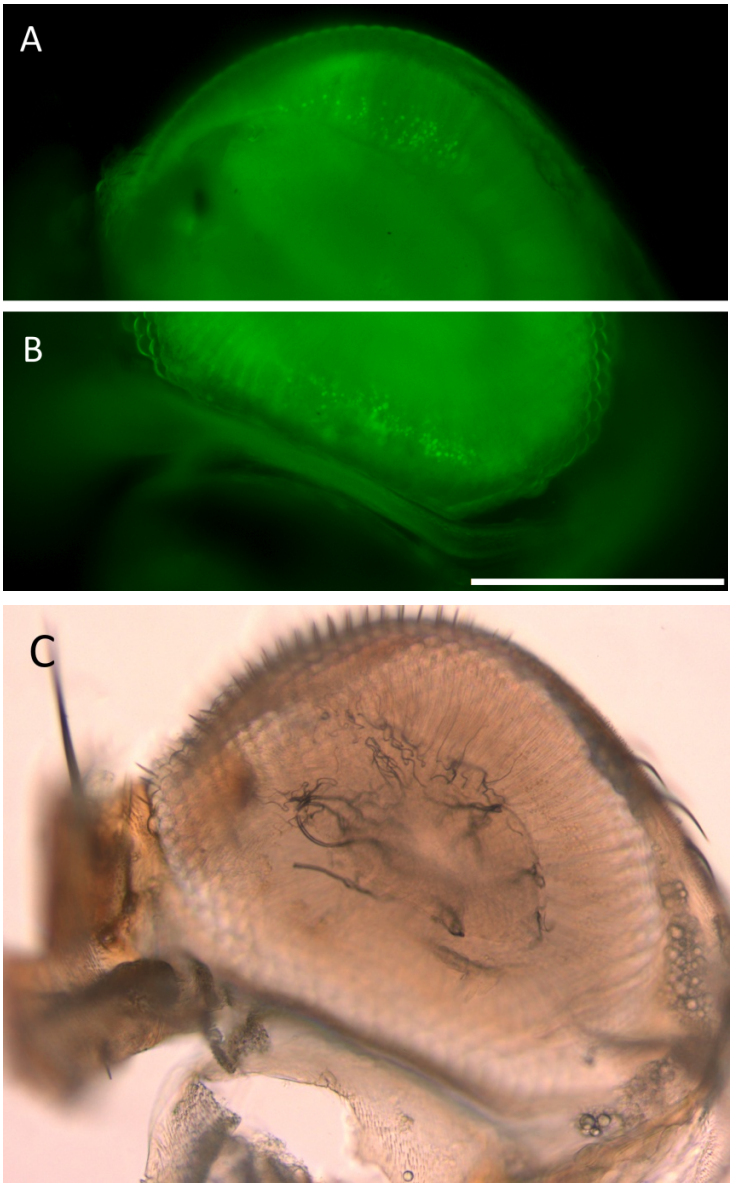

Figure 13: HDC-FLAG displays a novel pattern in the photoreceptors. Eye whole mounts *of gHdc-SacIFLAG*$^{135\text{-}1}$ stained used the FLAG antibody A) and B) FITC filter images of the same eye at two different focal planes and regions of the eye, superimposed for better visualization of the staining pattern. HDC-FLAG is sequestered into punctuations, present up and down the more proximal regions of the photoreceptor cartridges, around the entire eye. Two planes that represent this are presented here. C) The bright-field image of the above. Scale bar = 100 µm.

Developmental Profile

In an effort to demonstrate the usefulness of HDC-FLAG as a tool to detect and localize HDC, the localization of HDC-FLAG at various points in development was attempted. As part of the initial assessment of HDC-FLAG localization, many larval brains at the second instar stage were analyzed. Adult brains were observed as well, as shown above (Fig. 12). Stages that had not been observed include the first instar larvae, third instar larvae, embryo and pupal stages. The brains of those stages, except for pupae and embryos, are presented below. Third instar brain localization did not differ greatly from second instar localization, despite changes in brain morphology and size. The only difference was the relative intensity of the immunofluorescence (Fig. 14), which appears to be reduced. First instar larval brains also had similar HDC-FLAG localization when compared to second instar larvae. They varied by the appearance of the cells themselves, with distribution of fluorescence around the edges of the cells being even more pronounced. Projection staining from the cell also appeared to be absent, with the overall background increased in first instar brains (Fig. 14). In both the positive control, as well as experimental samples, the intensity of the histamine staining within the histaminergic cells was noticeably reduced when compared with second instar larval brains. This reduction was more pronounced in the experimental lines which suggests that HDC's activity may not yet be fully established in newly hatched larvae and could imply that HDC is not present at significant levels until after hatching, although this still needs to be rigorously examined.

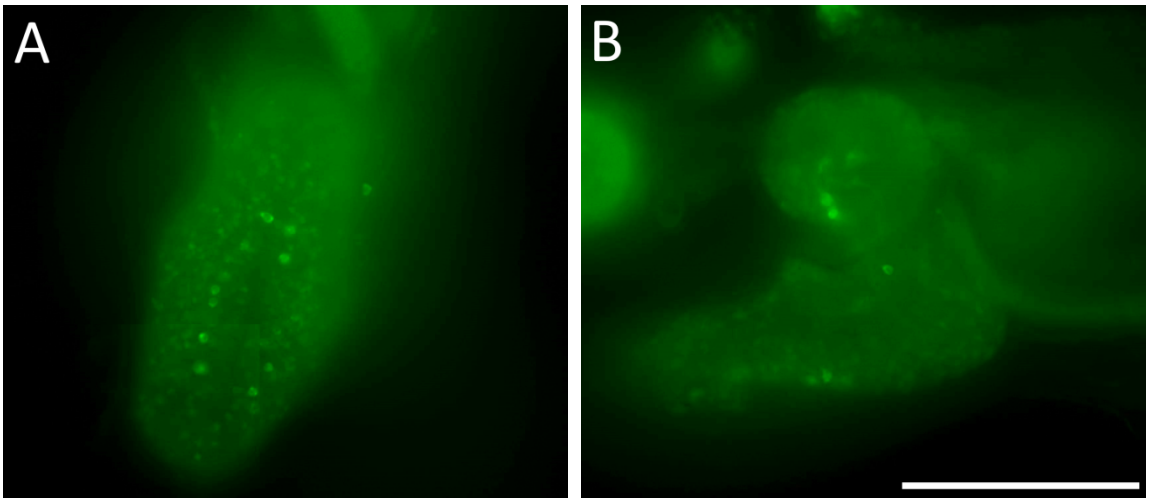

Figure 14: First instar brains look similar to second instar for FLAG immunostaining.

FLAG immunofluorescent labeling of *gHdc-SacIFLAG*$^{135\text{-}1}$ first instar larval brain. The first instar brains have a staining pattern that is relatively the same as later stages. However, the intensity of the staining is slightly lower and neurite projections cannot be seen. A) Ventral view. B) Lateral view. Scale bar = 100 µm.

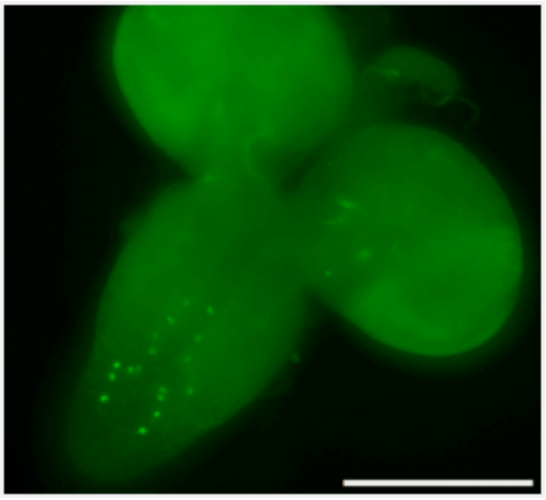

Figure 15: Third instar brains show no change in pattern from second instar. FLAG immunofluorescent labeling of *gHdc-SacIFLAG*$^{135\text{-}1}$ third instar larval brain. The localization pattern of HDC in brains of third instar larval brain does not differ greatly from the second instar brain, only displaying somewhat less protein, judging by the reduced signal. Otherwise, almost all the cells from the earlier stage can be identified (except for the posterior-most cell pair, which always stains dimmer than the others). Scale bar = 100 µm.

Embryonic Localization

RNAseq expression data obtained from FlyBase showed that expression of HDC in the embryo began 12 hours into development, all the way until hatching of the larva (Graveley et al., 2010). This data was used to determine what stages to examine for HDC-FLAG detection in embryonic stages. Our prediction was that the histaminergic cells, seen in the larvae, would be seen in analogous regions in the embryonic developing nervous system. Later embryonic stages were chosen, particularly, stages 14-16, as the central nervous system was better formed at that point.

Initial experiments resulted in no differences between the experimental HDC-FLAG specimen and negative controls, so the next objective was to determine the earliest point at which HDC was expressed, as stage 16 may have been too early. HDC-FLAG was observed in first instar larvae, so embryos were revisited. Embryos were incubated at a warmer ambient temperature of 25° C, in order to acquire the final stages of embryonic development: stages 15-17. The samples observed did not display any staining patterns similar to the larval patterning in their developing nerve cords. However, a new staining pattern was observed in these later samples. Along the lateral edges of the larvae, tiny points of punctate staining were observed (Fig. 16). These points appear to come in sets of 3-5, in a linear fashion (Fig. 17). At least one set of points is visible in each segment, though in some specimens, it appears that there are two sets. This staining was seen in a segmentally-based pattern, across many specimens. It would appear that HDC is present in the late embryonic peripheral nervous system, in small amounts.

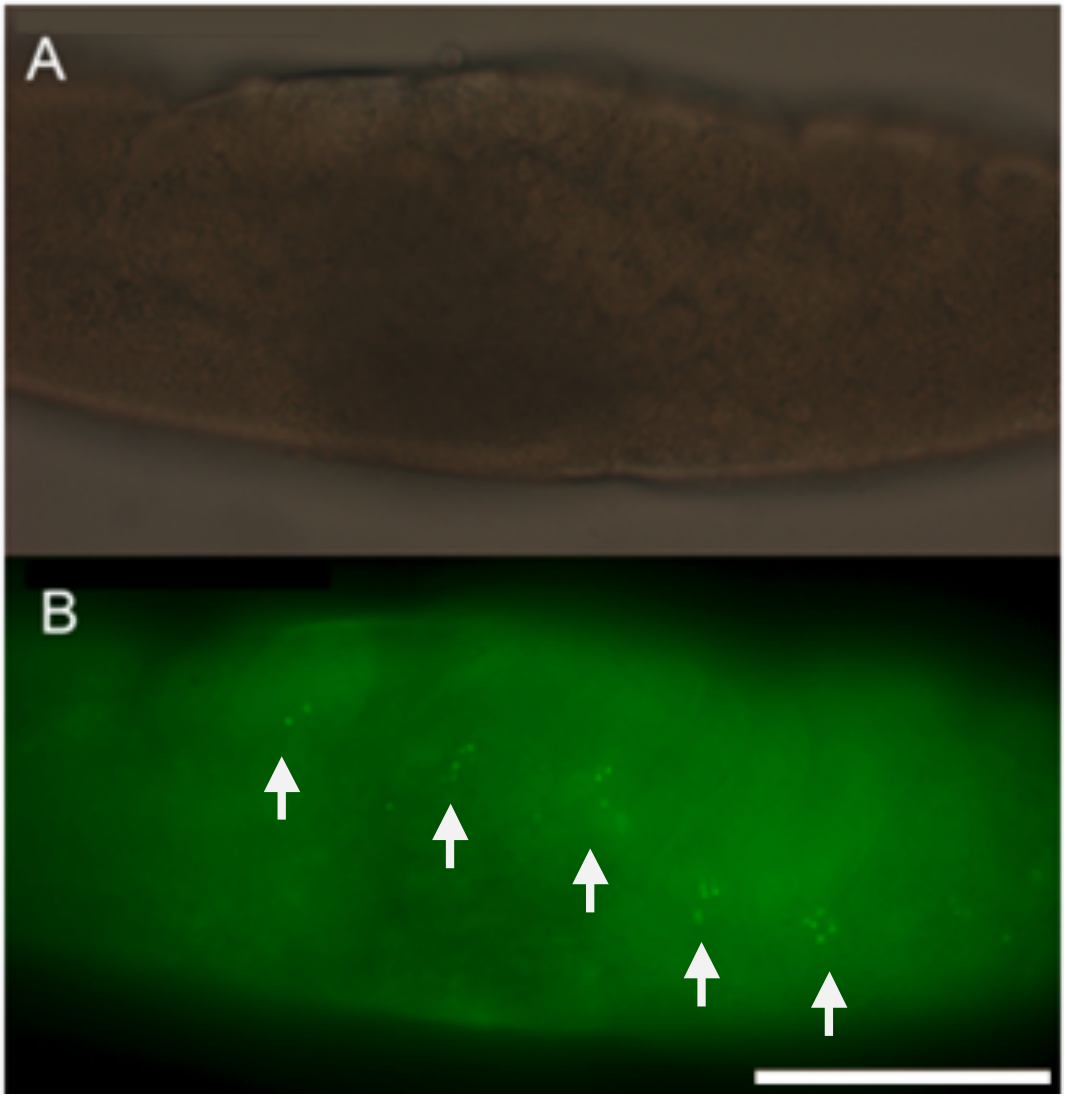

Figure 16: Late stage embryos display a unique localization of HDC-FLAG. Embryo FLAG immunostain of *gHdc-SacIFLAG*$^{135\text{-}1}$ approximately stage 16. A) Bright field image. B)FITC filter image. This image demonstrates the segmental distribution of a signal detected by the FLAG antibody that was not present in non-HDC-FLAG transformant flies.

Scale bar = 100 µm.

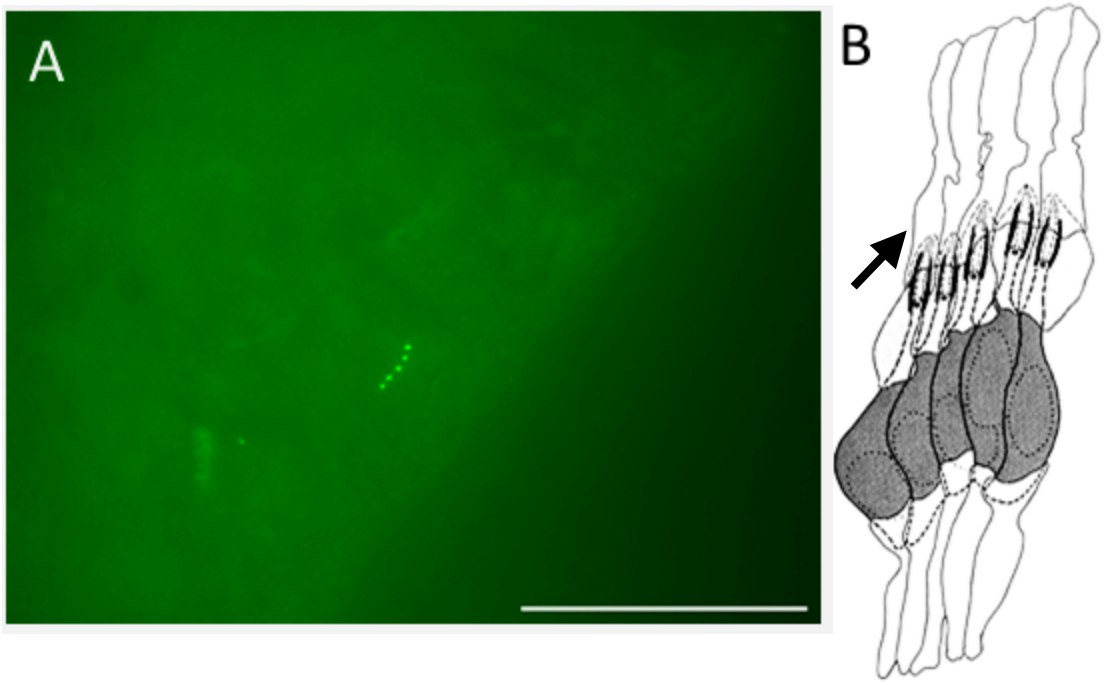

Figure 17: The embryonic HDC-FLAG staining pattern resembles that of chordotonal organs. A) Anti-FLAG immunostain of *gHdc-SacIFLAG*$^{78\text{-}5}$ stage 16 embryo. B) Stage 16 abdominal lateral pentascolopidial chordotonal organ lch5. The highest magnification of the embryo localization pattern clearly demonstrates the 5 points of immunofluorescence in each section. This pattern very closely resembles the chordotonal organ, seen in this illustration of the organ. Scale bar= 50 µm. Image credit for (B): FlyBase, originally from Hartenstein, 1998.

Gut Localization

Upon observing HDC-FLAG in adult tissue sections, what appeared to be localization of HDC in a distinct region of the midsection of the midgut was seen (Fig. 22). To demonstrate the presence of HDC in the intestines of *Drosophila*, the digestive tracts were dissected out and immunocytochemistry was performed on second instar larval digestive tracts. These experiments showed no FLAG immunofluorescence in the digestive tracts, but whole mount immunofluorescent labeling of adult guts has not yet been attempted.

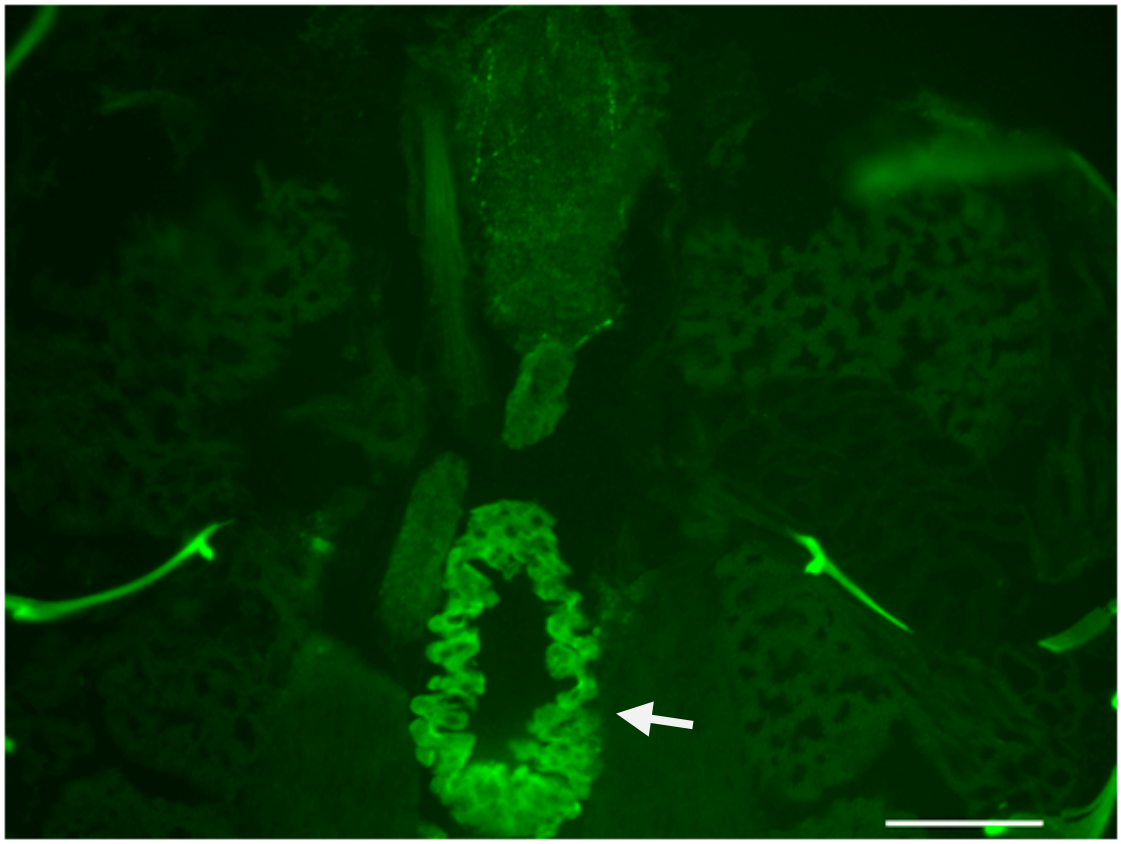

Figure 18: Gut staining is present in *gHdc-SacIFLAG*$^{135\text{-}1}$ FLAG immunofluorescent labeling of tissue sections. This sample was the impetus for further investigation of the gut for HDC-FLAG signal in larvae. The gut tissue is adult tissue sections displays strong fluorescent signal (arrow), which was not seen in wild-type. Scale bar = 100 µm.

Ectopic Expressing HDC-FLAG Transgenic Lines

The insertion sites of the P-element in germline transformation experiments are not random, favoring GC-rich regions and particular secondary structures of DNA (Liao et al., 2000) and are preferentially in the 5' UTRs of certain genes (Spradling et al., 1995). Because of this, there is a possibility that a HDC-FLAG bearing transgene could be inserted into a position under the control of another gene's promoter or enhancer region, leading to ectopic expression of the HDC protein. Due to this possibility, several of the distinct transformant fly lines were examined to determine the most common expression pattern that is overlapping between several lines. Several lines were examined that displayed localization of HDC-FLAG in locations that differed from the majority of the lines examined with immunohistochemistry. In most lines, the ectopic localization is additive and does not appear to disrupt the normal expression patterns. Each ectopically expressing line had a unique pattern, suggesting the transgene was being controlled by another gene. None of the unique ectopic patterns are present in more than one line.

gHdc-SacIFLAG$^{6\text{-}1}$, located on the X chromosome, features the most dramatic ectopic localization of the ectopically expressing lines. In the larval brain, it displays a bright staining of segmented neuropil, running down the center of the ventral nerve cord (Fig. 18). The FLAG immunoreactivity has been seen in nerve roots emanating from the ventral nerve cord as well (Fig. 18). In addition, staining has been seen in cells and nerve projections running across the cuticle. Also of note, histamine has been observed

in locations where HDC-FLAG is also present (Fig. 10). This provides additional evidence showing that what is being detected actually is HDC. Ectopically localized HDC-FLAG appears to produce histamine, which also is present in these ectopic locations. However, the ectopic histamine stain is less intense than the FLAG signal (usually it is the other way around), likely because the locations of expression do not have the capability to properly store histamine properly. *gHdc-SacIFLAG*$^{6\text{-}1}$ is also the only ectopic line to exhibit a reduction in staining intensity in the expected histaminergic cell pairs (Fig. 18). The cells still appear to contain HDC and histamine, although the level of immunoreactivity appears to be reduced. Some samples appeared to have normal intensity, while in others the signal strength rendered the cells nearly invisible.

gHdc-SacIFLAG$^{55\text{-}4}$,with its insert located on the X chromosome, at first appeared to be a typical-staining line, but further experiments revealed it had a small amount of aberrant HDC-FLAG localization. Additional HDC-FLAG signal, consisting of two symmetric pairs of cells, was discovered between the ventral ganglia and the central brain complex (Fig. 19). However, it has shown to be a strongly expressing line, displaying bright staining in the typical regions and working well with double stains. For this reason, it may be used in further experiments, despite the atypical expression pattern (also see Fig. 10).

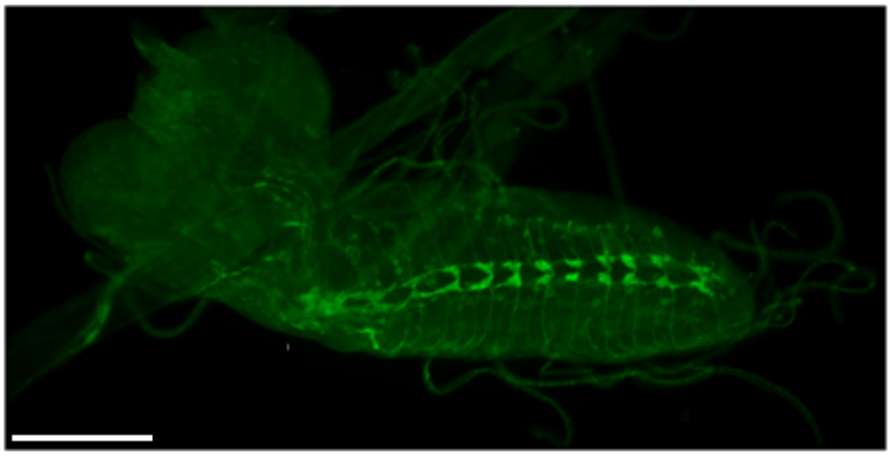

Figure 19: *gHdc-SacIFLAG*$^{6-1}$ displays a dramatic ectopic localization of HDC-FLAG in the segmented neuropil. FLAG immunofluorescence labeling of *gHdc-SacIFLAG*$^{6-1}$ second instar larval brain. This is a two-dimensional image, as the sample was squeezed between the cover glass and slide, rendering all structures into the same focal plane. The segmented neuropil is very obvious, with nerve projections originating from it extending towards the lateral edges of the ventral ganglia, as well as tiny punctuations of staining visible in nerve roots trailing off the brain. The 10 cell pairs seen in other samples have greatly reduced intensity here. Scale bar = 100 µm.

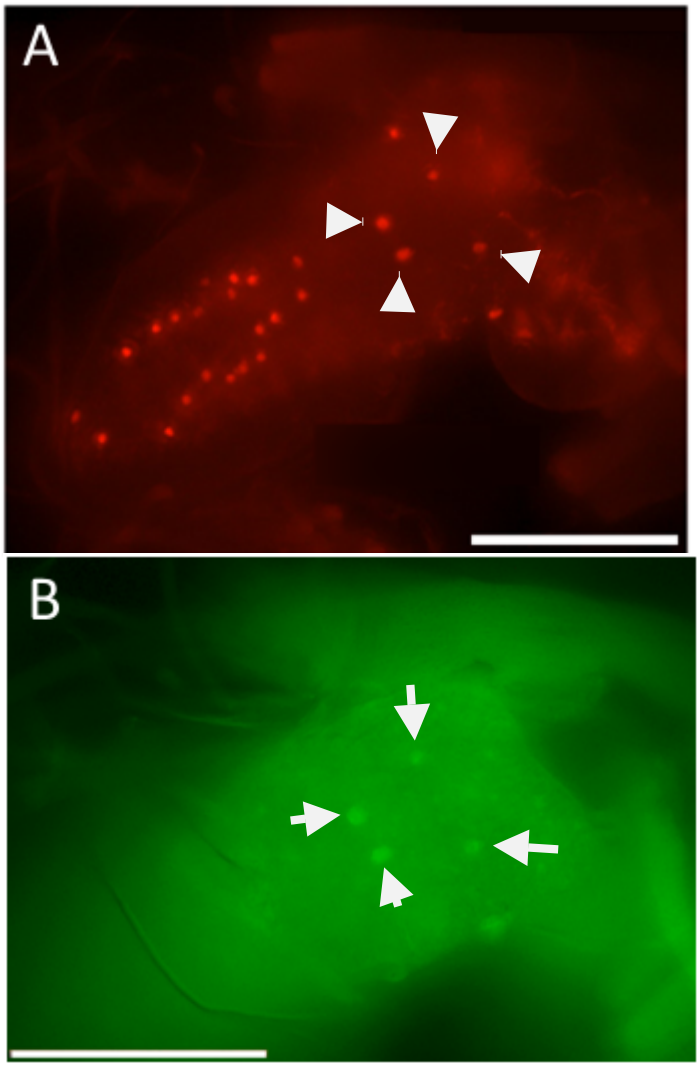

Figure 20: Four extra cells are present near the central brain of *gHdc-SacIFLAG$^{55-4}$*.

Histamine and FLAG double immunofluorescent labeling of *gHdc-SacIFLAG$^{55-4}$* second instar brain. A) Histamine immunofluorescence. B) FLAG immunofluorescence. The arrows point out the additional staining near the central brain complex. 10 cell pairs are visible in the histamine immunostain. Scale bar = 100 µm; image a result of Z-stack of 5 images.

The *gHdc-SacIFLAG*$^{132\text{-}6}$ insert is present on the third chromosome and features a dramatic ectopic localization. It has an extra pair of intensely staining cells set above the other histaminergic cells in the ventral nerve cord, several cells that stain on the lateral edges and three extra cells that stain on the posterior tip of the ventral nerve cord. There is also staining seen in the nerve roots, which appears in more than one of the ectopic lines (Fig. 20).

The *gHdc-SacIFLAG*$^{57\text{-}6}$ line produced yet another dramatic staining pattern. It has additional staining in a set of roughly 6 large, lateral cells on the sides of the ventral ganglia, as well as punctate staining in nerve roots trailing off the brain. The 10 histaminergic cell pairs seem to be present and stain with above average intensity (Fig. 21).

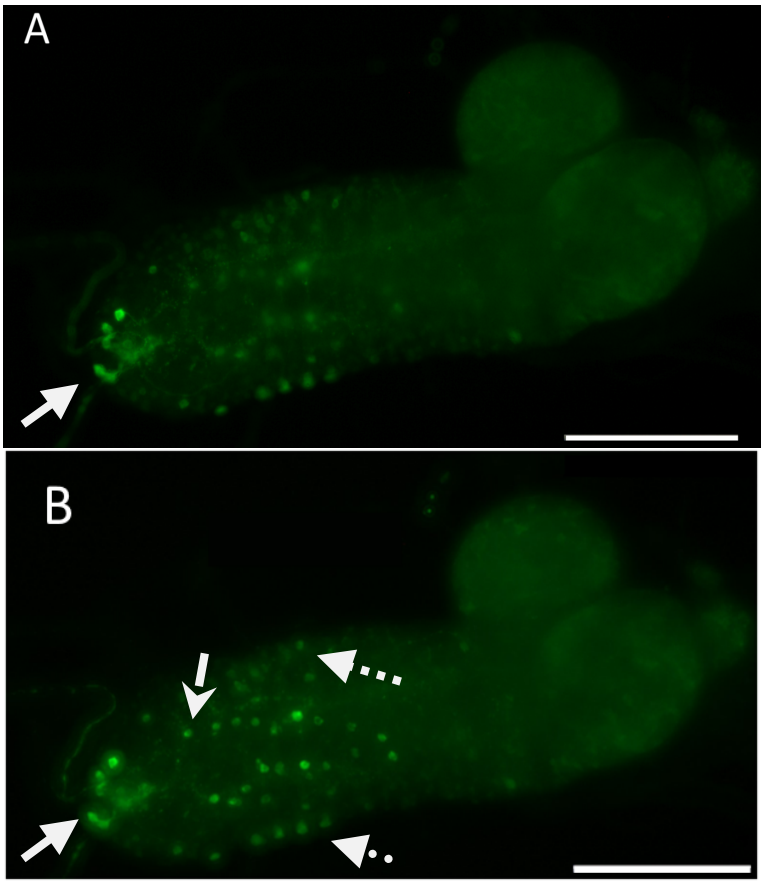

Figure 21: *gHdc-SacIFLAG*$^{132\text{-}6}$ has many extra cells with HDC-FLAG. FLAG immunofluorescence labeling of *gHdc-SacIFLAG*$^{132\text{-}6}$ second instar brain. A) Single focal plane. B) Z-stack of 4 images from the same sample. There is a significant number of extra cells that are stain for FLAG. Additional cells can be seen on the posterior end of the ventral ganglia (solid arrow), along the lateral edges of the ventral ganglia (dotted arrow), as well as in the same general area as the 10 histaminergic cell pairs (dashed arrow). Also note that trailing nerves have HDC-FLAG present in them as well (dash-dotted, stealth-tipped arrow.) (A) Shows the cells at the tip of the ventral ganglia without the halo effect from the out-of-focus images in the Z-stack. Scale bar = 100 µm.

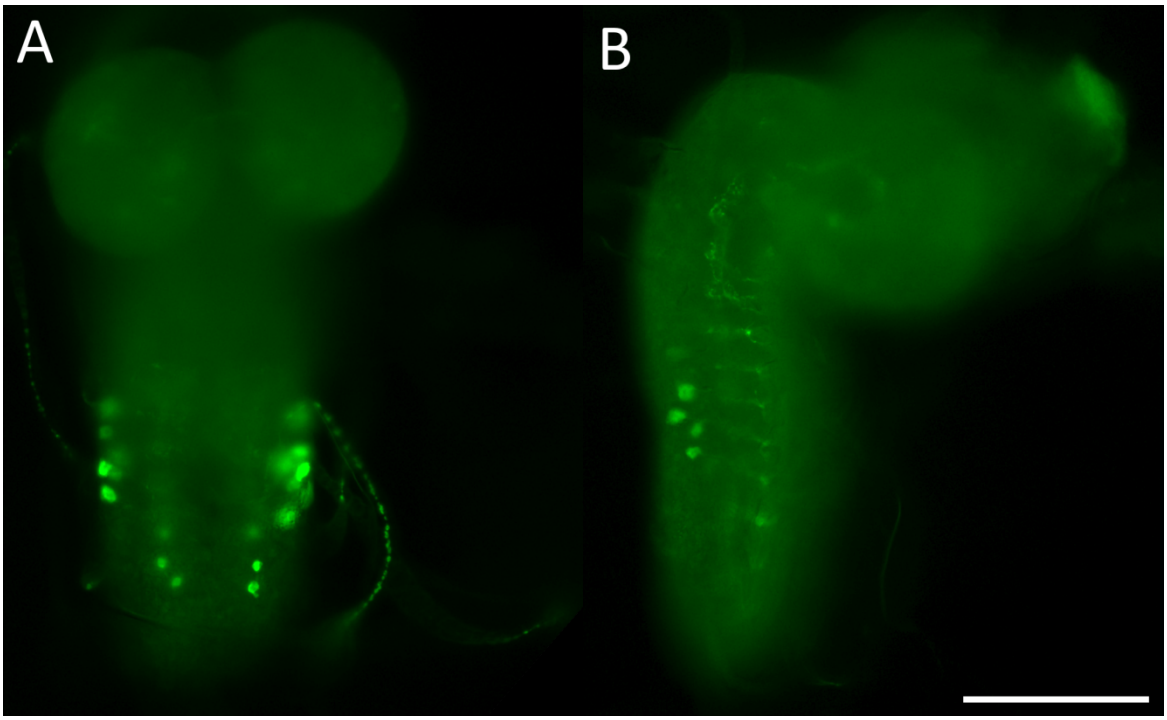

Figure 22: *gHdc-SacIFLAG*$^{57\text{-}6}$ has extra cells with HDC-FLAG on its lateral edges. FLAG immunofluorescence labeling of *gHdc-SacIFLAG*$^{57\text{-}6}$ second instar brain. A) Ectopically expressing lateral cells are visible here. There appears to be at least 6 cells per side. B) The lateral cells seen in the previous figure are shown to be above the 10 histaminergic cell pairs. Z-stack of 6 images. Scale bar = 100 µm.

Conclusions and Discussion

The primary goal of this project was to produce an epitope-labeled HDC protein that is expressed normally in the fly that can be detected in tissue using immunofluorescence labeling. The data presented has shown this to be the case. HDC localization can be detected with this tool. The internally-tagged HDC-FLAG enzyme retains its capability to synthesize histamine. Co-localization with known histaminergic cells was demonstrated, providing evidence that the immunofluorescence seen with FLAG is actually HDC. The co-localization of ectopic HDC-FLAG with ectopic histamine in other derived transgenic lines also provides strong evidence that it is HDC that it is being detected. Subcellular localization of HDC in histaminergic cells can be observed, apparently gathering around the edges of the cells. This should be further explored, utilizing antibodies with specificity against organelles and subcellular structures to determine where in the cell HDC is being stored, paired with confocal microscopy that can also be used to better visualize cells.

Double immunofluorescent labeling techniques were tweaked to allow for immunofluorescent visualization of histamine and FLAG in the same specimen. The double labeling procedure was not without difficulty, however. It required two fixation periods using carbodiimide for histamine and paraformaldehyde for HDC-FLAG. While the signal for histamine appeared unaffected for the most part, the FLAG signal strength was reduced in comparison to a single immunostain (see methods section for details). Despite this, a handful of specimens were able to produce a clear, strong FLAG signal

when double stained with histamine. Because of the disparity of signal strength between the histamine and FLAG immunostains, image editing was occasionally required to ensure that all structures were visible.

A secondary goal was to also demonstrate the use of this new tool for potentially uncovering new locations of HDC in the fly. The adult eye was analyzed, revealing that HDC was punctate, running up and down what appears to be the edges of the photoreceptors. This is a novel observation and different from the predicted outcome- that HDC would be present throughout the cell, localized more diffusely. There is a possibility that this is not real: it could just be an artifact of the detection process. Confocal microscopy will be needed to determine exactly how these punctations are localized in the photoreceptor. These vesicle-like structures seem to be much more numerous in whole mounts of the eye- apparently running along the length of the photoreceptor, while in the tissue sections, they are localized on the edges, indicating that they may only be present in a certain layer of the eye tissue. There are different possibilities as to what this pattern represents. It is a possibility that they are vesicles or clusters of vesicles that transport the HDC protein to where it needs to be. It also needs to be determined whether these packets are mobile or stationary in the photoreceptor. Determining the nature of this localization would be aided by doing double immunostains using antibodies against FLAG and antibody-specific organelles. If HDC-FLAG colocalizes with markers specific to the Golgi or other organelles, the HDC-FLAG localization could be better understood. The photoreceptor localization of HDC-FLAG is the only instance of the FLAG immunofluorescence not matching the histamine

localization. In all other locations examined, HDC-FLAG and histamine where found in the same cells. This may call into play the histamine recycling system of the Tan/Ebony pathway. Ebony expression is found primarily in the photoreceptors and other components of the visual system (Hovemann et al., 1998) and *ine* expression is also only detected in the head of the adult (Romero-Calderon et al., 2007). The different localizations of histamine and HDC-FLAG in the photoreceptors, along with the specific expression of histamine recycling pathway components suggests a different type of histamine regulation in the eye. This is also supported by the exogenous histamine feeding experiments (Melzig et al., 1998), which show recovery in histamine localization in the photoreceptors, but not the brain cells. What other tissues make use of the histamine recycling pathway and why it is needed to supplement HDC could be the subject of a future line of research.

Another major set of analyses made using HDC-FLAG is an addition to the developmental profile of HDC localization in *Drosophila*. Differences between larval instars are minor, with localization patterns being very similar and major differences only being the amount of fluorescence (which may or may not correspond to different amounts of protein being present). Adult localization has been characterized using tissue section analysis. Pupal localization of HDC-FLAG has not yet been attempted. Embryonic analysis has shown no FLAG signal in the ventral nerve cord cells. This does not necessarily mean that there is no HDC there. The method of fixation and permeabilization in embryos differs from the methods used on larval brains. It is possible that the tissue was not fixed or permeabilized properly to allow for

immunofluorescent signal. Further experiments could attempt to optimize these conditions to potentially allow visualization of tissue deeper into the embryo.

In the embryo, there is a stereotyped, segmental staining pattern, consisting of 5 points per segment. From analyzing the arrangement of the points, a hypothesis has been developed that these points are part of the chordotonal organs (Fig. 17). These organs are known to be positioned in clustered arrays (Campos-Ortega & Hartenstein, 1997), matching what was seen in our data. In addition, it has been shown that mechanosensory cells (which are histaminergic) and chordotonal organs share a developmental (and possibly evolutionary) lineage (Merrit, 1997). Known chordotonal organ sites will have to be examined in larvae and adults to acquire evidence for them being histaminergic. Monoclonal antibodies against chordotonal organs exist, enabling future research into this identification of HDc-FLAG localization. There is a possibility that HDC being present in the chordotonal organs could just, in fact, be an evolutionary artifact that does not carry over to later developmental stages, or it could be a cross-reactive protein.

The digestive tract displayed strong staining in an adult tissue sections. That data lead to the attempt of whole mount FLAG immunofluorescent labeling of the larval digestive tracts. All attempts resulted in no discernible difference in fluorescence between the transgenic lines and wild-type in larval digestive tracts. Histamine immunofluorescent labeling was also attempted. There appeared to be no difference between wild-type or $HdcJ^{K910}$, suggesting that histamine has no role at all in the

digestive tract of *Drosophila*. However, the level of intensity of fluorescence is somewhat subjective and there may be a subtle difference in the histamine labeling. A more systematic approach could be attempted, using the same levels of magnification and exposure time, then utilizing an image program to compare intensity levels. There is also the possibility that the immunochemistry utilized in the experiments wasn't properly optimized. The same methods used with the brain were employed. Methods optimized specifically for analyzing the digestive tract should be studied and adapted for future experiments, before any final statement can be made regarding the presence or absence of histamine and/or HDC in the fly digestive tract. There is also the possibility that larval digestive tracts simply don't utilize HDC or histamine. Adult whole mount digestive tracts were not attempted and would likely be the subject of future experiments before trying larval guts again.

With this tool available, there is a wide breadth of research that can be done, aside from the follow-up experiments suggested above. The pupal stage could be analyzed for the presence of HDC, completing the developmental profile. A major use for the HDC-FLAG detection method is Western Blot detections. Previous work in the Burg lab attempted to make use of the 6xHIS-HDC to detect HDC with immunoblots, but was unsuccessful. Success in this area means that the actual size of translated HDC can be determined. Discovering new variants of HDC, made from different paths of post-translational processing could be found using Western Blotting. What functions these variants could have, as well as what tissues they are found in could also be determined

All these points taken together, it is clear that the internal FLAG epitope tag insert in HDC is effective as a tool for detection, enabling many future avenues of research. This is a powerful tool that should greatly help in the study of the fly's histamine biology.

Appendix I: Summary of Transformant Lines

Second Transformation Summary

This table summarizes the chromosomal linkage of analyzed lines.

X Chromosome	2nd Chromosome	3rd Chromosome
11-2	127-1	55-5
55-4	127-2	57-6
62-1		68-1
62-2		68-2
62-3		78-2
62-4		78-5
		79-4
		127-7
		132-7
		132-11
		134-1
		134-3

The following table is a summary of the lines used in experiments. Information on which chromosome the transgene was inserted into, whether or not the immunofluorescence pattern was typical or ectopically localized, brief details on the immunofluorescence and whether or not the line was put into an *Hdc*^{JK910} background is included.

Line Designation	Chromosome	Typical or Ectopic	Details	*Hdc*^{JK910} background?
135-1	third	Typical	Relatively strong signal. Benchmark for typical localization pattern.	Yes
78-5	third	Typical	Strongest signal of all typical lines. Flies may have problems with balance and survivability.	Yes, although most experiments done with it have not used the *Hdc*^{JK910} background.
79-4	third	Typical	Signal is weaker than	Yes.

			135-1, probably the weakest of all lines acquired.	
127-7	third	Typical	Signal slightly weaker than 135-1.	Yes.
62-3	first	Typical	Slightly weaker signal than 135-1.	No.
55-4	X	Aberrant	Has two extra pairs of cells near the central brain. These cells stain very lightly. This line may still be used for experiments despite this.	Yes.
6-1	X	Aberrant	Very dramatic atypical localization in segmented neuropil on the ventral ganglia. Staining in nerves. Normal 11 histaminergic cell pairs have greatly reduced signal.	No.
57-5	third	Aberrant	Very bright lateral cells. HDC-FLAG in nerves.	No
132-6	third	Aberrant	Extra cells on the lateral edges of the ventral ganglia, posterior end of ventral ganglia and above the typical 11 histaminergic cell pairs.	No.
11-2	third	Null	No signal. Anti-FLAG stain appeared as	No.

			wild type.	
3-1	first	Null	No histamine present, as HDC^{JK910}.	Yes.
4-3	third	Null	No histamine present, as HDC^{JK910}.	Yes.
35-1	third	Null	No histamine present, as HDC^{JK910}.	Yes.
39-2	third	Null	No histamine present, as HDC^{JK910}.	Yes.
49-1	third	Null	No histamine present, as HDC^{JK910}.	Yes.

Appendix II: Solutions

PBS

1.48 g $Na_2HPO_4 \cdot H_20$

0.43 g $KH_2PO_4 \cdot H_20$

7.2 g NaCl

pH 7.4, bring to 1000 mL with dH20

TBS

0.5 M Tris-HCl pH 7.4

PBS (See above)

1 part Tris-HCl + 9 parts PBS, bring to pH 7.4

1% Normal Goat Serum (50 mL)

0.5 mL 100% Normal Goat Serum

1.5 mL 10% Triton X-100

48.0 mL TBS

5% Normal Goat Serum (10 mL)

0.5 mL 100% Normal Goat Serum

0.3 mL 10% Triton X-100

9.6 mL TBS

Drosophila Ringer's Solution (1 L)

0.249 Anhydrous CaCl2

13.6 g KCl

2.7 g NaCl

1.21 Tris Base

1 M HCl to adjust pH to 7.2

4% Paraformaldehyde Solution

1. Weigh out 4.0 g of granulated paraformaldehyde.
2. Pour 40 mL of distilled H_2O into a 200 mL Erlenmeyer flask. Place a small stir bar inside and heat, while stirring on a hot plate. Set hot plate to heat level 3. Place a temperature probe into the beaker.
3. When temperature reaches 60° C, add the paraformaldehyde to the flask.
4. Stir 5 minutes.
5. Add, at roughly 300 µL increments, 1 M NaOH to the flask. Wait several minutes between each addition and observe the flask to see how much of the granulated paraformaldehyde has dissolved. (Typically it takes at least 1 mL of NaOH to dissolve all of it for me.) Be patient. You do not want to increase the pH of the solution too much.
6. When all or most of the paraformaldehyde has dissolved, turn off the heat. Place the flask on ice to cool it down to room temperature.

7. While waiting for the contents of the flask to cool, mix 11.5 mL of Solution A (0.1 M KH_2PO_4) with 38.5 mL of Solution B (0.1 M Na_2HPO_4). This is the buffer solution.
8. After flask contents have cooled, add it to the buffer solution in the graduated cylinder. Place a piece of parafilm over the cylinder and invert to mix (or alternatively, add the buffer solution to the flask and swirl to mix.) Return to graduated cylinder. Bring to 100 mL with dH_2O.
9. Take the pH and record. It should be around 7.0.
10. Filter the paraformaldehyde solution using a 0.5 micron filter and a syringe. Filter will need to be replaced at least once or twice.
11. Keep paraformaldehyde on ice or at 4° C until ready to use.

4% Carbodiimide Fixative Solution

1. Put 0.50 g of carbodiimide (stored at -20° C) into a conical falcon tube.
2. Add 3 mL of distilled H_2O to the tube. Invert the tube gently to dissolve. Add more water if this has caused the total volume to drop to bring to 3 mL.
3. Add an additional 0.25 mL of distilled water to bring the total volume to 3.25 mL.
4. Add 5 mL of 0.1 M Na_2HPO_4. Invert gently to mix.
5. Bring to 12.5 mL with 0.1 M KH_2PO_4.
6. Check pH with a test strip. Ensure that pH is around 7.0.
7. Keep on ice. Fixative solution should be used within 12 hours of making it.

Appendix III: Multiple Sequence Alignment of HDC

Multiple sequence alignment of the histidine decarboxylase amino acid sequences of the following:

Rattus norvegicus (NP_058712.2)

Mus musculus (NP_032256.3)

Homo sapiens (NP_002103.2)

Drosophila melanogaster Isoform B (AGB93388.1)

```
CLUSTAL O(1.2.1) multiple sequence alignment


gi|440214257|gb|Fly|        ----MDF----KEYRQRGKEMVDYIADYLENIRERRVFPDVSPGYMRQLLPESAPIEGEP
gi|92110055|ref|Human|      MMEPEEY-------RERGREMVDYICQYLSTVRERRVTPDVQPGYLRAQLPESAPEDPDS
gi|158186734|ref|Rat|       MMEPSEYREY----QARGKEMVDYICQYLSTLRERQVTPNVKPGYLRAQIPSSAPEEPDS
gi|28173556|ref|Mouse|      MMEPCEYREYREYYRARGKEMVDYISQYLSTVRERQVTPNVQPGYLRAQLPASAPEEPDS
                                 ::       : **:******.:**..:***:* *:*.***:*  :* *** : :

gi|440214257|gb|Fly|        WPKIFSDVERIVMPGITHWQSPHMHAYFPALNSMPSLLGDMLADAINCLGFTWASSPACT
gi|92110055|ref|Human|      WDSIFGDIERIIMPGVVHWQSPHMHAYYPALTSWPSLLGDMLADAINCLGFTWASSPACT
gi|158186734|ref|Rat|       WDSIFGDIEQIIMPGVVHWQSPHMHAYYPALTSWPSLLGDMLADAINCLGFTWASSPACT
gi|28173556|ref|Mouse|      WDSIFGDIERVIMPGVVHWQSPHMHAYYPALTSWPSLLGDMLADAINCLGFTWASSPACT
                            * .**.*:*:::***:.**********:***.* **************************

gi|440214257|gb|Fly|        ELEIIVMNWLGKMIGLPDAFLHLSSQSQGGGVLQTTASEATLVCLLAGRTRAIQRFHERH
gi|92110055|ref|Human|      ELEMNVMDWLAKMLGLPEHFLHHHPSSQGGGVLQSTVSESTLIALLAARKNKILEMKTSE
gi|158186734|ref|Rat|       ELEMNIMDWLAKMLGLPDFFLHHHPSSQGGGVLQSTVSESTLIALLAARKNKILEMKAHE
gi|28173556|ref|Mouse|      ELEMNIMDWLAKMLGLPEYFLHHHPSSRGGGVLQSTVSESTLIALLAARKNKILAMKACE
                            ***: :*:**.**:***: ***   .*:******:*.**:**:.***.*.. *  ::  .

gi|440214257|gb|Fly|        PGYQDAEINARLVAYCSDQAHSSVEKAALIGLVRMRYIEADDDLAMRGKLLREAIEDDIK
gi|92110055|ref|Human|      PDADESCLNARLVAYASDQAHSSVEKAGLISLVKMKFLPVDDNFSLRGEALQKAIEEDKQ
gi|158186734|ref|Rat|       PNADESSLNARLVAYASDQAHSSVEKAGLISLVKIKFLPVDDNFSLRGEALQKAIEEDKQ
gi|28173556|ref|Mouse|      PDANESSLNARLVAYTSDQAHSSVEKAGLISLVKIRFLPVDDNFSLRGEALQKAIEEDKQ
                            *  ::: :******* ***********.**.**::::: .**::::**: *::***:* :

gi|440214257|gb|Fly|        QGLVPFWVCATLGTTGSCSFDNLEEIGIVCAEHHLWLHVDAAYAGSAFICPEFRTWLRGI
gi|92110055|ref|Human|      RGLVPVFVCATLGTTGVCAFDCLSELGPICAREGLWLHIDAAYAGTAFLCPEFRGFLKGI
gi|158186734|ref|Rat|       QGLVPVFVCATLGTTGVCAFDKLSELGPICAREGLWLHVDAAYAGTAFLCPELRGFLKGI
gi|28173556|ref|Mouse|      QGLVPVFVCATLGTTGVCAFDRLSELGPICASEGLWLHVDAAYAGTAFLCPELRGFLEGI
                            :****.:********* *:** *.*:* :** . ****:******:**:***:* :*.**

gi|440214257|gb|Fly|        ERADSIAFNPSKWLMVHFDATALWVRDSTAVHRTFNVEPLYLQHENSGVAVDFMHWQIPL
gi|92110055|ref|Human|      EYADSFTFNPSKWMMVHFDCTGFWVKDKYKLQQTFSVNPIYLRHANSGVATDFMHWQIPL
gi|158186734|ref|Rat|       EYADSFTFNPSKWMMVHFDCTGFWVKDKYKLQQTFSVNPIYLRHANSGVATDFMHWQIPL
gi|28173556|ref|Mouse|      EYADSFTFNPSKWMMVHFDCTGFWVKDKYKLQQTFSVNPIYLRHANSGAATDFMHWQIPL
                            * ***::******:*****.*.:**:*.  :::**.*:*:**:* ***.*.*********

gi|440214257|gb|Fly|        SRRFRALKVWFVLRSYGIKGLQRHIREGVRLAQKFEALVLADHRFELPAKRHLGLVVFRI
gi|92110055|ref|Human|      SRRFRSVKLWFVIRSFGVKNLQAHVRHGTEMAKYFESLVRNDPSFEIPAKRHLGLVVFRL
gi|158186734|ref|Rat|       SRRFRSIKLWFVIRSFGVKNLQAHVRHGTDMAKYFESLVRSDPVFEIPAERHLGLVVFRL
gi|28173556|ref|Mouse|      SRRFRSIKLWFVIRSFGVKNLQAHVRHGTEMAKYFESLVRSDPSFEIPAKRHLGLVVFRL
                            *****::*:***:**:*:* ** *:*.*. :*: **:**  *  **:**:*********:

gi|440214257|gb|Fly|        RGDNEITEKLLKRLNHRGNLHCIPSSLKGQYVIRFTITSTHTTLDDIVKDWMEIRQVAST
gi|92110055|ref|Human|      KGPNCLTENVLKEIAKAGRLFLIPATIQDKLIIRFTVTSQFTTRDDILRDWNLIRDAATL
```

```
gi|158186734|ref|Rat|       KGPNCLTESVLKEIAKTGQVFLIPATIQDKLIIRFTVTSQFTTKDDILRDWNLIREAANL
gi|28173556|ref|Mouse|      KGPNCLTESVLKEIAKAGQLFLIPATIQDKLIIRFTVTSQFTTKEDILRDWHLIQEAANL
                            :* * :**.:**.: : *.:. **:::: : :****:** .** :**::**  *::.*.

gi|440214257|gb|Fly|        VLEEMNITISNRVY---LK-ETKEKNEAFGSSLLLSNSPLSPKVVNGSFAAIFDADEFLA
gi|92110055|ref|Human|      ILSQHCTSQPSPRVGNLISQIRG--ARAWACGTSL-------QSVSGAGDDPVQARKI--
gi|158186734|ref|Rat|       VLSQHCTSQPSPRAKNLIPPPVTRDSKDLTNGLSL-------ESVNEGGDDPVQVRKI--
gi|28173556|ref|Mouse|      VLSQHCTSQPSPRAKNVIPPPPG------TRGLSL-------ESVSEGGDDPAQARKI--
                            :*.:   :  .     :             .  *       : *. .     :. ::

gi|440214257|gb|Fly|        KTYAGVRIAHQESPSMRRRVRGILMSGKQFSLDSHMDVVVQTTLDAGNGATRTSTTNSYG
gi|92110055|ref|Human|      -----IKQPQRVGAGPMKRENGLH-------LETLLDPVDDCFSEEAPDATKH-KLSSF-
gi|158186734|ref|Rat|       -----FRLPG---DS----------------LETTMDPFDDCFSEEASDTTKH-KLSSF-
gi|28173556|ref|Mouse|      -----IKQPG---ASLARREGGSD-------LETMPDPFDDCFSEEAPNTTKH-KLSSF-
                                 .:       .               *::  * . :   : .  :*:  . .*:

gi|440214257|gb|Fly|        HTTSAAQANSERQASIQEDNEESPEETELLSLCRTSNVPSPEHAHSLSTPSRSCSSSSHS
gi|92110055|ref|Human|      ----------------------------LFSYLSVQT-------KKKTVRSLSCNSVPVS
gi|158186734|ref|Rat|       ----------------------------LFSYLSVQN-------KKKTMRSLSCNSMPMS
gi|28173556|ref|Mouse|      ----------------------------LFSYLSVQN-------RRKTTRSLSCNSVPMS
                                                      *:*   ...       :  :  * **.*   *

gi|440214257|gb|Fly|        LIHSLTQSSPRSSPV---NQFRPITLCAVPSQSQLSMPLAMPLPNRNVTVSVDSLLNPVT
gi|92110055|ref|Human|      AQKP----LPTEASVKNGGSSRVRIFSRFPEDMMMLKKSA--FKKLIKFYSVPS----FP
gi|158186734|ref|Rat|       AQKS----PPPDASVKHGGFFRARIFSGFPEEMMMMKKGG--FKKLIKFYSVPS----FP
gi|28173556|ref|Mouse|      AQKS----LPADASLKNGGSFRARIFSGFPEQMMMMKKGA--FKKLIKFYSVPS----FP
                              :      * .: :      *   :. .*.:  :    .  : :     ** *    .

gi|440214257|gb|Fly|        TCNVYHGKRF--LEPLENLAQTSASFSSSIFRLPTPIATPTRESPEDPDWPAKTFSQLLL
gi|92110055|ref|Human|      ECSSQCG-LQLPCCPLQAMV----------------------------------------
gi|158186734|ref|Rat|       ECSSQCGTLQLPCCPLQAMV----------------------------------------
gi|28173556|ref|Mouse|      ECSSQCAR-QLPCCPLEAMV----------------------------------------
                             *.   .       **: :.

gi|440214257|gb|Fly|        ERYSSQSQSLGNNSSTESSSLSGGATPTPTPMSSLDELVTPLLLSFASPSQPMLSAHGIG
gi|92110055|ref|Human|      ------------------------------------------------------------
gi|158186734|ref|Rat|       ------------------------------------------------------------
gi|28173556|ref|Mouse|      ------------------------------------------------------------


gi|440214257|gb|Fly|        EGQREQGSDSDATVCSTTSSMESL
gi|92110055|ref|Human|      -------------------------
gi|158186734|ref|Rat|       -------------------------
gi|28173556|ref|Mouse|      -------------------------
```

Appendix IV: Cross Schemes

The following cross schemes detail how linkage was determined, as well as how lines were put into an *HdcJK*^{910} background.

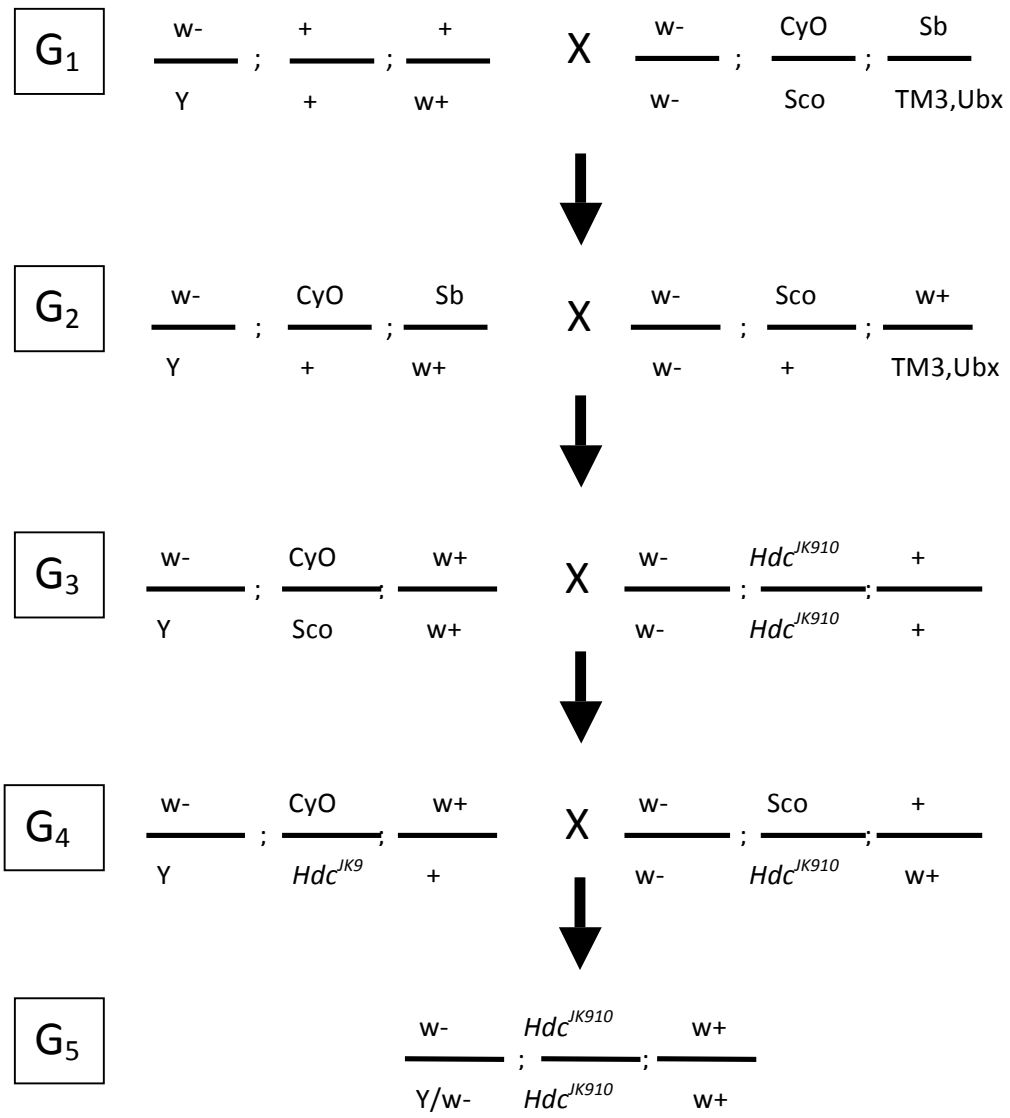

Figure A1: Cross scheme used to identify linkage of transgenic inserts and introduce it into a HDC^{JK910} background. The transgene is indicated as w^+ (referring to the *mini-white* marker followed in the cross scheme). G1 and G2 demonstrate the cross of a hypothetical insert on the third chromosome with a multiply marked balancer stock. The markers that remain after *mini-white* containing flies emerge determine what chromosome the transgene is on. G3, G4 and G5 demonstrate the subsequent cross scheme of introducing the transgene into an Hdc^{JK910} background.

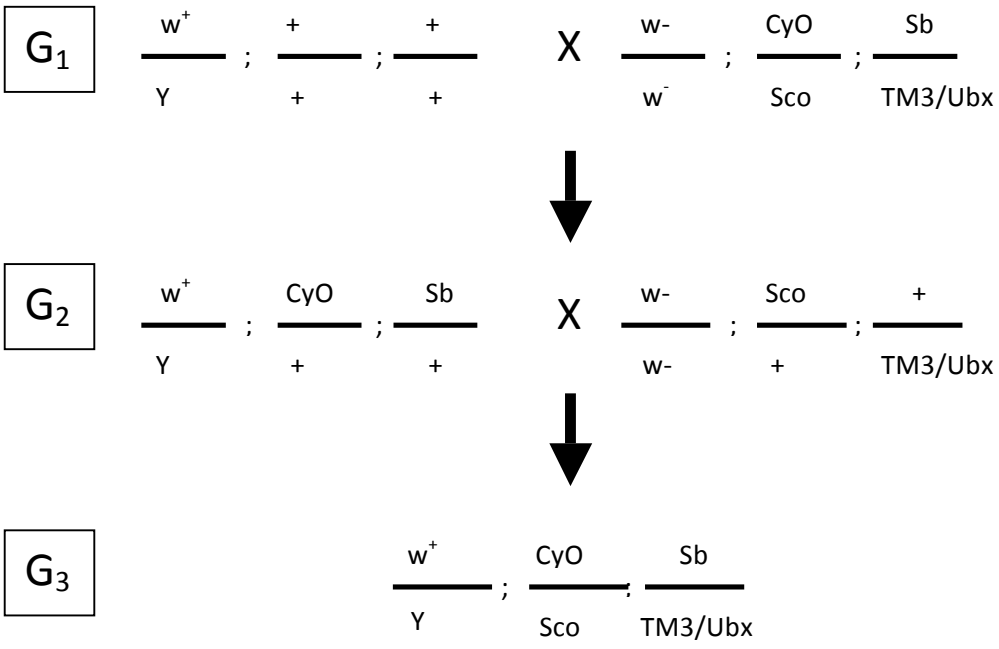

Figure A2: Cross scheme for determining if the insert is on the first chromosome. If all markers are present in the fly and *mini-white* is still present, it can be concluded that the insert is on the X chromosome. In addition, male flies will always have darker eye color if the insert is on the X.

Glossary

Abbreviation	Meaning
Carbodiimide	1-ethyl-3(3-diamethylaminopropyl)-carbodiimide
DDC	Dopa Decarboxylase
HDC-FLAG	Refers to the HDC construct with the *SacI* FLAG insert
HDC	Histidine decarboxylase protein
Hdc	Histidine decarboxylase gene
NGS	Normal goat serum
PBS	Phosphate-buffered saline
TBS	Phosphate-buffered saline with Tris

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