IDENTIFICATION OF DROSOPHILA DOPAMINERGIC NEURAL CIRCUITS USING MARCM

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By

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Abstract

Parkinson's disease is a devastating, progressive neurodegenerative disorder, characterized by degeneration of the dopaminergic neurons and the presence of Lewy bodies in the basal ganglia of the midbrain. The disease results in disordered movement such as tremors, bradykinesia, rigidity, and postural instability. Animal models of Parkinson's disease currently offer a way to study the disease's causes, progression, and possible treatments, much of which is currently unknown. The *Drosophila melanogaster* model is particularly useful, because fruit flies develop Parkinson's symptoms and respond to treatment much like humans do. However, knowledge about Parkinson's disease mechanisms in *Drosophila* is relatively limited, just as it is for the human disease. There are several dopaminergic neural circuits in *Drosophila*, and the cell bodies are arranged in several distinct clusters. A closer look at disease progression in the individual clusters, as well as a better understanding of the normal functioning of the clusters, could open doors to more advanced research in the *Drosophila* model. This study seeks to do just that: to single out individual DA neuron clusters in *Drosophila* for more specified study. The technique used to accomplish this goal is MARCM (mosaic analysis with a repressible cell marker). In this study, I attempt to develop the MARCM technique for use in targeting specific dopaminergic neuron clusters, so that it can be reliably used in future Parkinson's disease research.

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Introduction

Basic Human Neurology

The human brain is composed of three main parts: the brainstem, cerebellum, and cerebrum (Nolte, 2009). The cerebrum is the largest part of the brain and is divided into two hemispheres by the longitudinal fissure. The corpus callosum, a huge nerve fiber bundle, connects the two hemispheres. The outermost part of the cerebrum is called the cerebral cortex. The cortex is covered in ridges called gyri and grooves called sulci which increase the overall surface area, and therefore space for nerve cells and connections. The cortex is subdivided by distinctive sulci into the frontal, parietal, temporal, occipital, and limbic lobes. The main roles of the frontal lobe are motor initiation and cognition, the parietal lobe is involved in sensory perception, the temporal lobes are involved in hearing, the occipital lobe is involved in vision, and the limbic lobe is connected with the limbic system, which is important for emotional responses, drive-related behavior, and memory (Nolte, 2009). The brainstem, which lies inferior to the cerebrum, is responsible for transmitting information to and from the spinal cord. It also is the command center for many basic bodily functions, such as heartbeat and breathing rate. The brainstem is divided into the midbrain, pons, and medulla (superior to inferior). The cerebellum, which lies behind the brainstem and beneath the cerebrum, is crucial for the fine-tuning of motor commands from the frontal lobe, as well as coordinating repetitive movements like walking. Altogether, the human brain is incredibly large for the human body size. This size helps to explain the immense cognitive capacities of human beings relative to other vertebrates.

The brain and spinal cord together make up the central nervous system (CNS), while the cranial nerves and spinal nerves which exit and enter the CNS structures to innervate the body make up peripheral nervous system (PNS) (Funk & Wagnalls, 2015). The nervous system is also divided into two main functional systems, the somatic and autonomic. While the somatic system controls the skeletal muscles and sensations which we are consciously aware of (somatosensation), the autonomic system controls muscle actions and sensations that are visceral and therefore not susceptible to conscious control (e.g. the respiratory, circulatory, digestive, and urogenital systems). That autonomic division is subdivided into the sympathetic and parasympathetic systems, which coordinate stress responses and rest/normal functioning, respectively (Funk & Wagnalls, 2015).

The nervous system is composed of a wide variety of cell types, but the types can be divided into two main categories – nerve cells (neurons) and neural glia (Funk & Wagnalls, 2015). The glial cells are the most numerous and are responsible for the protection, nourishment, and up-keep of the neurons. Neurons are the cells which receive stimuli, transmit electrical impulses, and activate effectors such as muscle cells. There are two main types of neurons: efferent, which transmit motor commands away from the CNS, and afferent, which transmit sensory information towards the CNS. Neurons each contain a soma/cell body, dendrites to receive information from other neurons, and axons to send information to other neurons.

The transmission of a signal along an axon is done through an action potential, which is coordinated through the opening and closing of sodium and potassium ion

channels. However, the transmission of information across a synapse is coordinated by neurotransmitters, chemicals that relay information from the terminal of an axon to the dendrites on another neuron (*Neurogistics,* 2015). Some neurotransmitters, such as serotonin and GABA, are inhibitory, and prevent the initiation of an action potential in the post-synaptic neuron. Other neurotransmitters, such as epinephrine and norepinephrine, are excitatory, and stimulate the initiation of action potentials. Dopamine, which is discussed further below, is one of many neurotransmitters which can act as both excitatory and inhibitory. The excitatory or inhibitory nature of a neurotransmitter is not determined by the chemical structure of a neurotransmitter, but rather that of the receptor which binds it on a given post-synaptic neuron or effector (*Neurogistics,* 2015).

Dopamine

Dopamine (DA), also known as dihydroxyphenylethylamine, is a catecholamine neurotransmitter which serves a variety of functions in the central nervous system, such as movement initiation, cognition, attention, reward, reward anticipation, addiction, and stress (Avramut, 2015). Dopamine also plays a role as a hormone in kidney function, heart function, breast milk flow cessation, and nausea regulation, and it acts as an antipsychotic agent (Brookshire, 2013).

Neurons which produce dopamine are called dopaminergic neurons. These neurons only exist in a few places in the brain, mainly in the substancia nigra of the midbrain. However, they establish connections with numerous other brain areas

(Avramut, 2015). For example, dopaminergic neurons exert influence over movement initiation through associations with the motor cortex in the frontal lobe (Brookshire, 2013). Additionally, DA neurons play a role in cognition through associations with the prefrontal cortex. The DA pathway that is heavily involved in reward, reward anticipation, and addiction is called the mesolimbic pathway. This pathway begins with DA neurons in the ventral tegmental area of the midbrain, and sends projections to the nucleus accumbens and the cortex (Brookshire, 2013). Due to associations with the cerebral cortex and the limbic systems, dopamine plays a huge role in learning and memory, particularly in learning that is reward-driven (Avramut, 2015).

Parkinson's Disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder marked by the death of dopaminergic neurons. It is the 14th leading cause of death in the United States (Murphy, Xu, & Kochanek, 2013), and the second most common neurodegenerative disorder after Alzheimer's disease (De Lau & Breteker, 2006). The major clinical manifestations of the disease can be summarized by the TRAP acronym, which stands for tremors, rigidity, akinesia, and postural instability (Frank, Pari, & Rossiter, 2006). Diagnosis of Parkinson's disease based on these symptoms can be challenging: about 15% of patients diagnosed with PD do not actually fulfill the correct pathological criteria, and 20% of diagnosed PD cases had been missed upon a previous examination.

Additional motor symptoms of PD include micrography (changes in handwriting), changes in gait, and hypomimia (masklike face which causes less frequent blinking and drooling). PD patients also develop dementia, but typically only after the disease has increased in severity for a number of years. Many non-motor symptoms have also been discovered, including impaired olfaction, constipation, and disordered sleep (Shulman, Jager, & Feany 2011). Interestingly, these symptoms can manifest up to 20 years before the major motor symptoms begin.

Parkinson's disease is characterized by the death of dopaminergic neurons in the basal ganglia of the midbrain, particularly in the substancia nigra (figure 1). The basal ganglia are deep nuclei which assist in the initiation and execution of movement (Shulman, Jager, & Feany, 2011).

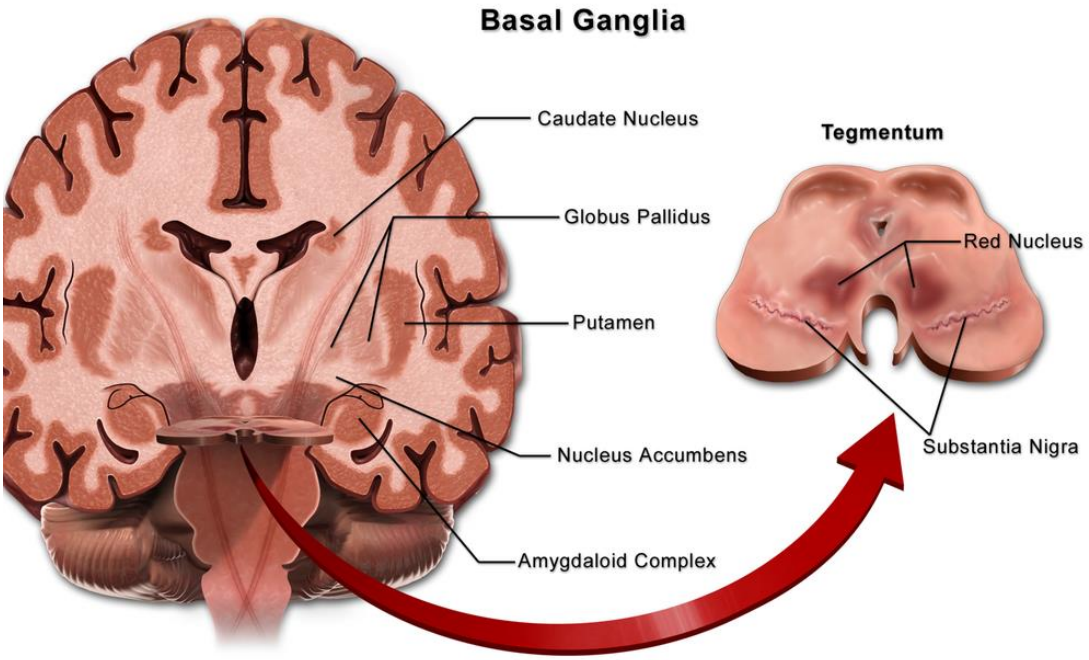


Figure 1: Basal ganglia (Blaunsen.com staff, 2014)

The degeneration of the dopaminergic (DA) neurons in the basal ganglia is accompanied by the presence of Lewy Bodies, which are abnormal aggregates of filamentous proteins (Spillantini et. al, 1998). These Lewy bodies are also found in individuals with Lewy Body dementia, but they form in the cerebral cortex rather than in the basal ganglia. Parkinsonianism (PD-like symptoms, not necessarily caused by PD) often occurs in people with Lewy Body dementia, but is relatively mild (Frank, Pari, and Rossiter, 2006). In both diseases, the protein α-synuclein can be found in the Lewy bodies (Spillantini et. al, 1998). A point mutation in the gene for α-synuclein has been found as a cause for the rare familial form of Parkinson's disease. In fact, several causative monogenetic mutations have been found (Blau & Greteler, 2006). However, 90% of cases seem to be sporadic, rather than hereditary.

In addition to genes which cause mutations in the alpha-synuclein protein, other PD-associated genes affect pathways involved in mitochondria function, morphology, and dynamics (Winklhofer, Haass, 2002). For example, in the 1980's, it was found that an inhibitor of complex I in the electron transport chain could induce Parkinsonism. Mitochondrial dysfunction can be found in both sporadic and genetic cases of PD, and is a central component of the disease's pathogenesis.

Current PD Therapies

Currently, the most effective therapy for Parkinson's disease is Carbidopa/Levodopa (L-dopa). No drugs have been developed which can slow or halt the progression of PD, but L-dopa can help to relieve symptoms (Xia & Mao, 2012).

L-dopa, a dopamine precursor, is taken up by dopaminergic neurons and undergoes decarboxylation at the synaptic terminal. Carbidopa is combined with L-dopa to prevent L-dopa from converting to dopamine before it passes the blood brain barrier by inhibiting the enzyme which catalyzes L-dopa's decarboxylation. This allows for amplification of L-dopa delivery to the central nervous system and decreases the adverse effects of dopamine in circulation (Münchau & Bhatia, 2000).

Although it offers many benefits, L-dopa as a PD therapy is far from perfect. First, it only relieves symptoms for 5-10 years, after which it causes serious motor complications, mainly dyskinesia (random, involuntary movement). Also, as the DA neurons disappear, a smaller number of DA neuron terminals results in less storage space for the drug, resulting in severe symptom fluctuations (Münchau & Bhatia, 2000). Additionally, L-dopa has little effect on certain motor symptoms such as gait and balance problems, as well as non-motor symptoms such as dementia, disordered sleep, and mood fluctuations (Sethi, 2008).

Although several PD-related genes have been found and many mechanisms of the disease's pathogenesis have been identified, the cause of death of the dopaminergic neurons still remains largely a mystery. It is also currently unknown how the disease's effects spread beyond the basal ganglia to affect other brain functions in the disease's later stages. With little understanding of the disease, we are left with imperfect therapies and no permanent treatments or cures. A vast amount of research is currently being performed in an attempt to combat this disease.

Animal Models of PD

For decades, animal models of PD have allowed researchers to study the cellular and molecular pathology of the disease. The animals used include mice, rats, *Drosophila*, *C. elegans*, zebrafish, and primates (Blandini & Armentero, 2012). Parkinsonism has classically been elicited in these animal models through either systemic or local administration of neurotoxins known to cause PD symptoms. In the past decade, a number of transgenic models of PD have also been used to mimic familial, rather than sporadic, PD. The toxin models only allow for late-stage PD symptom research, but they offer a means by which to test pharmacological treatment of those symptoms (Blesa et. al, 2006). Transgenic models can better model early stages of the disease, but still do not fully demonstrate the complexity of human PD. Systemically-administered toxins used for animal models include 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and the pesticides rotenone and paraquat (Blandini & Armentero, 2012). Locally administered neurotoxins include 6-hydroxydopamine (6-OHDA) and lipopolysaccharide. Transgenic models include α-synuclein (mutant genes A53T or A30P), leucine-rich repeat serine/threonine kinase 2 (LRRK2), PTEN-induced putative kinase 1 (PINK1), parkin, and DJ-1.

Drosophila Model of PD

The use of *Drosophila melanogaster* to study PD offers many benefits over other animal models. *Drosophila* have a very short generation time and lifespan, and have, consequentially, rapid disease progression. This rapid disease progression and

high reproduction rate provide researchers with vast amounts of data, and allows genetic manipulations to be done relatively quickly.

The most developed transgenic PD models in *Drosophila* are A53T and A30P, which produce a mutated form of human α-synuclein, a protein found in Lewy bodies (Feany and Bender, 2000). These models allow for an analysis of pathogenesis, which is not as obtainable from the toxin models. In has been found that old, but not young, A30P flies exhibit walking abnormalities (a variety of features which are analogous to bradykinesia in humans), as well as centrophobism (a sign of anxiety, which presents in 40-69% of PD patients) (Chen et. al, 2014). Additionally, an *in vitro* model of PD in *Drosophila* demonstrated that the presence of defective human α-synuclein in primary *Drosophila* neuronal cultures resulted in the death of DA neurons and the formation of cellular aggregations (Park & Lee, 2006).

Mosaic Analysis with a Repressible Cell Marker (MARCM)

In order to better understand the *Drosophila* model of PD, it is necessary to understand the physiology of the *Drosophila* DA neuronal circuits. The *Drosophila* brain is highly complex and organized with thousands of neurons, on the order of 10^5 (Blanco et. al, 2011). The DA neurons exist in bilaterally symmetrical, spatially arranged clusters, which are named based on their anatomical location and axonal positioning. Little is known about the functional differences of these different DA clusters, as well as the ways that PD affects the individual clusters. This study seeks to

add to that knowledge through a technique called MARCM (mosaic analysis with a repressible cell marker).

MARCM is a genetic technique which allows for the generation of labelled clones (either single cells or multiple cells sharing a single progenitor) within an animal (Wu & Luo, 2006). These marked cells are homozygous for certain genes in an otherwise heterozygous, marker-suppressed animal. The genome of the animal contains a transgene or marker of interest, but each cell also contains one copy of a gene which represses the marker's driver. The homozygous clones do not have a copy of the repressor, so they are the only cells in the organism which express the mutant gene or marker. This homozygosity is achieved in the clones through mitotic recombination, driven by heat-shock activation of the gene for the heat-shock flippase protein (hs-FLP). FLP catalyzes double strand DNA breaks and recombination at flippase recognition target (FRT) sites, which are located near the centromeres of both the chromosome that contains the repressor gene and its homolog.

To generate labelled clones in a MARCM-testable animal, heat-shock is applied to the developing embryo (Wu & Luo, 2006). During this time, the FLP/FRT-mediated recombination will occur in the cells which are mitotically active, or undergoing rapid development. After DNA replication and chromosome condensation in the cell, heat-shock can induce DNA breaks at any of the four FRT sites (2 sister chromatids for each of 2 homologous chromosomes). When a chromatid from either homolog recombines with a chromatid from another homolog, the resulting chromosomes in the daughter cells will no longer be heterozygous, but rather,

homozygous for the genes located distal to the FRT site. Therefore, one daughter cell will be homozygous for the repressor, and will still be phenotypically marker-suppressed. The other daughter cell will be homozygous with no repressor, and the transgene or cell label used will be expressed in that cell as well as its mitotic progeny. The major uses of MARCM include lineage analysis, gene function investigation in single or small populations of cells, and neuronal circuit tracing (Wu & Luo, 2006).

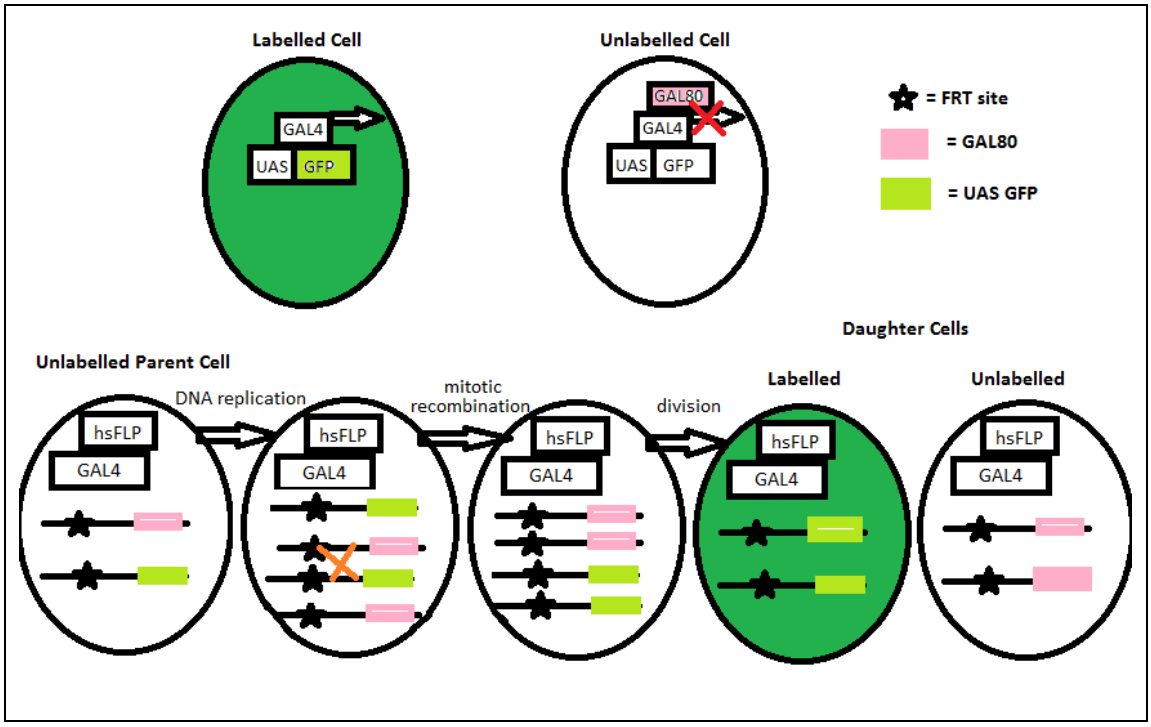


Figure 2: General MARCM Process

Experimental Goals

The goal of this study was to use MARCM to target individual DA neuron cells or cell clusters in *Drosophila* larvae. The hypothesis was that, through experimental trials, optimum heat-shock timing (developmental timing and duration of

heat shock) that would reliably induce mitotic recombination in each of the DA neuron clusters could be found.

Through a series of crosses, a MARCM-testable *Drosophila* line was generated. The marker gene used was UAS- mCD8::GFP, which labels the neurolemma with green fluorescent protein (GFP). The driver used for this gene was TH-GAL4. GAL4 is a yeast protein which is inserted into the *Drosophila* genome to act as a transcriptional activator (Brand & Perriman, 1993). The UAS promoter contains GAL4 binding sites. Therefore, GAL4 is necessary to drive any UAS-promoted transgenes, including UAS-mCD8::GFP. In this study, TH-GAL4 was used because the *Drosophila* TH (tyrosine hydroxylase) gene is uniquely expressed in DA neurons (Grelin et. al, 2003). TH is the enzyme that catalyzes the rate-limiting step in dopamine biosynthesis. Consequentially, TH-GAL4 is only expressed in DA neurons, and mitotically-recombined clones in this study only expressed mCD8::GFP if they were DA neurons. The repressor used was tubP-Gal80, which potently represses the activity of GAL4 (Wu & Luo, 2006). One copy of hs-FLP was incorporated into the genome as well, and the mCD8::GFP and GAL80 genes existed on homologous chromosomes distal to FRT sites.

Materials and Methods

Fly Maintenance

Drosophila melanogaster fruit flies were raised and kept in bottles containing media made of the following ingredients:

Ingredients	Gram/liter ddH_2O
Dry yeast	21
Agar	9
Dextrose	48.75
Sucrose	21
Cornmeal	60

Additionally, the media contained 0.4% propionic acid as a preservative (Podolsky, 2015). The flies were grown at 20-25°C and were transferred to new bottles on a weekly basis.

Egg Collection

Eggs for MARCM experimentation were collected on the lids of CytoOne 35mm culture dishes. About 3mL media, taken from empty bottles and melted in a microwave, were poured into these dishes. Yeast paste was lightly smeared over the surface of the media to promote egg-laying. The desired crossing adults were

transferred to new bottles without media, inverted over the dishes and left in a 22°C incubator for one hour at a time. The bottles contained roughly 150-200 females and 50-100 males. Since developmental stages of the larvae needed to be as synchronized as possible, egg-laying was limited to one hour in order to allow for the collection of a reasonable number of eggs that were minimally varied in age.

After egg collection, adult flies were returned to their original bottles and any dead or trapped adults were removed from the dish and discarded. Egg-laying plates were then stores in a 22°C incubator until heat shock.

Heat Shock

Eggs were heat-shocked either 3, 5, 7, 9, 11, 13, or 15 hours after egg laying (AEL). The hours were counted starting at the initiation of egg collection, rather than its completion, so eggs heat-shocked at 3 hours AEL varied in age from 2-3 hours, eggs heat-shocked at 5 hours AEL varied in age from 4-5 hours, and so on. The egg-laying plates, lightly moistened with distilled water to prevent drying, were placed in lidded petri dishes and immersed in a 37°C water bath for 10, 20, or 30 minutes. This duration and timing was chosen based on results in Blanco et. al (2011), which indicated that, when heat-shocked 3-7 hours AEL, nearly 100% of brains contained MARCM-labelled DA clones, and that after 13 hours, only 10% or fewer brains contained such clones. After heat shock, egg-laying plates were returned to the 22°C incubator and moistened once per day with distilled water.

Fly Sorting

Flies needed to be sorted for purposes of virgin female collection and phenotyping, as described below. Sorting was performed at a fly-sorting station, where a CO_2 tank was connected to both a foam-stopped funnel set in PVC pipe where the flies were dumped and knocked out, and also a stage set under a dissection microscope where the flies were observed and sorted with a paint brush. Unwanted flies were discarded into a fly morgue, a bottle containing a mixture of water ethanol, apple cider vinegar, and dish detergent to trap and kill the flies.

Virgin Female Collection

Virgin females needed to be collected for all crosses, since non-virgin females can carry the sperm of the males in their original bottles and contaminate future crosses. Flies begin mating around 6-7 hours old, so it is nearly impossible to distinguish virgin females from non-virgin females if pupae have been hatching for more than 6 hours. However, females which are younger than about 2 hours can be visibly distinguished from the rest due to the presence of a meconium (first defecation), which can be seen as a dark, off-center mass in their abdomens (shown below). These very young females also typically have lighter-colored, slightly swollen bodies.

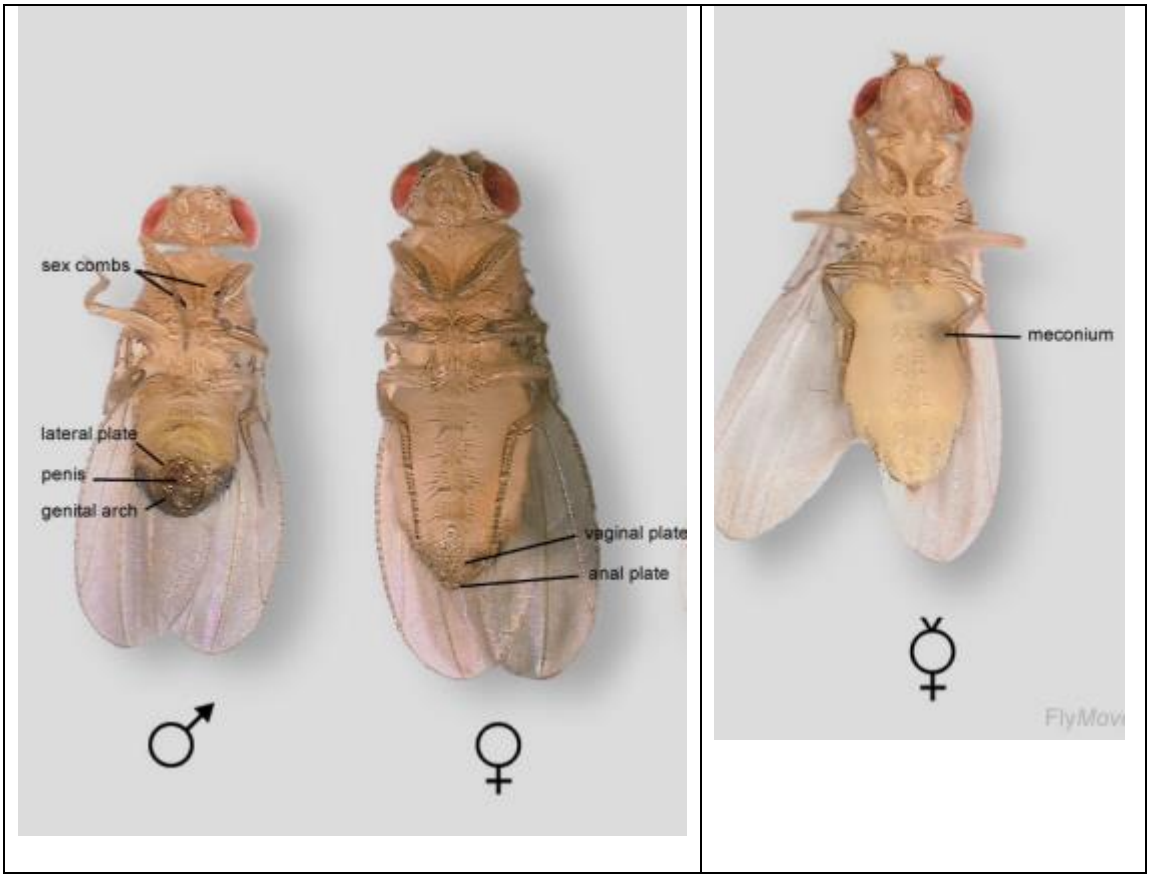


Figure 3: Male, female, and virgin female (<2hrs old, meconium present) drosophila (Tauber Lab, 2013).

During the first sort of each day (following sorting procedure as described above), only females with the meconium were collected, since the presence of the meconium is the only clear indication that a female is younger than 6 hours. The bottles were then thoroughly cleaned out to ensure that all adult flies were removed. Once or twice again throughout the day, sorts were performed at intervals of 6 or fewer hours, so that all females could be collected.

Phenotype Selection

At nearly every stage in the MARCM line creation, specific balancer chromosomes were selected for by careful selection of their corresponding phenotypes. The balancer line used was Cyo/Sco; TM2/TM6b. Each of these alleles is homozygous lethal, allowing them to be used as effective genetic markers. The phenotypes that were used as indication of each of the alleles are shown below.

All images are from the Genesis "Learning to Fly" poster (Childress, Behringer, Halder).

Figure 4: CyO (Curly O) flies have wings that curl upward rather than lie flat.

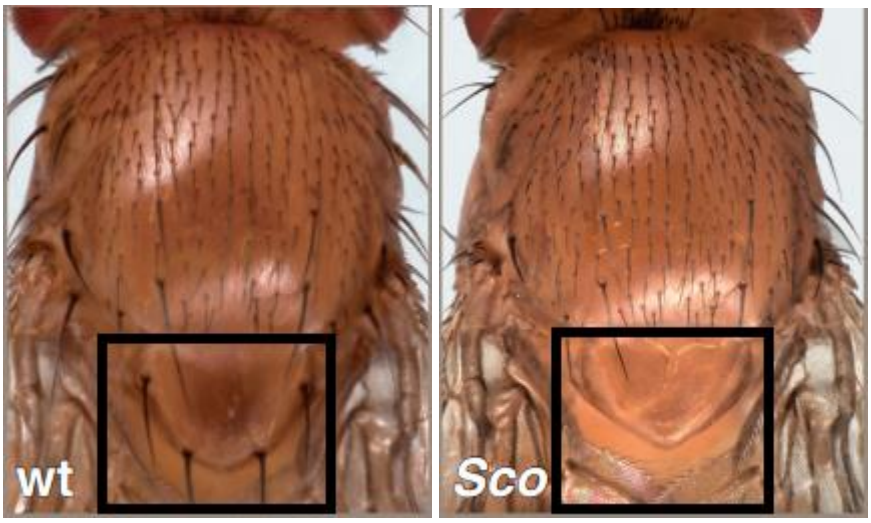


Figure 5: Sco (Scutoid) flies have fewer than hairs on their scutellum, rather than the typical four hairs.

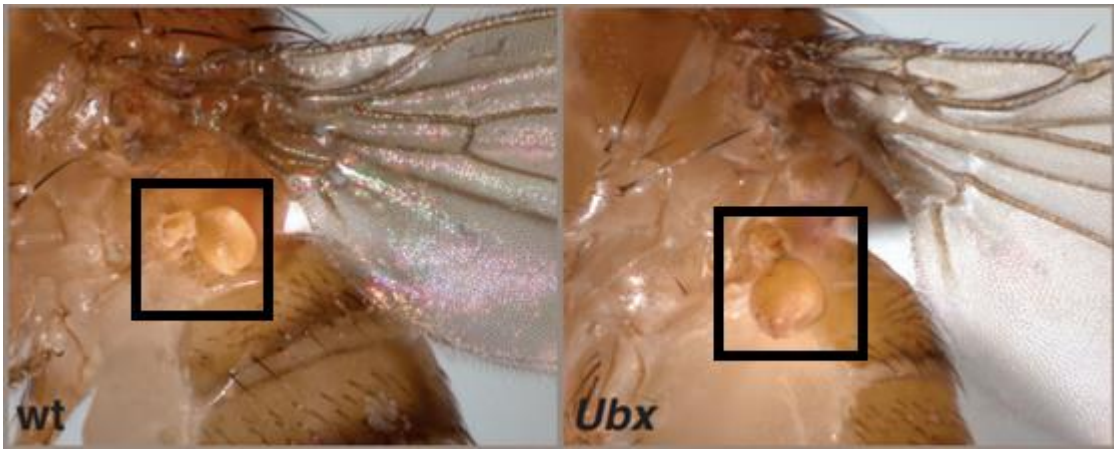


Figure 6: TM2 flies present the Ubx (Ultrabithorax) phenotype, in which the haltere organ is bent and club-shaped, often with a single hair at its base.

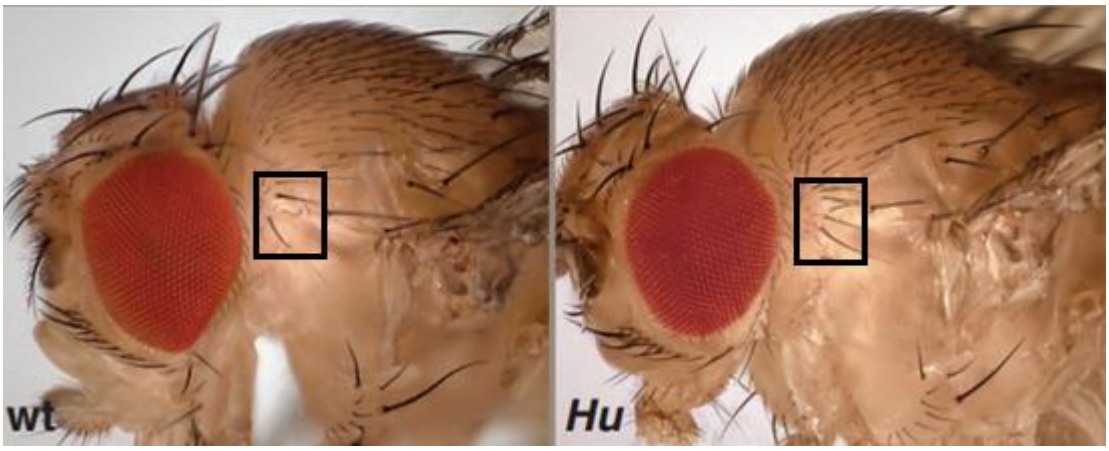


Figure 7: TM6b flies present the Hu (Humeral) phenotype, in which the bristles on the shoulder are shorter and greater in number than in the wild type flies.

Brain Dissection

Larvae were dissected 92-96 hours after egg collection to ensure that all were third instar. Under a dissection microscope, larvae were moved from the egg laying plates to the lids of CytoOne 35mm culture dishes containing roughly 1mL dissection solution. To remove the brain from the body, fine-tipped tweezers were used to grab both the mouth of the larvae and the body at approximately one-third the body length from the mouthpiece. The body was then pulled apart and the brain located and removed from the surrounding tissues. The optic lobes, unnecessary for the MARCM experiments, were removed from the brain as well. The brains were then placed on ice in lidded CytoOne 35mm culture dishes containing 2mL of the dissection solution.

Antibody Staining

Solutions for brain staining:

a) Fixative solution (for 2.5mL): 0.5mL PFCHO (4% final from 20% stock)

0.25mL PBS

1.75mL ddH_2O

b) Washing solution (for 50mL): 1mL BSA

5mL PBS

44mL ddH_2O

c) Blocking and Permeability (for 20mL):

0.5mL NGS

300µL Titron X-100

2mL PBS

17 mL ddH_2O

Procedure

The dissected brains were fixed for 60 minutes on ice in fixative solution. They were then washed three times for 20 minutes at room temperature in washing solution. The brains were then placed in blocking solution at room temperature on a slow rocker for 60 minutes. After the blocking solution, the brains were incubated overnight in primary antibody at 4ºC. Two primary antibodies were used at a 1:1000 concentration

in washing solution: Rabbit anti-GFP to tag the mosaic cells and Mouse anti-TH to tag all dopaminergic neurons.

The following day, the brains were washed three times for 25 minutes in washing solution. The primary antibody was recycled and used a total of three times before being discarded. After being washed, the brains were incubated in secondary antibody in a foil-wrapped dish on a rocker at room temperature for two hours. The secondary antibodies (Alexa Mouse Red and Rabbit Green) were also in a 1:1000 dilution in washing solution. After secondary staining, the brains were washed again three times for 25 minutes.

During all staining procedure, brains with different heat shock timing were kept in separate CytoOne 35mm culture dishes. Solutions were changed by pipette while the brains remained in a single dish. For all solutions except the antibodies, 2mL were used for each dish. 1mL of the antibody solutions were used for each dish. For later experiments, Alexa Rb Red and Ms Green were used as 2° antibodies instead of Ms Red and Rb Green.

Mounting

Brains were mounted in mounting gel on pieces of 22x50mm cover glass. Brains were carefully moved by tweezers to the bottom of the gel and positioned with the dorsal side against the bottom glass. Any air bubbles were removed to prevent diffraction of light under the microscope. Circular glass spacers were placed on both

sides of the gel to prevent smashing. Nail polish was applied to all four corners of the slide to adhere to the top piece of cover glass, which was applied carefully to prevent the creation of air bubbles. Slides were stored in the dark at 4ºC for 1-2 days before being imaged to allow for slight drying of the media.

Confocal Microscopy

The Zeiss confocal microscope was set to GFP/TRITC settings to produce a red signal in the dopaminergic neurons (TH-stained) and a green signal in the MARCM-labelled neurons (GFP-stained). The Zen software by Zeiss Microscope Software was used to take pictures of the immunofluorescent larvae brains. Confocal microscopy was used because it allows for visualization of all fluorescent neurons, not just those on one focal plane, enables the simultaneous examination of two immunofluorescent stains, and displays the 3D nature of structures such as axonal projections (Lundell & Hirsh, 1994).

Analysis

In order to determine exactly which neuron clusters had been mitotically recombined by the MARCM technique, the GFP-expressing neurons were compared to those detailed in Figure 8, shown below. Since brain positioning varied from slide to slide, locations of the clusters relative to other dopaminergic neurons, as well as direction of axonal projection, were carefully examined.

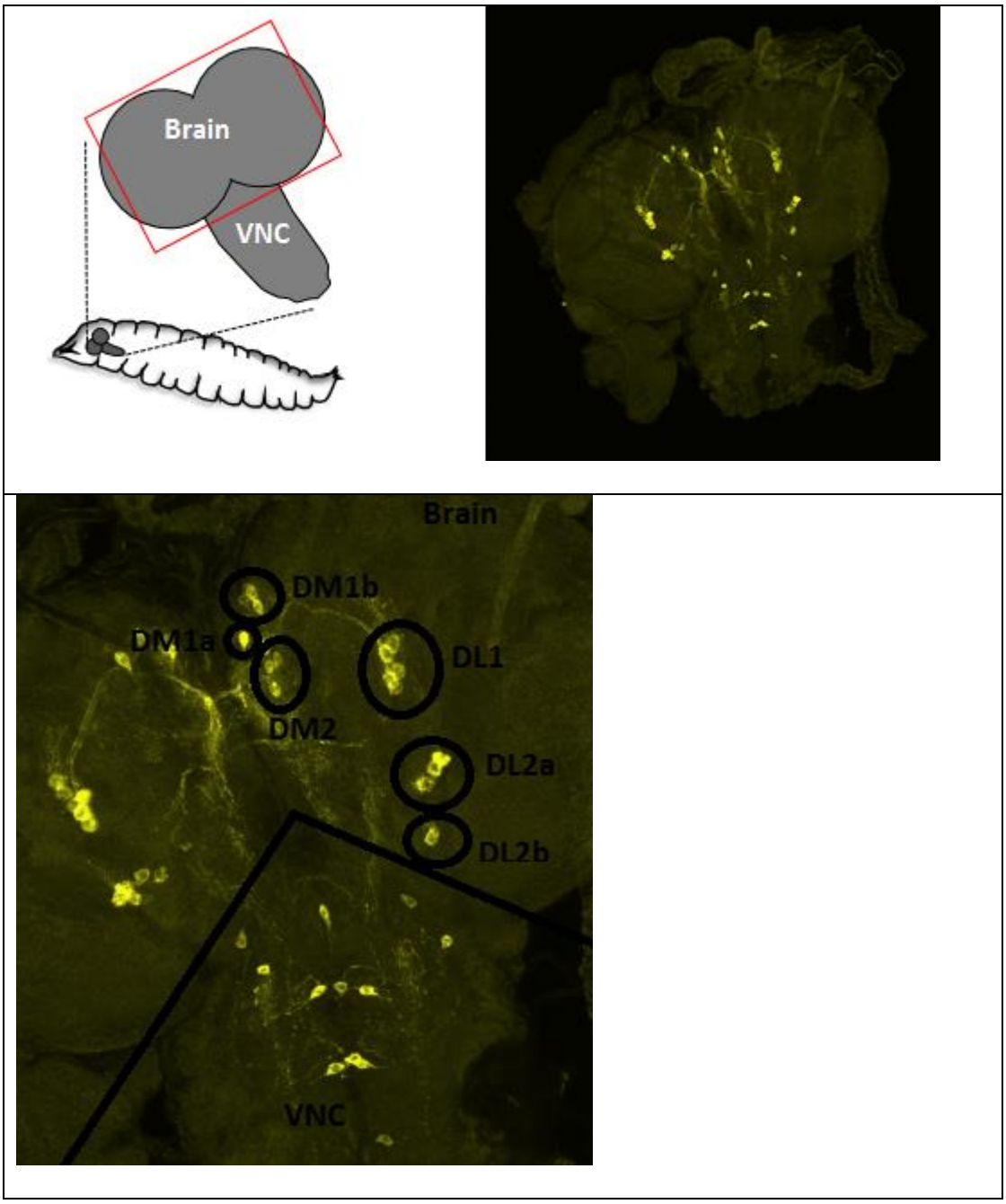


Figure 8: DA neuron clusters in Drosophila larvae brain

Fly Strains Used

Genotype	Strain Number	Source
P{w[+mW.hs]=GawB}elav[C155], P{ry[+t7.2]=hsFLP}1, w[*]; P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=tubP-GAL80}LL2/CyO	5145	Bloomington Drosophila Stock Center (Indiana University)
y[1] w[*]; P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=UAS-mCD8::GFP.L}LL5	5139	Bloomington Drosophila Stock Center (Indiana University)
w[*]; P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=piM}45F P{w[+mC]=tubP-GAL80}LL2; P{w[+mC]=tubP-GAL4}LL7/TM6C, Sb[1] Tb[1]	5143	Bloomington Drosophila Stock Center (Indiana University)
P{ry[+t7.2]=hsFLP}1, y[1] w[1118]; P{w[+mW.hs]=FRT(w[hs])}G13 P{w[+mC]=UAS-mCD8::GFP.L}LL5	5131	Bloomington Drosophila Stock Center (Indiana University)
CyO/Sco; TM2/TM6		Dr. Soichi Tanda (Ohio University)
TH-GAL4(III)		Dr. Jay Hirsh (University of Virginia)

Results

MARCM Test Cross

5145♂x5139♀	y[1]w[*]; FRTmCD8; +
elav,FLP,w[*]; FRTGAL80; +	elav,FLP,w[*]/y[1]w[*]; FRTGAL80/FRTmCD8; +/+
elav,FLP,w[*]; CyO; +	elav,FLP,w[*]/y[1]w[*]; CyO/FRTmCD8; +/+

The highlighted offspring had all the necessary components of MARCM, and the elav-GAL4 driver works in all neurons. Therefore, one half of the offspring had neurons with the capability to recombine under heat shock. Larvae from this cross were heat-shocked for one hour, three hours AEL. The brains were dissected out and imaged to ensure that MARCM worked as expected in this instance before complex creation of TH-driven MARCM-ready lines began. An image of one of these test brains, with GFP expression in random neurons, is shown below.

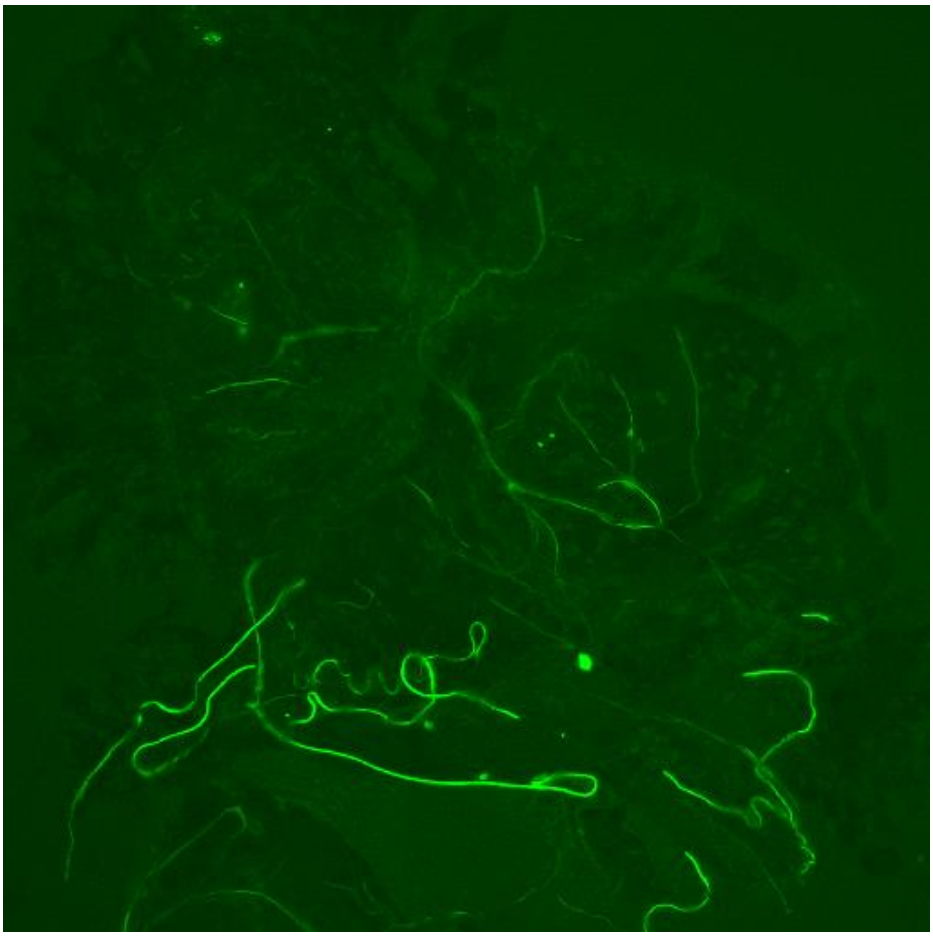


Figure 2: MARCM test with elav driver, 1hr hs 3hrs AEL

Creation of MARCM-Ready Line

Part A

5143♂xBalancer♀	CyO; TM2	CyO; TM6	Sco; TM2	Sco; TM6
FRTGAL80; GAL4	FRTGAL80/CyO; GAL4/TM2	FRTGAL80/CyO; GAL4/TM6	FRTGAL80/Sco; GAL4/TM2	FRTGAL80/Sco; GAL4/TM6
FRTGAL80; TM6b,Tb	FRTGAL80/CyO; TM6b,Tb/TM2	FRTGAL80/CyO; TM6b,Tb/TM6	FRTGAL80/Sco; TM6b,Tb/TM2	FRTGAL80/Sco; TM6b,Tb/TM6

Male offspring presenting phenotypes of the highlighted genotype were selected and saved for part D.

Part B

Balancer♂xTHGAL4(III)♀	THGAL4(III)
CyO; TM2	CyO/+; THGAL4/TM2
CyO; TM6	CyO/+; THGAL4/TM6
Sco; TM2	Sco/+; THGAL4/TM2
Sco; TM6	Sco/+; THGAL4/TM6

The offspring (males of one type, females of the other) presenting the phenotypes of the highlighted genotypes were collected and kept separate until part C.

Part C

♀CyO/+; THGAL4/TM2 x ♂Sco/+; THGAL4/TM2	CyO; THGAL4	CyO; TM2	+; THGAL4	+; TM2
Sco; THGAL4	CyO/Sco; THGAL4	CyO/Sco; THGAL4/TM2	Sco/+; THGAL4	Sco/+; THGAL4/TM2
Sco; TM2	CyO/Sco; THGAL4/TM2	CyO/Sco; TM2	Sco/+; THGAL4/TM2	Sco/+; TM2
+; THGAL4	CyO/+; THGAL4	CyO/+; THGAL4/TM2	+; THGAL4	+; THGAL4/TM2
+; TM2	CyO/+; THGAL4/TM2	CyO/+; TM2	+; THGAL4/TM2	+; TM2

Virgin females presenting the phenotypes of the highlighted genotype were collected and used in part D.

Part D

FRTGAL80/CyO; TM2/TM6,Tb♂XCyO/Sco; THGAL4♀	CyO; THGAL4	Sco; THGAL4
FRTGAL80; TM2	FRTGAL80/CyO; TM2/THGAL4	FRTGAL80/Sco; TM2/THGAL4
FRTGAL80; TM6b,Tb	FRTGAL80/CyO; TM6b,Tb/THGAL4	FRTGAL80/Sco; TM6b,Tb/THGAL4
CyO; TM2	CyO; THGAL4/TM2	CyO/Sco; THGAL4/TM2
CyO; TM6,Tb	CyO; THGAL4/TM6b,Tb	CyO/Sco; THGAL4/TM6b,Tb

Males and virgin females presenting phenotypes of the highlighted genotype were collected, allowed to mate, and maintained as a permanent line.

Part E

The final cross performed for the purpose of egg collection for MARCM experimentation was repeated many times as follows: males from the permanent line produced from part D were crossed with virgin females from Bloomington line #5131. These crossing adults were kept for 3-4 weeks and used throughout that time for egg collection. They were changed to new bottles weekly to prevent mixing with the F1 generation.

5131♀ x +; FRTGAL80/CyO; TM2/THGAL4♂	+; FRTGAL80; TM2	+; FRTGAL80; THGAL4	+; CyO; TM2	+; CyO; THGAL4
hsFLP; FRTmCD8::GFP; +	hsFLP/+ or Y; FRTGAL80/FRTmCD8; TM2/+	hsFLP/+ or Y; FRTGAL80/FRTmCD8::GFP; THGAL4/+	hsFLP/+ or Y; CyO/FRTmCD8::GFP; TM2/+	hsFLP/+ or Y; CyO/FRTmCD8; THGAL4/+

The results of this final cross are shown above. The highlighted genotype, making up 1/4 of the offspring, was the only complete set of MARCM genetic components. This ¼ was expected to have heat-shock induced mosaic brain tissue. ½ of the offspring lacked the THGAL4 driver, and were therefore expected to lack GFP expression altogether. The final ¼ of the offspring lacked the GAL80 repressor, and were therefore expected to express GFP signal in all dopaminergic neurons.

About halfway through all MARCM experimentation, the male line used in part E was purified in order to ensure that all offspring of the final cross were MARCM-testable: males and virgin females without the balancers CyO and TM2 were collected from this line and crossed together to create a homozygous line FRTGAL80; THGAL4.

MARCM Testing

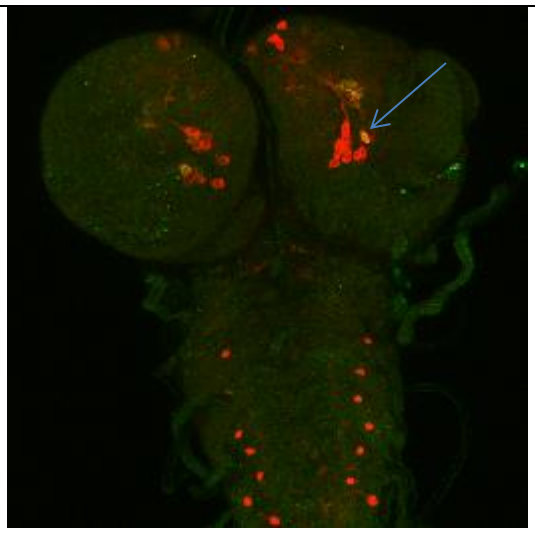
Each time eggs were collected for MARCM experimentation, they were collected on three separate plates. All three plates were heat-shocked at the same time (3, 5, 7, or 9 hours after egg-laying), but with varying duration (10, 20, or 30 minutes) Additionally, plates were run for just 10 minutes at 11, 13, and 15 hours after egg-laying.

With the original male line used for the MARCM crosses (shown in Part E above), only ¼ of the larval brains were expected to show mosaic expression of GFP. ½ were expected to lack GFP expression completely, and the final ¼ were expected to express GFP in all dopaminergic neurons. These expectations held true, indicating that the genetic makeup of the larvae was likely as planned and that the MARCM elements of the genome were functioning as expected. However, once the male line was made homozygous for the MARCM elements, 100% of the larval brains had mosaic expression of GFP, as expected, since they then all contained the necessary genetic machinery: the TH-Gal4 driver, UAS:mCD8::GFP, and the Gal4 repressor, Gal80, as well as properly positioned FRT sites and one copy of hsFLP.

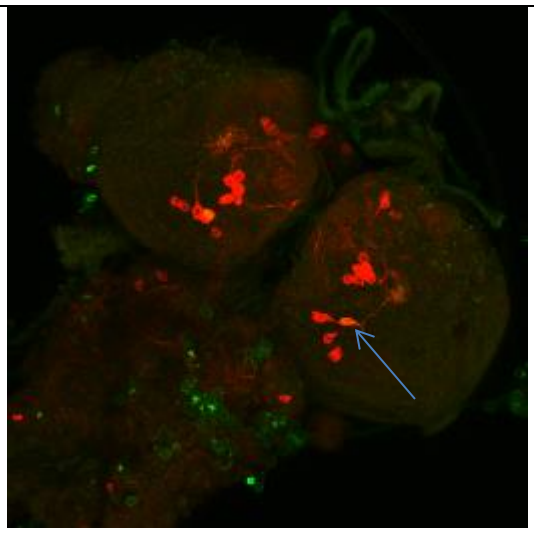
Pictures were taken of all mosaic brains under a confocal microscope at either 250x or 400x magnification, depending on the size of the brain. Neurons expressing GFP were identified by cluster. The dopaminergic neuron clusters are dorso lateral 1 (DL1, 7 neurons), dorso lateral 2a (DL2a, 4 neurons), dorso lateral 2b (DL2b, 2 neurons), dorso medial 1a (DM1a, 1 neuron), dorso medial 1b (DM1b, 3 neurons), and

dorso medial 2 (DM2, 4 neurons). Typically, only one neuron in the identified cluster expressed GFP.

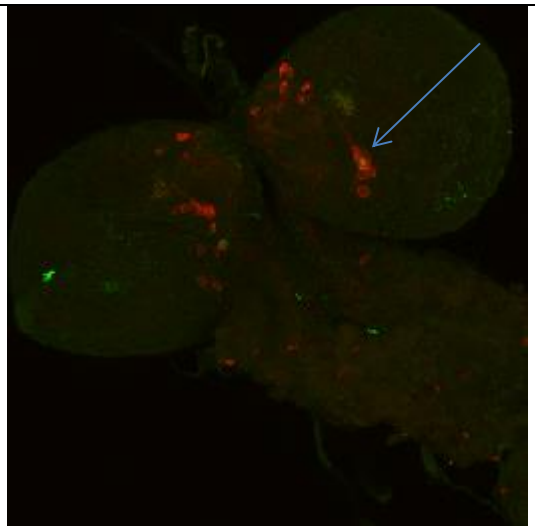
Figure 9: Heat shock at 3hrs AEL for 10 min



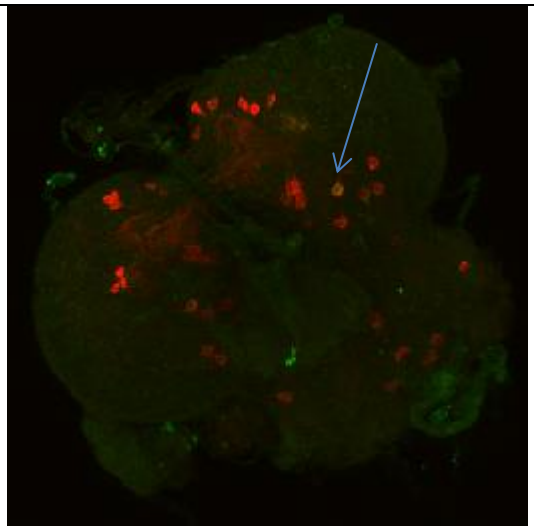
DL2a



DL2a



DL2a



DL2a

Figure 10: Heat shock at 3hrs AEL for 20min

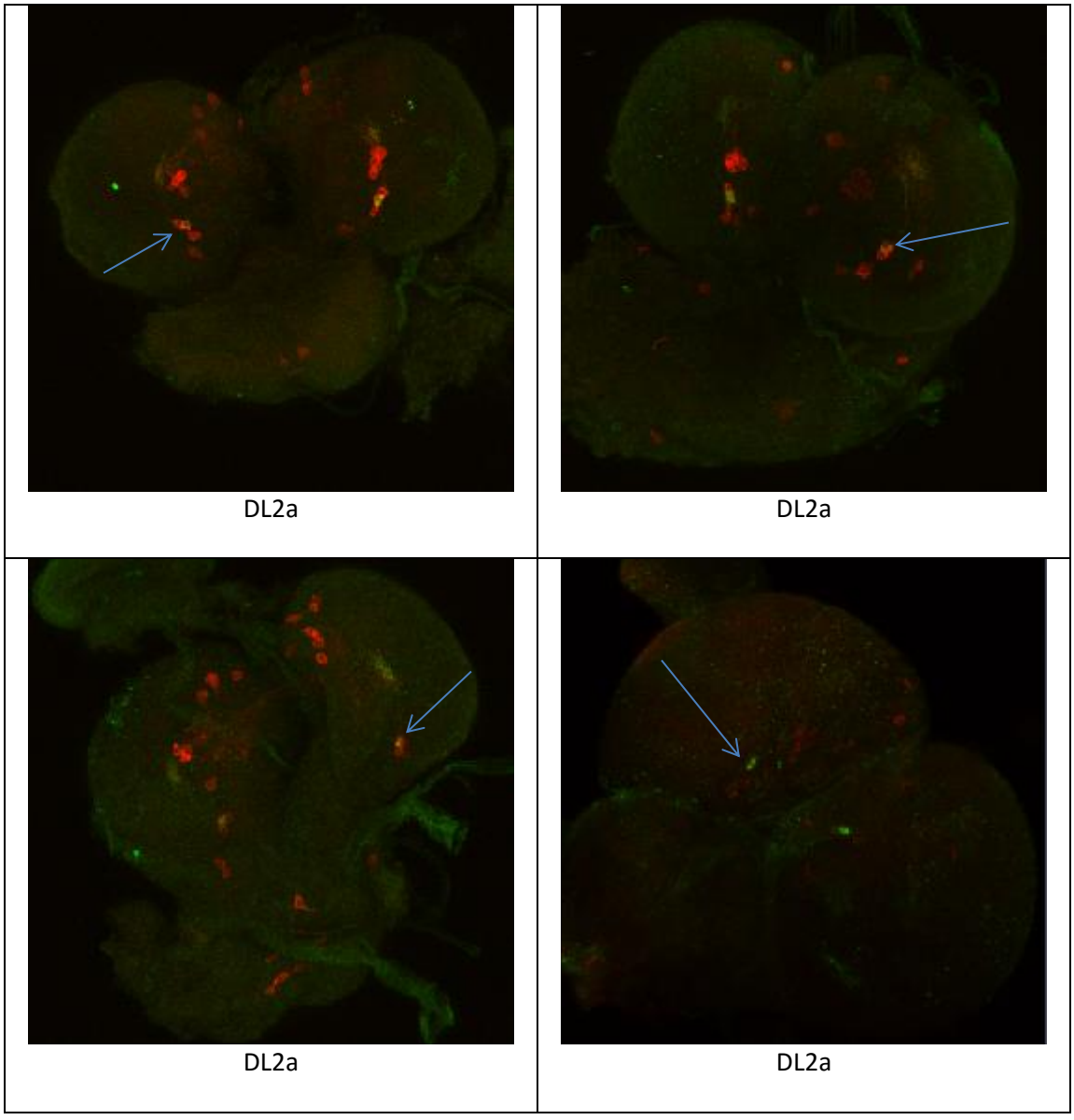
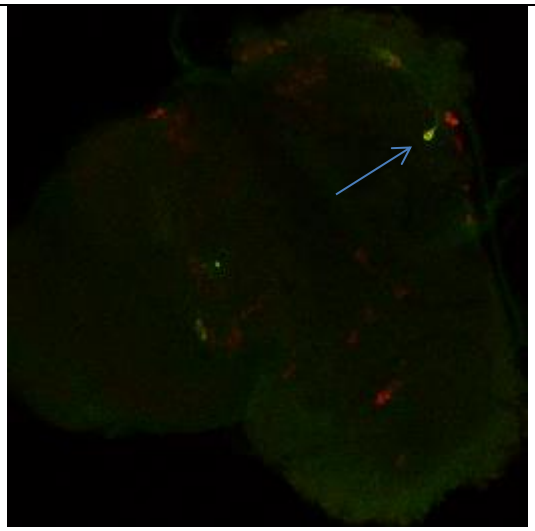
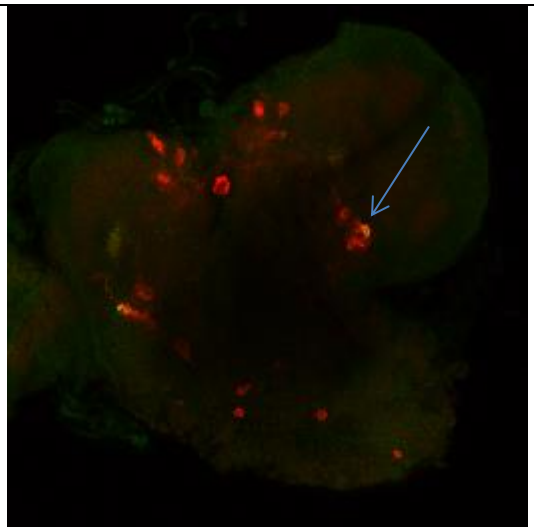


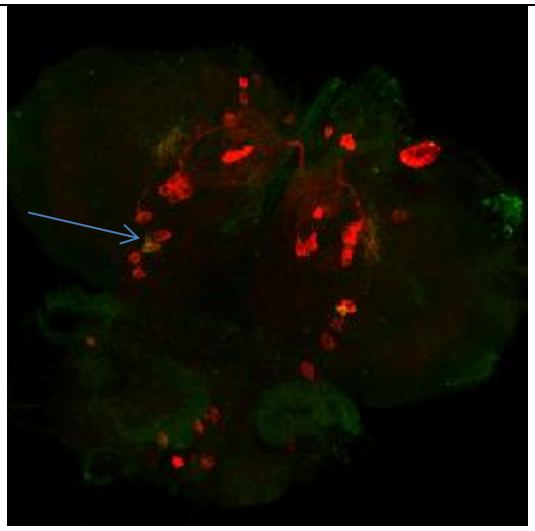
Figure 11: Heat shock at 3hrs AEL for 30min



DL2a

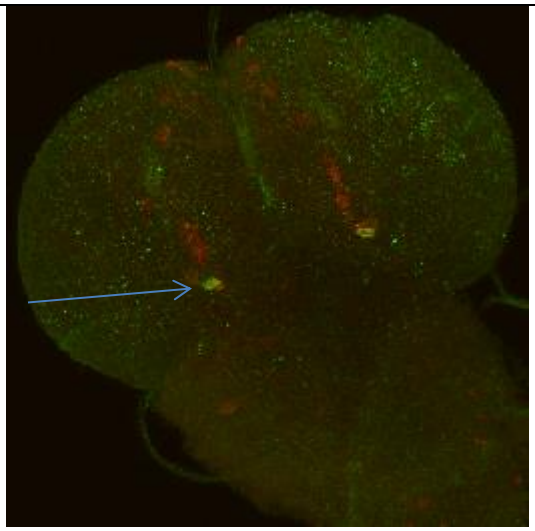


DL2a

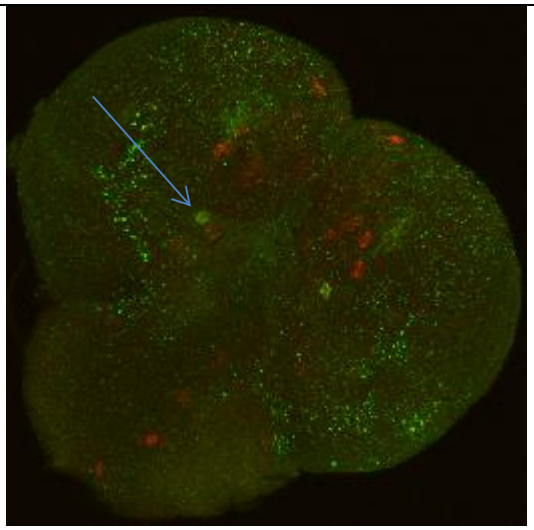


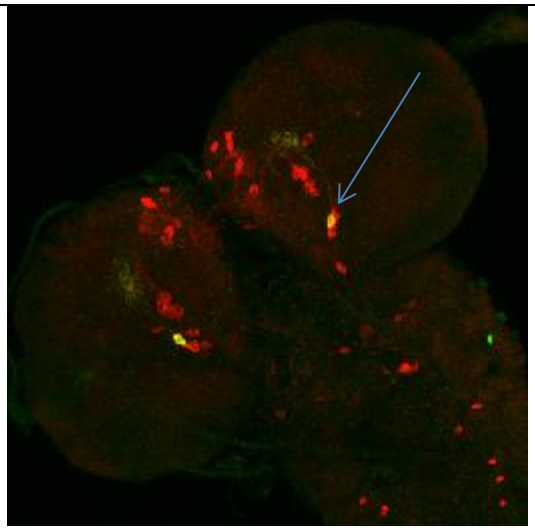
DL2a (400x)

Figure 12: Heat shock at 5hrs AEL for 10min



DL2a





DL2a (enlarged in next image)

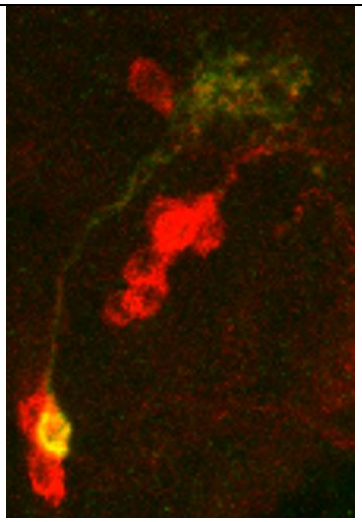
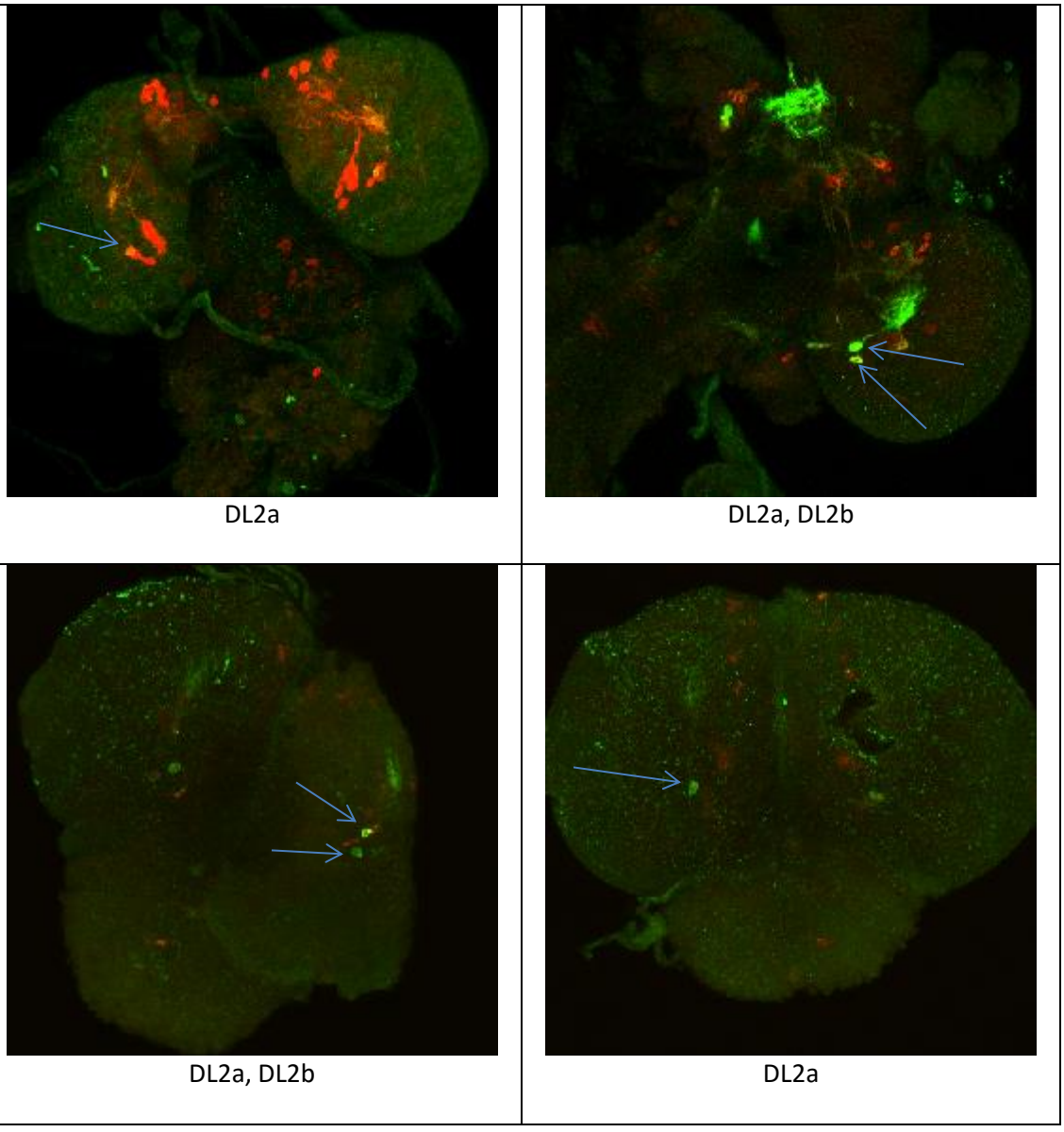


Figure 13: Heat shock at 5hrs AEL for 20min



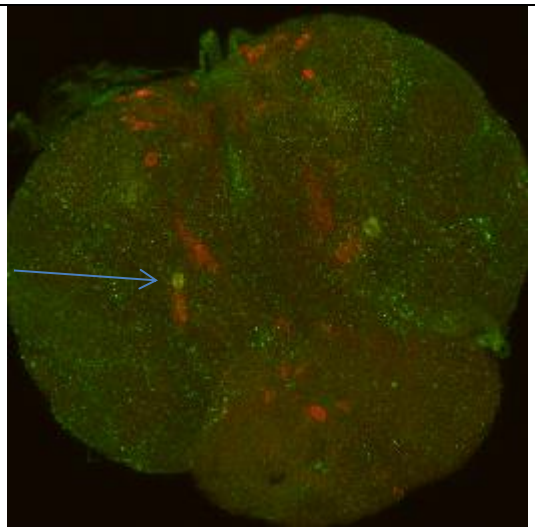
DL2a

DL2a, DL2b

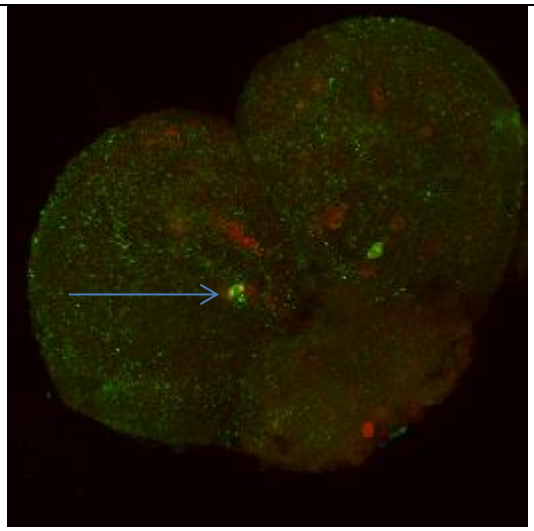
DL2a, DL2b

DL2a

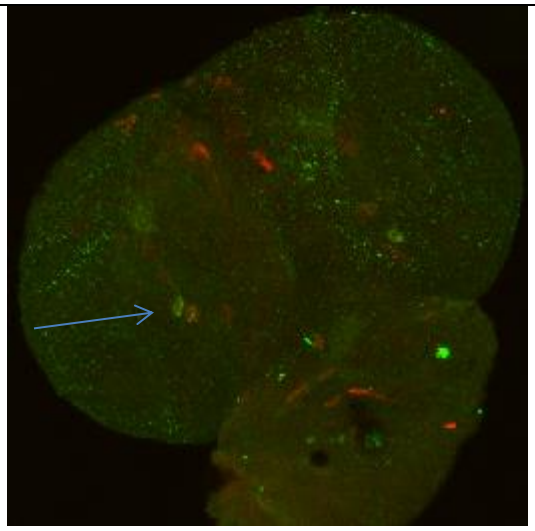
Figure 14: Heat shock at 5hrs AEL for 30min



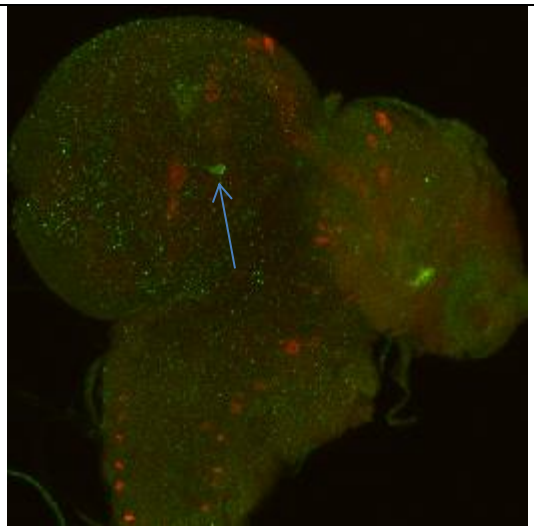
DL2a



DL2a

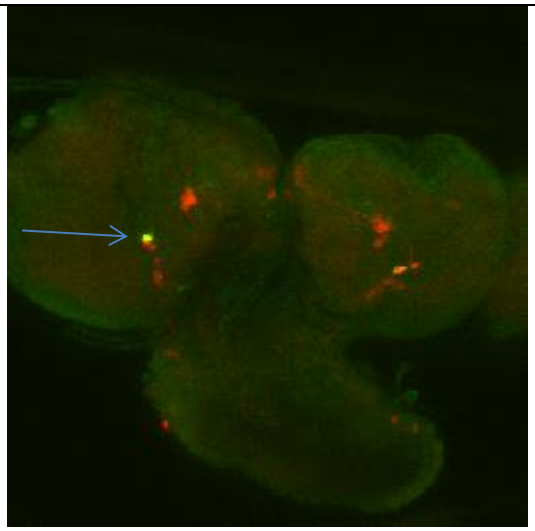


DL2a



DL2a

Figure 15: Heat shock at 7hrs AEL for 10min



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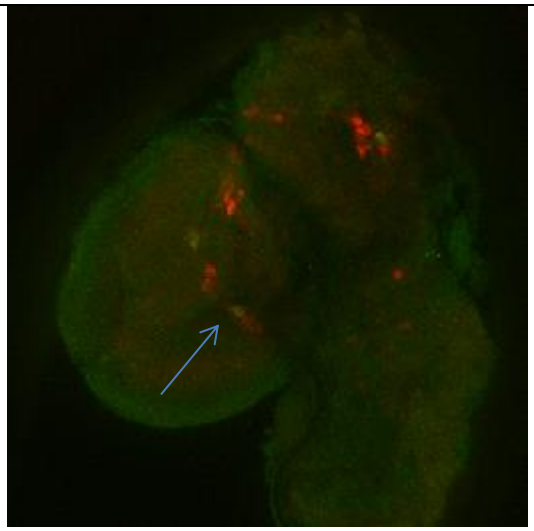
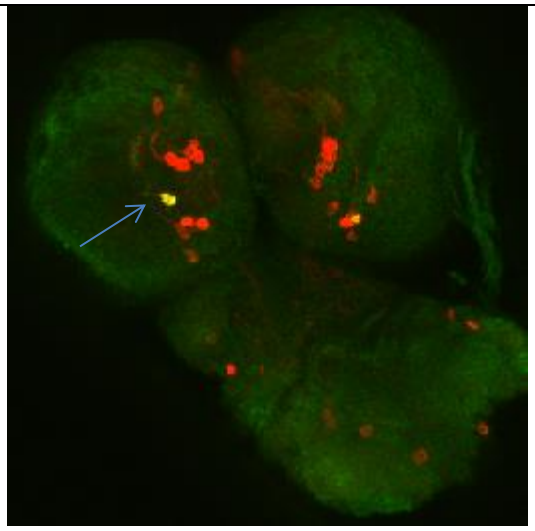
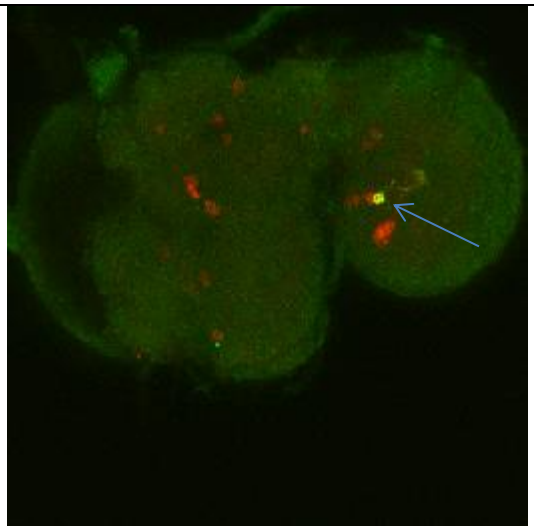


DL2a

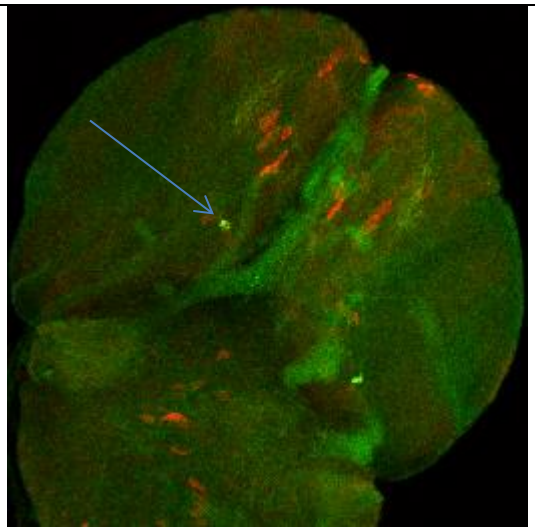


DL2a(400x)

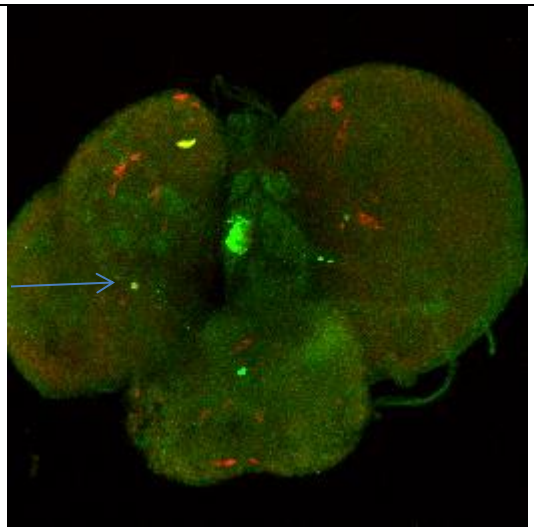


DL2a

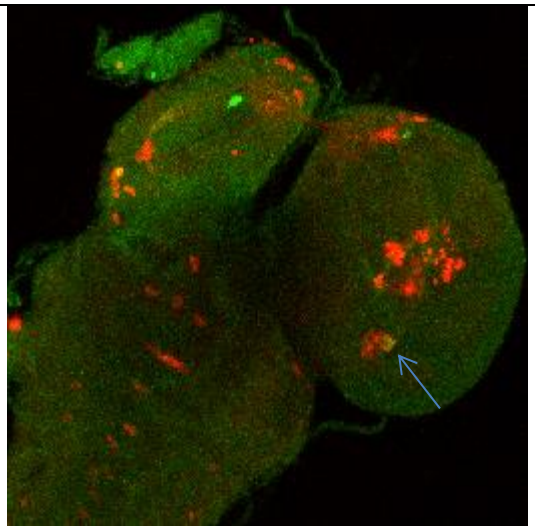
Figure 16: Heat shock at 7hrs AEL for 20min



DL2a

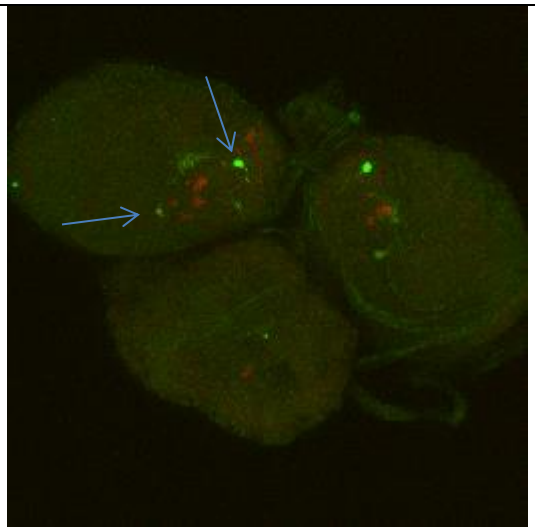


DL2a

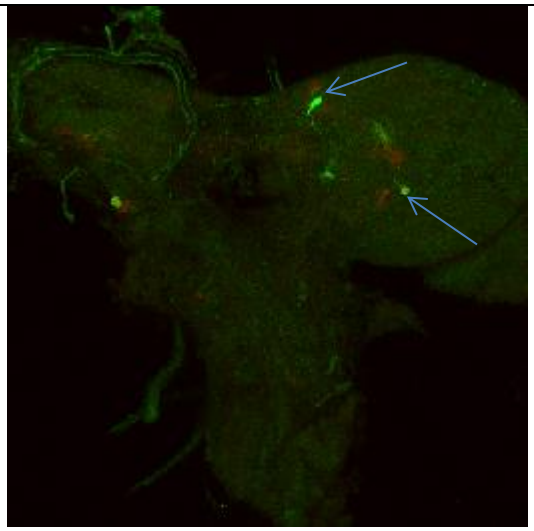


DL2a

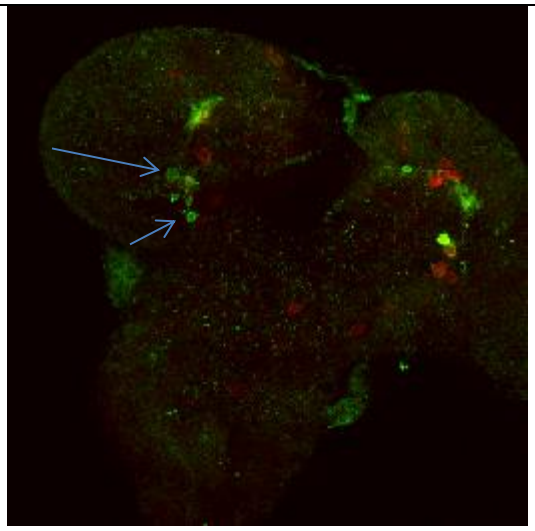
Figure 17: Heat shock at 7hrs AEL for 30min



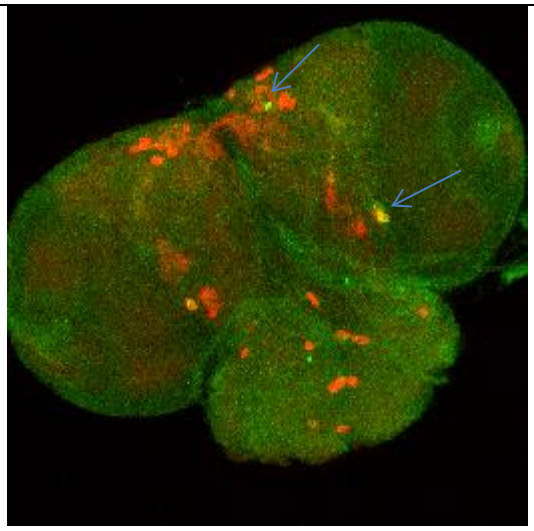
DL2a, DM1b



DL2a, DM1b



DL2a, DL2b



DL2a, DM1b

Figure 18: Heat shock at 9hrs AEL for 10min

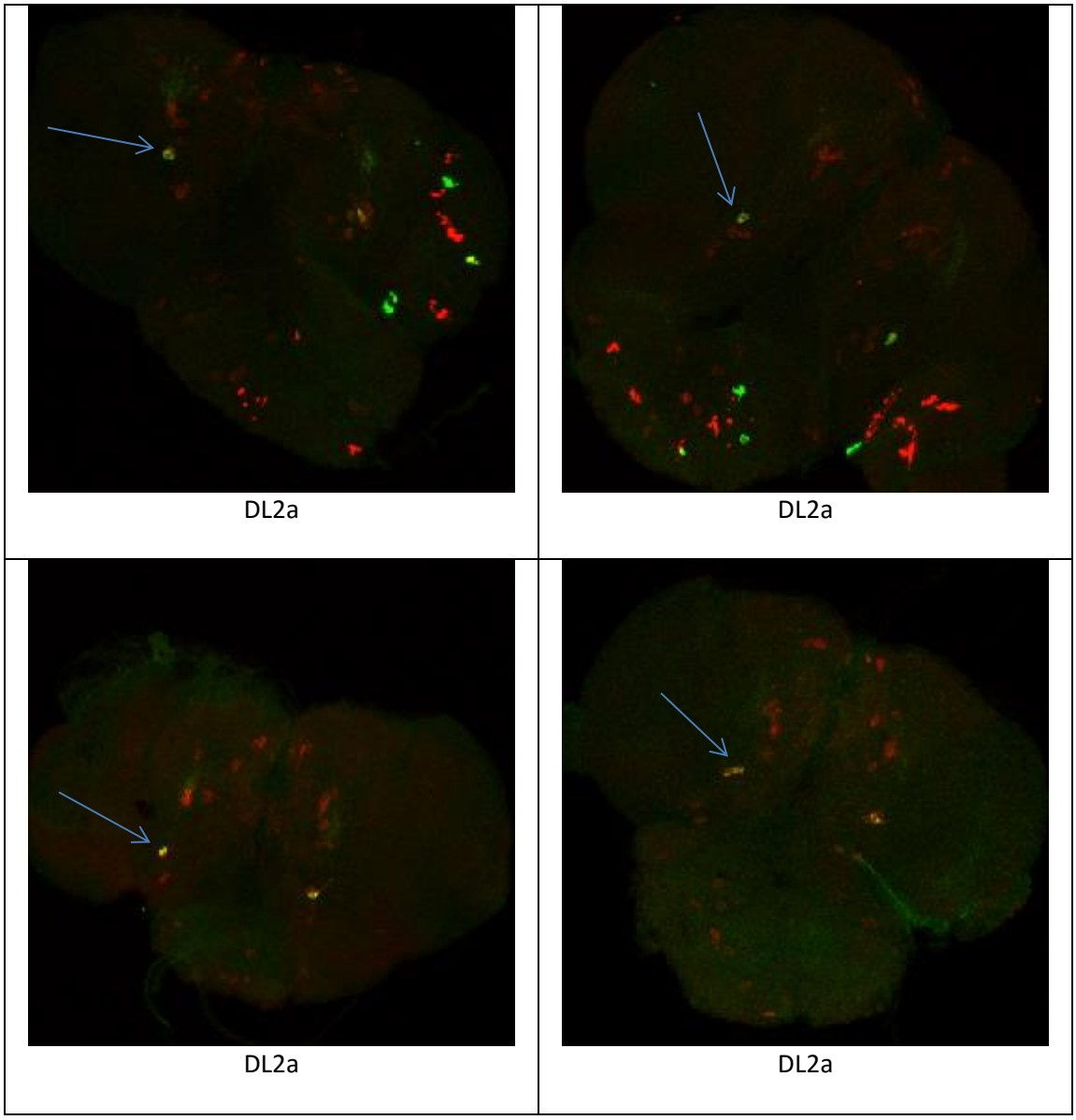
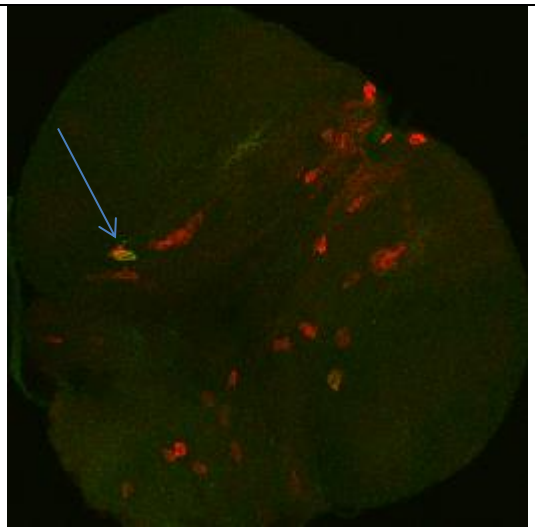
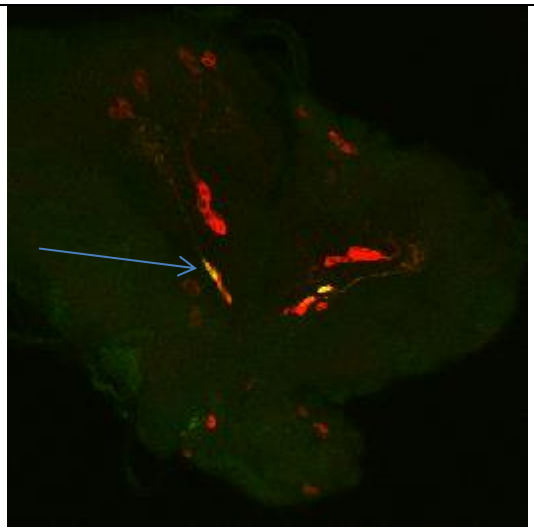


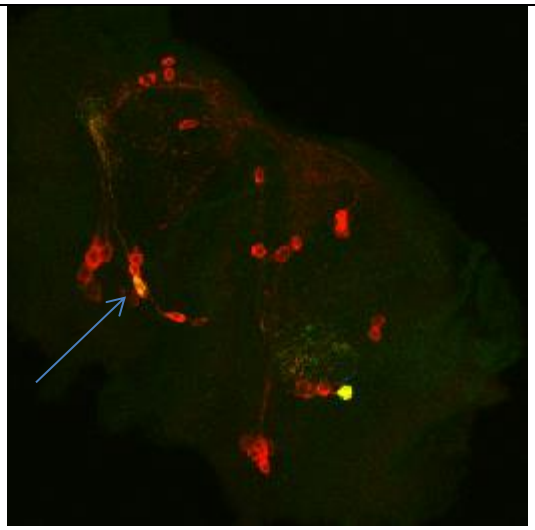
Figure 19: Heat shock at 9hrs AEL for 20min



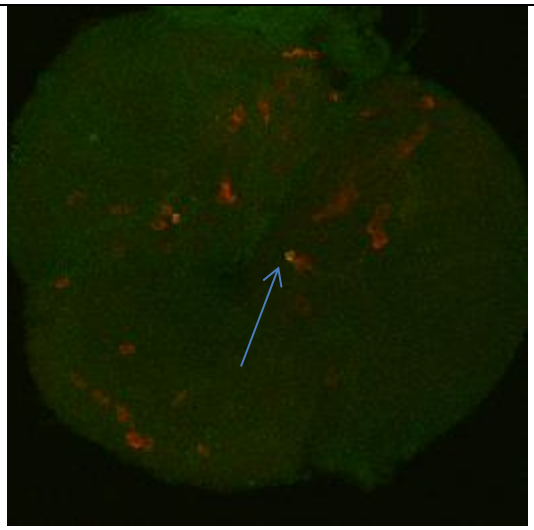
DL2a



DL2a(400x)

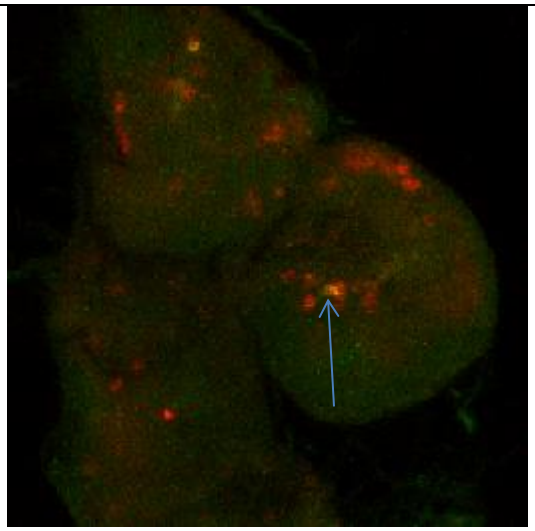


DL2a

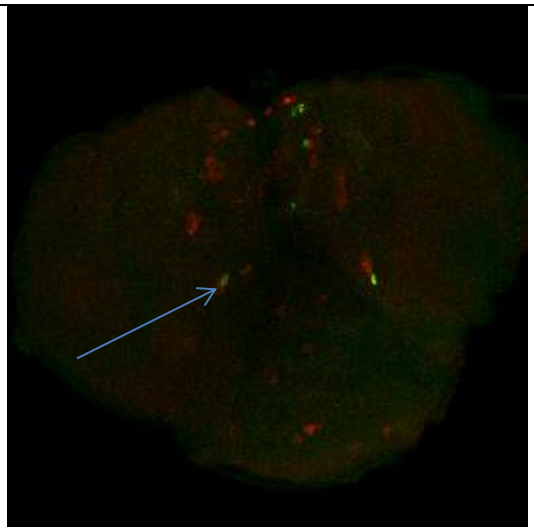


DL2a

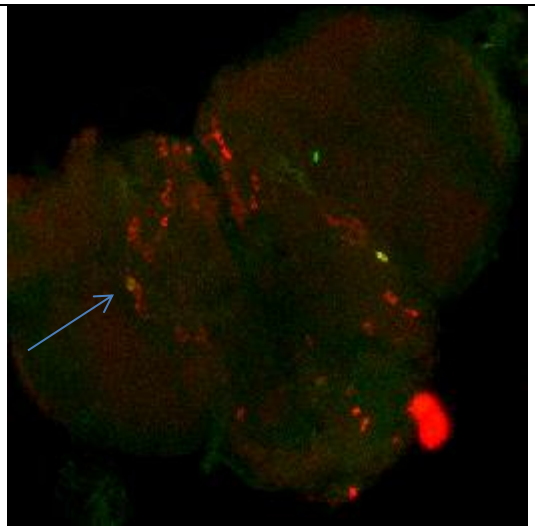
Figure 20: Heat shock at 9hrs AEL for 30min



DL2a

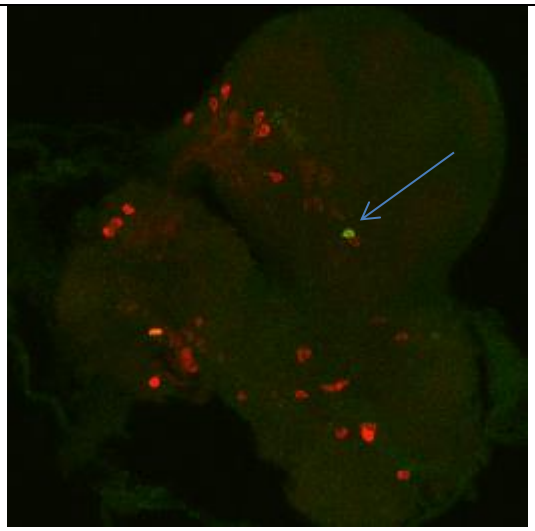


DL2a

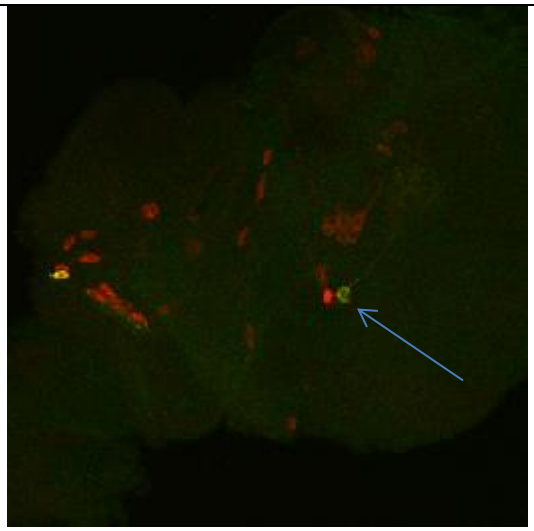


DL2a

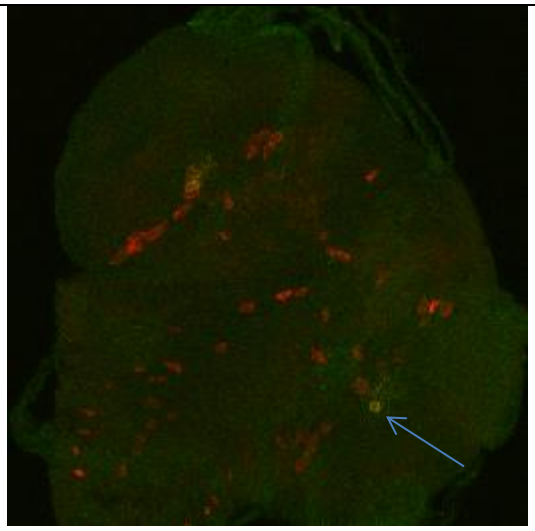
Figure 21: Heat shock at 11hrs AEL for 10min



DL2a

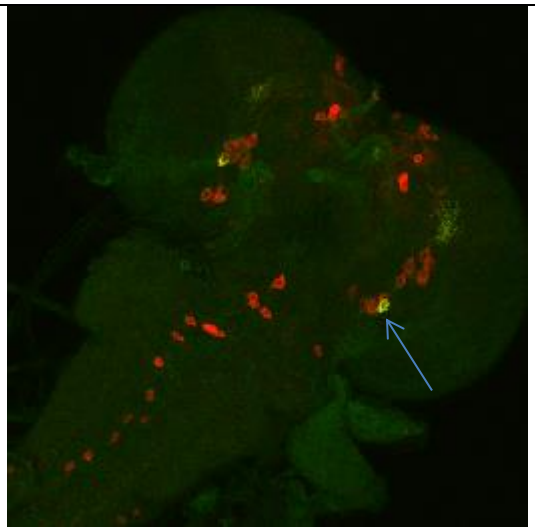


DL2a

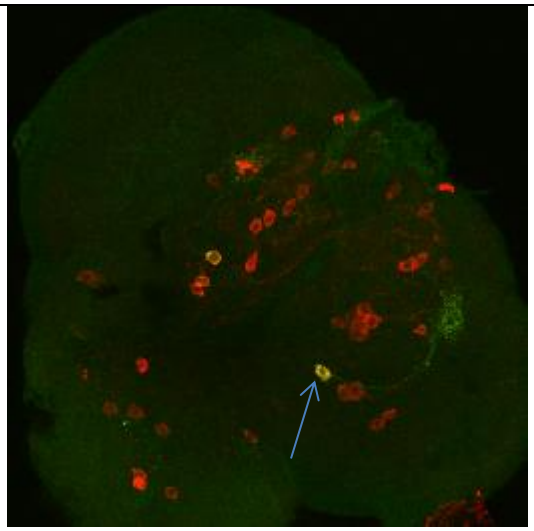


DL2a

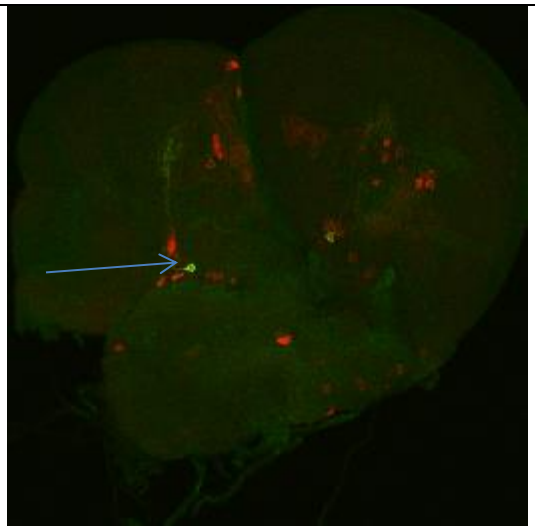
Figure 22: Heat shock at 13hrs AEL for 10min



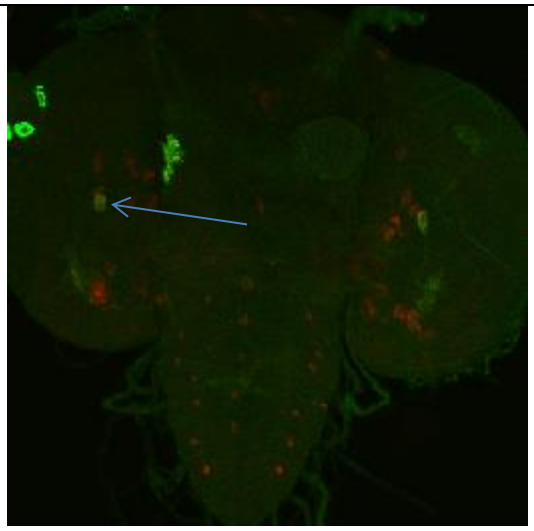
DL2a



DL2a

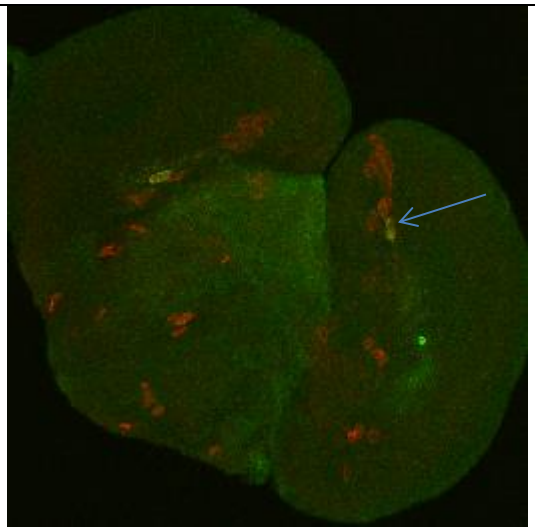


DL2a

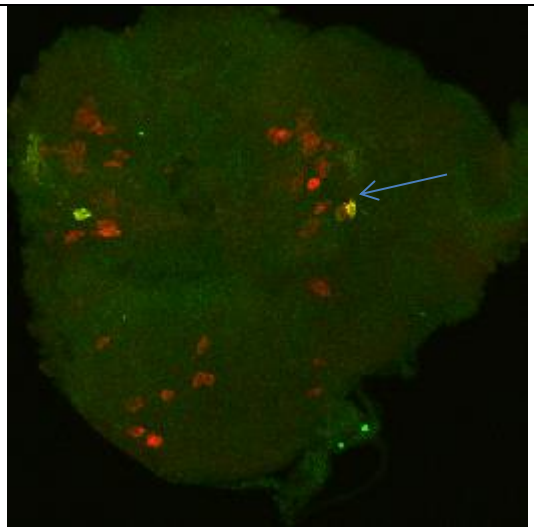


DL2a

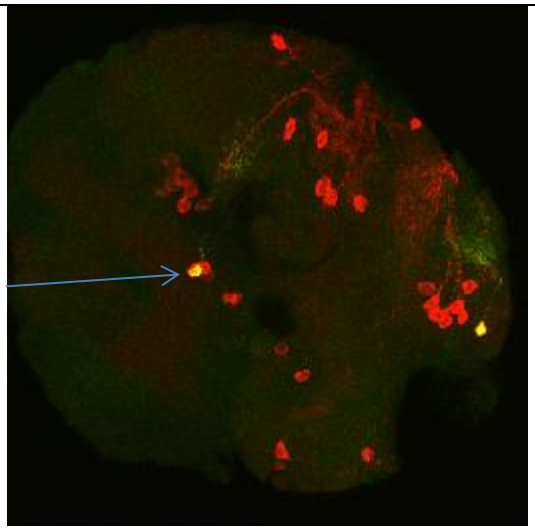
Figure 23: Heat shock at 15hrs AEL for 10min



DL2a

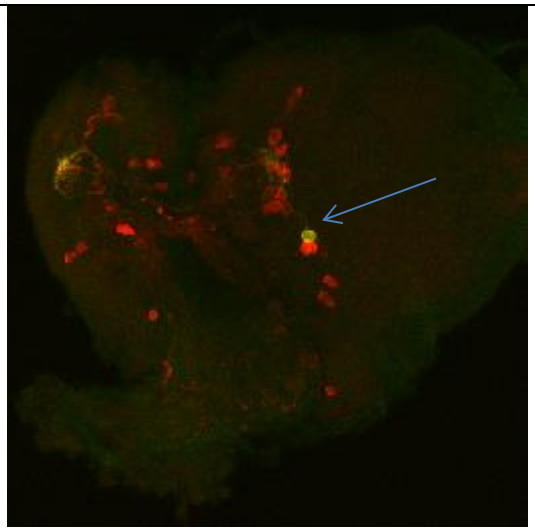


DL2a

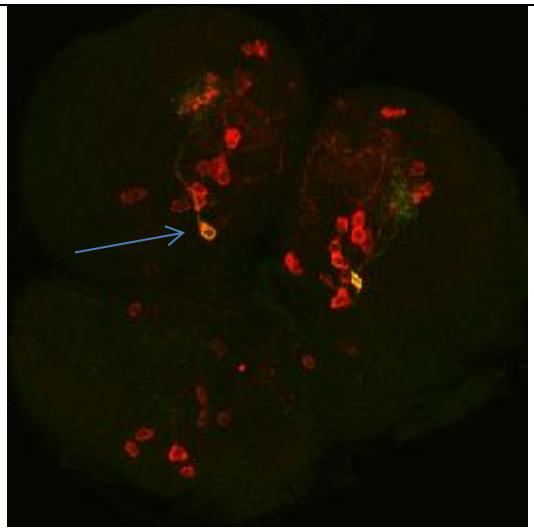


DL2a

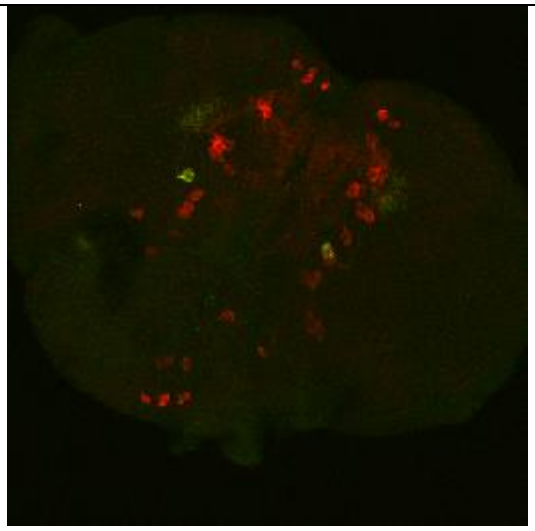
Figure 24: control - no heat shock



DL2a



DL2a(400x)



DL2a

heat shock information			fequency of expression by DA neuron cluster					
time AEL(hrs)	Duration (min)	sample size	DL1	DL2a	DL2b	DM1a	DM1b	DM2
3	10	4	0	1	0	0	0	0
	20	5	0	1	0	0	0	0
	30	3	0	1	0	0	0	0
5	10	9	0	1	0	0	0	0
	20	8	0	1	0.25	0	0	0
	30	6	0	1	0	0	0	0
7	10	4	0	1	0	0	0	0
	20	3	0	1	0	0	0	0
	30	6	0	1	0.17	0	0.67	0
9	10	8	0	1	0	0	0	0
	20	3	0	1	0	0	0	0
	30	3	0	1	0	0	0	0
11	10	3	0	1	0	0	0	0
13	10	7	0	1	0	0	0	0
15	10	3	0	1	0	0	0	0
No heat shock	N/A	3	0	1	0	0	0	0

Table 1: A summary of MARCM test results
For each combination of heat shock timing and duration, the entire sample was evaluated together to calculate the frequency of GFP expression in each neuron cluster (0 being 0% of the time, and 1 being 100% of the time).

The mosaic results did not vary much: every larval brain with all MARCM components expressed GFP in a DL2a neuron, regardless of heat shock timing or duration. The DL2a cluster expressed GFP even in the absence of heat shock (control). Other clusters were rarely mitotically recombined. ¼ of the larvae heat-shocked at 5 hours for 20 minutes expressed GFP in the DL2b cluster. 1/6 of the larvae heat-shocked at 7 hours for 30 minutes expressed GFP in the DL2b cluster, and 2/3 of the same group of larvae expressed GFP in the DM1b cluster. In both cases where clusters

other than DL2a expressed GFP multiple times (DL2b at 20min heat shock 5hrs AEL and DM1b at 30min heat shock 7hrs AEL), instances of that expression occurred in not just one experiment, but two. Otherwise, there was no expression found outside of the ubiquitous DL2a cluster.

Discussion

This study did not quite accomplish the development of the MARCM technique for dopaminergic neurons in *Drosophila* as it intended to. The hope was to elicit GFP expression in varying neuron clusters depending on varying timing and duration of heat-shock during embryonic development. However, one cluster, DL2a, expressed GFP for every tested situation, including the control with no heat shock.

The ubiquitous expression of GFP in the DL2a cluster, even in the absence of heat shock, is a very unexpected result. Without the mitotic recombination induced by the heat shock, a single copy of the Gal80 repressor in every cell should have prevented GFP expression. If the genotype of the larvae was as expected, then there are two possible causes for this expression: 1. In the DL2a cluster, the Gal80 protein was not repressing the TH-Gal4 driver as it should have, allowing the mCD8::GFP gene to be expressed, or 2. The DL2a cluster was driven to mitotic recombination at the FRT sites by some force other than a 37ºC heat shock. The second possible explanation, that the DL2a cluster is simply highly sensitive and underwent recombination without heat shock, is the most likely explanation.

Further evidence that the ubiquitous expression of GFP in the DL2a cluster was caused by improper functioning of the MARCM technique is that, if the expression was indeed caused by heat-shock-induced mitotic recombination, then the DL2a cluster should have only been vulnerable to recombination during its mitotically-active developmental stage, meaning that only a small range of heat shock timing would have triggered its expression. Instead, it expressed at every stage of experimentation.

A couple other clusters expressed GFP after certain variation of heat shock. DL2b expressed GFP 25% of the time for brains heat-shocked at 5 hours for 20 minutes and 17% of the time for brains heat-shocked at 7 hours for 30 minutes. DL2b expression after 20min hs at 5hrs AEL occurred in 2 separate experiments. Even still, 25% expression is relatively small. This heat shock timing could be used in the future to target the DL2b cluster, but not very reliably.

The DM1b cluster expressed GFP 67% of the time for brains heat-shocked at 7 hours for 30 minutes. This result is very promising, and is what we had hoped to achieve for every DA neuron cluster. DM1b expression at this heat shock timing occurred in 2 separate experiments and is fairly reliable. If future research sought to target the DM1b cluster, it could use a 30min heat shock at 7 hours AEL.

Overall, these results show that the MARCM technique may be useable for *Drosophila* dopaminergic neurons, but requires more testing. Those neurons in the DL2b and DM1b clusters were likely expressing GFP due to heat-shock-induced

mitotic recombination, as expected. However, the sample sizes were relatively small, so reproducibility of these results could potentially be low. More larvae would need to be examined in order to rule out random or misleading GFP expression in these neurons (such as that seen in the DL2a cluster). The DL2b expression in the 20min heat shock 5hrs AEL group is especially questionable, since no such expression was found in the brains heat-shocked at the same time for 30 minutes. If 20 minutes was enough to induce recombination in the DL2b cluster, 30 minutes should have logically yielded the same results.

Clearly, some component of the MARCM technique was not functioning as expected in these experiments. One possible explanation for the unexpected results could be that the flies used in these experiments had significantly different developmental timing than those used in *Neural Development* (Blanco et. al 2011), which was the model by which heat shock timing and duration was chosen. The DA neurons in our flies (other than the DL2a cluster) could also be less sensitive to heat shock than in the Blanco study and could require longer heat shock time or higher temperature. The reality that the final lines used for this experimentation could have been incredibly genetically distinct from the lines used in the Blanco study means that an entirely new approach may be necessary. Further testing could include the addition of heat shocks earlier or later in development, as well as in increase in heat shock duration and/or temperature.

Another possible explanation for the less-than-optimal results is that the genotype of the flies was not as expected. With multiple steps taken to create the

MARCM-ready line from several other lines, it is highly possible that a mistake was made along the way, and some necessary genetic element was lost or diluted. However, this is highly unlikely. As discussed earlier, the initial MARCM-ready male line was expected to yield 50% larvae with no GFP expression, 25% with universal dopaminergic GFP expression, and 25% mosaic expression (larvae with the complete set of MARCM elements). This expectation was made based on the presence of both necessary MARCM elements and balancer chromosomes in the male line. Since these expectations seemed roughly to hold true until the MARCM-ready male line was purified to a homozygous state, it seems that the genotype of the flies was indeed as expected. No data was collected for the non-functioning brains, because they were initially seen as inconsequential, or unnecessary, data. It is possible that the MARCM flies have the incorrect genotypes; however, from this evidence, it seems more likely that something in that genotype simply was not functioning as expected, or the experimental conditions, such as the independent variables of heat shock timing and duration, were not set at optimum levels.

Throughout this study, it was difficult to collect enough eggs and, eventually, larvae, to produce large sample sizes. This problem was exacerbated by low survival of larvae to the 3rd instar stage, as well as the relatively short amount of time that eggs were collected (1 hour). Since the brief egg-laying duration was necessary to keep the larvae developmentally synchronized, a greater number of crossing adults per bottle could be used in the future in order in maximize egg collection.

Future Directions

Future studies could further investigate MARCM usability to target dopaminergic neuron clusters in *Drosophila.* New variations in heat shock timing, duration, and perhaps, temperature, could possibly yield more positive results.

Once the MARCM technique is developed, potential uses are vast. Parkinson's disease effects in the flies could be more closely examined in each neuron cluster, rather than within the whole brain. For example, the number of subcellular structures, such as mitochondria and synaptotagmin, could be compared between healthy and diseased neuron clusters through study of both wild-type and A53T Parkinsonian flies. One way this experimentation could be achieved is with the MARCM-ready line used in this experiment, with mosaic GFP expression in the neurolemma, overlapped with fluorescent stains for specific organelles (e.g. mitochondria) and proteins (e.g. synaptic marker). By examining the fluorescent overlap, one could quantify changes in Parkinsonian flies.

A far more precise, though more time-consuming, method for examination of subcellular proteins and organelles would be to create unique MARCM-ready lines for each area of interest which expressed mosaic GFP not in the neurolemma, as does the mCD8::GFP line, but rather, in the protein or organelle in question. This method would require a series of crosses, similar to those in the results section above, to insert genes such as mt-GFP and syt-GFP where mCD8::GFP was used in this study.

Another use of the MARCM technique in *Drosophila* dopaminergic neurons could be to examine the functionality of different neuron clusters. Optogenetic genes such as activator CHR2 and suppressor NpHR could be inserted into the MARCM-ready genome as the gene which is mosaically expressed. If the technique is developed to a point where specific clusters are predictably expressed after specific heat shocks, then eggs could be heat-shocked to elicit CHR2 or NpHR expression in specific clusters. Then, the resulting channels in the neurons could be opened through light stimulation (for an excitatory or inhibitory effect), and the larvae could be put through locomotion tests or learning/memory tests. These tests would help to determine the specific functions of the different dopaminergic neuron clusters in *Drosophila.*

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References

Avramut, M. P. (2015). Dopamine and addiction. Salem Press Encyclopedia Of Health.

Blanco, et. al. (2011). Orthodenticle is necessary for survival of a cluster of clonally related dopaminergic neurons in the Drosophila larval and adult brain. *Neural Development.* 6:34.

Blandini, F., & Armentero, M.-T. (2012). Animal models of Parkinson's disease. *FEBS Journal*, 279(7), 1156–1166. http://doi.org/10.1111/j.1742-4658.2012.08491.x

Blausen.com staff. (2014). Blausen gallery 2014. *Wikiversity Journal of Medicine*. http://doi.org/10.15347/wjm/2014.010

Blesa, J., Phani, S., Jackson-Lewis, V., & Przedborski, S. (2012). Classic and New Animal Models of Parkinson's Disease. *Journal of Biomedicine and Biotechnology*, 2012, 1–10. http://doi.org/10.1155/2012/845618

Brand, A. H., & Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, 118(2), 401–415.

Brookshire, B. (2013). Dopamine Is. . . Is it love? Gambling? Reward? Addiction? Slate. http://www.slate.com/articles/health_and_science/science/2013/07/what_is_dopamine_love_lust_sex_addiction_gambling_motivation_reward.html

Chen, A. Y., Wilburn, P., Hao, X., & Tully, T. (2014). Walking deficits and centrophobism in an α-synuclein fly model of Parkinson's disease. *Genes, Brain and Behavior*, 13(8), 812–820. http://doi.org/10.1111/gbb.12172

Childress, Behringer, Halder. Learning to Fly – Phenotypic Markers in Drosopila. *Genesis – The Journal of Genetics and Development.* Accessed Feb. 2016. http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1526-968X/homepage/free_posters.htm

De Lau, L. M., & Breteler, M. (2006). Epidemiology of Parkinson's disease. *The Lancet Neurology*, 5(6), 525–535.

Feany, M. B., & Bender, W. W. (2000). A Drosophila model of Parkinson's disease. *Nature*, 404(6776), 394–398. http://doi.org/10.1038/35006074

Frank, C., Pari, G., & Rossiter, J. P. (2006). Approach to diagnosis of Parkinson disease. *Canadian Family Physician*, 52(7), 862–868.

Grelin et. al. (2003). Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *Developmental Neurobiology.* 54.4:618-27.

Lundell, Hirsh. (1994). Temporal and spatial development of serotonin and dopamine neurons in the Drosophila CNS. *Developmental Biology.* 165(2):385-96.

Münchau, A., & Bhatia, K. P. (2000). Pharmacological treatment of Parkinson's disease. *Postgraduate Medical Journal*, 76(900), 602–610.

Murphy, S. L., Xu, J., & Kochanek, K. D. (2013). Deaths: Final Data for 2010. National Vital Statistics Reports, 61(4). Retrieved from http://www.cdc.gov/nchs/data/nvsr/nvsr60/nvsr60_04.pdf

Nervous System. (2015). Funk & Wagnalls New World Encyclopedia, 1p. 1.

Nolte, J. (2009). The human brain. [electronic resource] : an introduction to its functional anatomy. Philadelphia, PA : Mosby/Elsevier, c2009.

Park, S. S., & Lee, D. (2006). Selective loss of dopaminergic neurons and formation of Lewy body-like aggregations in α-synuclein transgenic fly neuronal cultures. *European Journal of Neuroscience*, 23(11), 2908–2914. http://doi.org/10.1111/j.1460-9568.2006.04844.x

Podolsky. (2015). Pharmacological Rescue of Parkinson's Disease Symptoms with *Drosophila* Larvae. *A Thesis Presented to the College of Arts and Sciences Ohio University*.

Sethi, K. (2008). Levodopa unresponsive symptoms in Parkinson disease. *Movement Disorders*, 23(S3), S521–S533. http://doi.org/10.1002/mds.22049

Shulman, J. M., De Jager, P. L., & Feany, M. B. (2011). Parkinson's Disease: Genetics and Pathogenesis. *Annual Review of Pathology: Mechanisms of Disease*, 6(1), 193–222. http://doi.org/10.1146/annurev-pathol-011110-130242

Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., & Goedert, M. (1998). α-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proceedings of the National Academy of Sciences*, 95(11), 6469–6473.

The Tauber Lab, University of Leicester (2013). The Fly Manual – a Guide to Working with Drosophila. *LiveGene!*. www.tinyurl.com/livegene

Winklhofer, Haass. (2002). Mitochondrial dysfunction in Parkinson's disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease.* 1802(1):29-44.

What are neurotransmitters? (2015). Neurogistics, Inc.

Wu, Luo. (2006). A protocol for mosaic analysis with a repressible cell marker (MARCM) in Drosophila. *Nature Protocol.* 1(6):2583-9.

Xia, R., & Mao, Z.-H. (2012). Progression of motor symptoms in Parkinson's disease. *Neuroscience Bulletin*, 28(1), 39–48. http://doi.org/10.1007/s12264 012-1050