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The Role of *Broad-Complex* in Determining Neuronal Composition of the Drosophila Brain

by

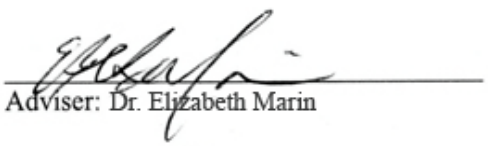
Alper Dincer

A Proposal Submitted to the Honors Council

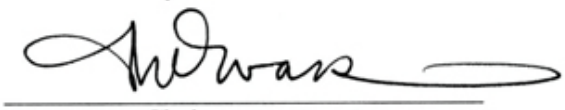
For Honors in Neuroscience

13 April 2010

Approved by:



Adviser: Dr. Elizabeth Marin



Department Chairperson: Dr. David Evans

Acknowledgements

I would first like to thank my research advisor, Dr. Marin, who had personally taught me the molecular biology techniques required for my experiment. Without her guidance and support, my research would have ended miserably and lasted only a few weeks into the project. My whole lab group actually needs an acknowledgement - someone always accounted for my absent-mindedness. I need to thank my close friends, both old and new, who were fundamental to surviving and enjoying Bucknell; my time was well spent only when spent with you.

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Most importantly, I would like to thank my family for their constant belief that I could succeed. During the worst of times, their support often meant the difference between quitting or persevering. I dedicate my work to them and I hope they know how much I truly appreciate all they have done for me. Beyond doubt, I would not have been capable of this if not for my family. Thank you, Dede, Nana, Anne, Baba, Çiğdem, and Aylin.

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Abstract

Developmental plasticity is the alteration of the nervous system throughout development influenced by environmental interactions and experience. The differentiation of neurons normally gives rise to the numerous types of specialized cells to perform specific functions within the nervous system. The modification of the number or ratio of specialized neuronal types is a category of developmental plasticity modulating neuronal composition. Changes in neuronal composition are especially conspicuous in holometabolous insects, which undergo radical changes in body plan during metamorphosis. The smaller and simpler nervous system of insects provides a model to study mechanisms altering neuronal composition.

A considerable transformation occurs in the mushroom body (MB) of the olfactory system of *Drosophila*. The mushroom body is a prominent structure in the insect brain essential to olfactory learning and memory, and serves as an excellent system to study the molecular mechanisms underlying the developmental plasticity. The MB's consists of three different classes of neurons (γ, α'/β', α/β) that are generated sequentially from four neuroblasts coincident with key stages in *Drosophila* development.

Evidence suggests there are environmental influences on mushroom body neuronal composition, likely mediated by hormones. Downstream of Juvenile Hormone (JH) and Ecdysone, key hormones regulating development and metamorphosis, is the

transcription factor *Broad-Complex* (*BR-C*), known to be critical in the transition of larvae into metamorphosis.

To elucidate the individual roles of the four *BR-C* isoforms, Z1-Z4, on neuronal composition in the mushroom body, I undertook a series of overexpression experiments and created tools for knockdown experiments. Specifically, I imaged and analyzed *Drosophila* brains from earlier experiments in which *BR-C* isoforms Z1 and Z3 were individually overexpressed in the MB. The knockdown experiments required the creation of the molecular tools necessary for isoform-specific RNA interference (RNAi). For these I performed PCR to amplify DNA sequences unique to each isoform and inserted those into the pWIZ vector, which will permit expression of loopless hairpin double stranded RNA to trigger the RNAi pathway in the fly.

Overexpression of BR-Z1 resulted in an increase in both γ neurons and total neurons in the mushroom body. Overexpression of BR-Z3 resulted in a decrease in γ neurons and no change in total neurons. These results suggest that individual isoforms of *BR-C* regulate distinct aspects of developmental plasticity in the neuronal composition of the MB. The generation of the pWIZ-based constructs is still in progress. Currently, the 1st inserts of BR-Z4 and BR-Z1 have been verified to be successfully ligated into pWIZ. Once complete, these RNAi constructs will be useful tools for investigation into the roles of individual *BR-C* isoforms in any tissue of interest in the fly and specifically will allow us to determine whether the isoforms are necessary in normal MB development.

Introduction and Background

Neural Plasticity

Nervous system development requires specific spatially and temporally regulated mechanisms to create a diverse population of neurons and connections. Developed neural circuits allow an organism to receive input from the environment, process it, and output certain behavioral or physiological responses. Importantly, an organism's nervous system must allow behavior appropriate to its particular environment. Innate nervous system development may control the construction of conserved neural circuits and connections, but the potential for a nervous system to be plastic, to alter structure and functional organization based upon experience, contributes to the survival and reproductive success of an individual animal.

Neural plasticity is a recent discovery, prior to which most had believed the brain remained static after childhood development. Ramon y Cajal, the founder of modern neuroscience, believed the nervous system was fixed and immutable. This opinion largely dominated the field of neuroscience until the 20th century. William James, a contemporary of Ramon y Cajal, gave the earliest documented proposal of plasticity. He stated, "Organic matter, especially nervous tissue, seems endowed with a very extraordinary degree of plasticity" (James, 1890).

Experimental evidence for the theory began as early as the 1920's, but was not truly considered definitive until the 1970's. In an attempt to allow blind people to see basic figures, Paul Bach-y-Rita created a device to substitute the visual system with the

sensory system (1969). Patients were soon able to recognize different shapes and figures. To be capable of visualizing figures through tactile stimulation involves the formation of novel connections, suggesting the brain adapts and changes. Evidence of plasticity was later found in many other mammals such as rats, cats, and songbirds (Guth, 1976; Brown, 1975; McEwan, 1980). Increasing evidence suggests almost all brain areas are capable of neural plasticity.

A known and commonly investigated mechanism underlying nervous system adaptation involves the alteration of strength and type of neural connections, or synaptic plasticity, by the modification of neurotransmitter release rate or receptor concentration. As the Hebbian theory suggests, “Cells that fire together, wire together.” The repeated stimulation of a neuron creates an increased synaptic strength and efficacy of the neurons at that synapse.

A broader view of variation in the nervous system is developmental plasticity, the alteration of the nervous system throughout development resulting from environmental interactions and experience. Developmental plasticity differs from hard-wired developmental mechanisms, which produce a common and organizationally conserved nervous system in species, by providing alteration of the nervous system throughout development specific to an individual organism. Although developmental plasticity encompasses synaptic plasticity, it also involves a less familiar type of plasticity mediating the numbers of neurons belonging to particular classes, termed neuronal composition. Throughout development, neural stem cells, or neuroblasts, differentiate and produce specialized neurons. Just as specialized cells exist from tissue to tissue,

classes and even subclasses of neurons have distinct roles in the nervous system and may be differentiated based on morphology and gene expression. The regulation of neuronal composition, or the number and ratio of types of neurons in the nervous system, may have profound effects on the organism. Mechanisms of neuronal composition remain largely unknown, leaving open opportunities for truly novel findings in this area.

Holometabolous Insects

The study of developmental plasticity remains difficult in mammalian organisms due to the size and complexity of the nervous system. Research is instead focused on insects, which exhibit smaller, simpler, yet still extremely complex nervous systems, allowing for studies of plasticity not feasible in mammals or larger organisms. Surprisingly, the insect brain has many regions analogous to those of mammals (Sweeney & Luo, 2010), suggesting that research on these areas could have direct relevance and significance to studies in humans.

The model genetic organism *Drosophila melanogaster* is ideal to study molecular mechanisms involved in the plasticity of neuronal composition. Important to developmental studies are the short life cycle of *Drosophila*, small number of chromosomes, and easily induced and preserved mutations. The nervous system has been studied extensively and many aspects of neuronal development have been investigated providing vast foundational research for intricate and specific studies of the organism.

Drosophila melanogaster, like all holometabolous insects, undergoes complete metamorphosis, a process in which an animal will abruptly change from an immature to adult form through cell death, growth, and differentiation. *Drosophila* females lay eggs while offspring are still in their embryonic stages. Larvae emerge after hatching and, due to their rigid exoskeletons, undergo ecdysis, the shedding of their outer cuticle layer, multiple times throughout development. Prior to the first shedding, larvae are termed 1st instar larvae. Two subsequent instances of shedding occur, allowing the larvae to progress to 2nd instar and finally 3rd instar. 3rd instar larvae ecdyse to form a pupal cuticle and undergo metamorphosis. Metamorphosis is dependent on a critical weight that must be achieved by the larva (Truman & Riddiford, 2005). Once this is reached, hormonal mechanisms induce widespread changes throughout the organism. After metamorphosis is complete, the adult fly emerges from its pupal case in a process termed eclosion.

Developmental plasticity is striking in holometabolous insects, which must prepare two nervous systems capable of distinct functions over the course of development: not only must neural circuits allow a larva to maneuver and find sources of food, but they must also confer flight, walking, and reproductive behavior on the morphologically distinct adult (reviewed by Levine & Restifo, 1995). Thus, the nervous system of a holometabolous insect must be reconstructed through programmed cell death, postembryonic neurogenesis, and the modification of persistent neurons. In one well-studied example, the olfactory system must undergo metamorphic changes to accommodate appropriate behavioral responses to volatile molecules for both larval and adult forms (Lee et al., 1999; Marin et al., 2005). Remarkably, the olfactory system

seems to have a highly conserved organization from insects to mammals while also exhibiting reduced numerical complexity in comparison to mammals (Couto et al., 2005), making it an especially attractive system in which to study plasticity in neuronal composition.

Olfactory System & Mushroom Body

The olfactory system is required for olfaction, or the sense of smell. Organisms are capable of discriminating between thousands of volatile organic compounds and associating certain compounds with appropriate outcomes and responses (reviewed by Firestein, 2001). The extreme diversity of compounds requires the olfactory system to be flexible and requires an intimate relationship with learning and memory.

In insects, the MB serves as an astonishing and unique brain structure in the olfactory system required for olfactory learning and memory. The MB, when first discovered in bees and ants, had been suggested as the seat of intelligence (Dujardin, 1850). Continued research on the structure has indeed supported a role in intelligence, as it is essential to learning and short-term memory (Heisenberg, 2003).The MB is also involved in other complex adaptive behaviors, such as modulation of food-seeking behavior during satiety and hunger, visual context generalization, choice behavior, courtship, and sleep **(**Krashes et al., 2009; Liu et al., 1999; Tang and Guo, 2001; McBride et al., 1999; Joiner et al., 2006).

Genetic and behavioral studies have shown that plasticity at the synapses of Kenyon Cells (KCs), the intrinsic mushroom body neurons, contribute to the acquisition

and storage of memory (Gu & O'Dowd, 2006). Therefore, the MB has been primarily implicated in the formation and recall of olfactory memory in both the larva and adult *Drosophila* (Pauls et al, 2010; Heisenberg, 2003), suggesting a high level of plasticity in this region.

The MB's are a pair of bilaterally symmetric brain structures that contain ~2500 Kenyon Cells (KCs) located in the dorsal protocerebrum (Figure 1A, B) (Gu & O'Dowd, 2006). KC's belong to one of at least three morphologically distinct subclasses of neurons (γ, α'/β', α/β) based upon their axonal projections into the lobe region of the MB and levels of expression of the cell-surface adhesion protein FasII (Crittenden et al., 1998). The dendrites and axons of KC's form the calyces and the peduncle, respectively, and also form five distinctive types of lobes (γ, α', α, β', β).

In the MB, four neural stem cells, or neuroblasts, produce the same set of neurons and glial cells (Ito et al., 1997) and are capable of generating the three distinct subtypes of neurons (Lee & Luo, 1999). The neuronal subtypes are generated sequentially by the divisions of neuroblasts, and their axons project into their respective lobe(s). The switch from one neuronal subtype is coincident with certain periods in development, particularly the time between the last larval molt and metamorphosis (Figure 1C) (Lee & Luo, 1999). During embryogenesis, ~50 γ neurons are generated, and after larval hatching, ~500 more neurons are produced. Neuroblasts generate γ neurons until the middle of the 3rd instar (Lee & Luo, 1999). α'/β' neurons are then generated between the middle of the 3rd instar and puparium formation, and α/β neurons are produced during metamorphosis.

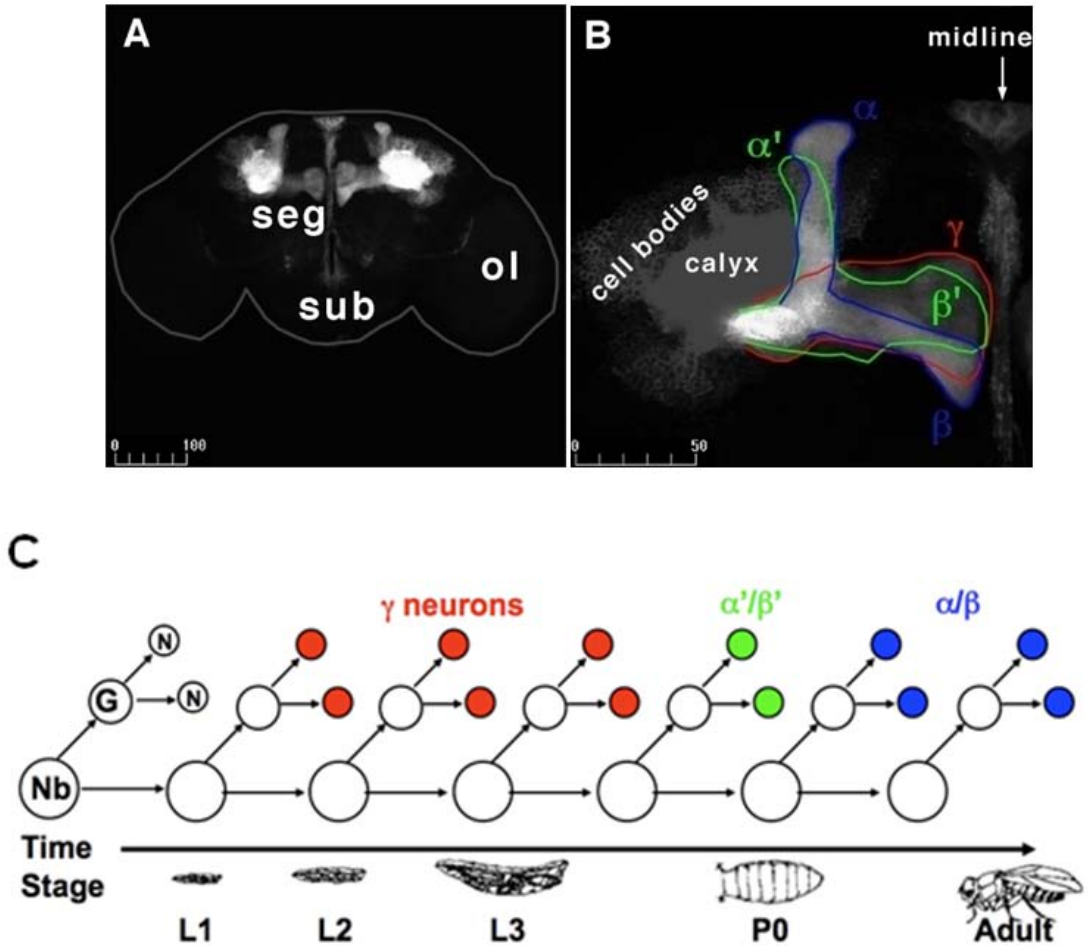

Figure 1 Adult *Drosophila* mushroom body anatomy and KC production. (A) Composite confocal image using immunohistochemistry to label the whole MB. (B) γ, α'/β', and α/β lobes are outlined in red, green, and blue, respectively. Distinct branching patterns identify each lobe. (C) The generation of KC subtypes is coincident to key developmental time periods. Neuroblasts (Nb) produce γ neurons until 3rd instar and then abruptly switch to creating α'/β' until P_0. During metamorphosis and in the adult, α/β neurons are generated.

Mechanisms of MB Developmental Plasticity

Although the nature of the switch from one MB neuronal subtype to another has not yet been elucidated, the timing of the production of MB neuron subtypes with development suggests hormonal influences are involved. Given the essential role of hormones in many aspects of development, a hormone-regulated mechanism for developmental plasticity in the MB is likely and has in fact been found in other insects. In the honeybee *Apis mellifera*, hormonally responsive KC's and their nuclear receptors are known to regulate MB structure and neuropil formation (Velarde et al., 2009). In *Drosophila*, ecdysone is responsible for γ neuron reorganization during metamorphosis (Lee et al., 2000).

Preliminary studies investigating the effects of environmental conditions on MB development have been conducted and suggest that neuronal composition is hormonally regulated. When *Drosophila* larvae are fed temporarily on a 20% sucrose diet, known to arrest larval growth but not affect MB neuroblast divisions, there is an increase in the numbers of the first born γ neuron subclass in the brain (Figure 2) (Marin, Apenteng, and Truman, unpublished). This regulation of composition due to dietary restrictions implies the possibility of MB-extrinsic factors, such as hormones, regulating proliferation rates of neuroblasts or survival of neuronal classes. Two key developmental timing regulators, ecdysone and Juvenile Hormone (JH), were therefore studied for their effects on MB neuronal composition during development.

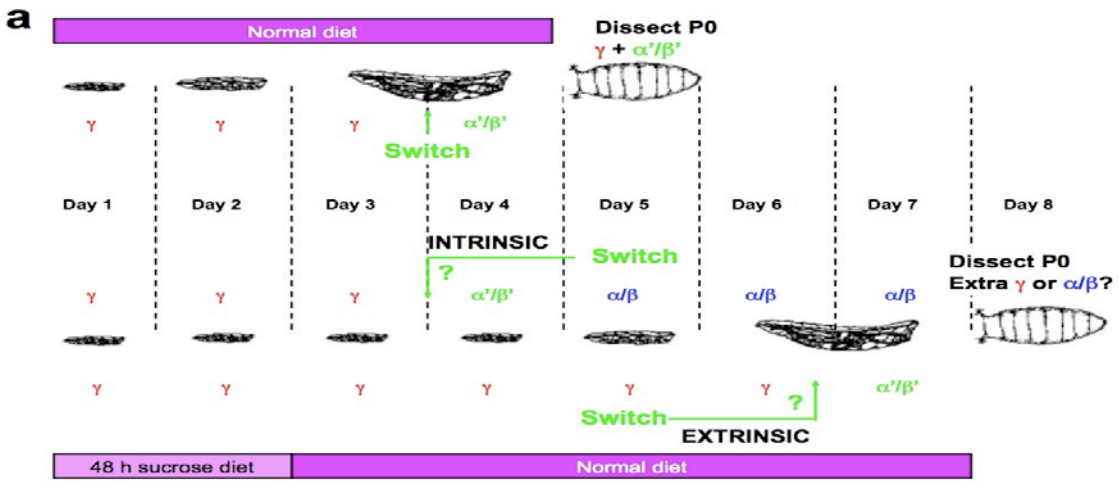

Figure 2 Determining Role of Environmental Factors in MB Development. On a normal diet, the switch from the production of γ neurons to α'/β' will occur after 3rd instar. When larva are fed on a sucrose diet for 48 hours, the number of γ neurons later in development will be indicative of an intrinsic or extrinsic cue governing the switch to α'/β'. If analysis of neuron number at P_0 indicates extra γ neurons, the cue is extrinsic. However, if there is no difference in γ neuron number, the switch is likely

Juvenile Hormone & Ecdysone

The timing of insect development is governed primarily by JH and Ecdysone. JH, released by the corpora allata, has classic 'status quo' action in preventing larvae metamorphosis while promoting larval growth and development (Riddiford, 1994; Riddiford et al., 2001). Before each larval ecdysis occurs, JH levels increase to counteract ecdysone and prevent metamorphosis. However, titers of JH decrease after 3rd instar has been reached to allow the insect to undergo metamorphosis. Both JH and ecdysone are known to regulate additional aspects of insect nervous system development (Bownes et al., 1989; Robinow et al., 1993; Lee et al., 2000).

In the MB, the ecdysone receptor (EcR) and its co-receptor ultraspiracle (USP) form a heterodimer expressed in γ neurons and mediates their reorganization and pruning

during metamorphosis (Lee et al., 2000). The surge in ecdysone before metamorphosis correlates with the transition of KC production from α'/β' to α/β. Using dominant negative ecdysone receptors and mutant USP alleles, the relationship between EcR and temporal MB neuronal subtype composition was investigated. Results did not support a role for the canonical ecdysone signal transduction pathway in the control of neuronal subtype composition (Marin, Apenteng, and Truman, unpublished).

Although ecdysone was not implicated as the extrinsic signal, another plausible candidate was JH. Recent results from loss and gain of JH function experiments suggest a direct relationship between JH levels and α'/β'neuron number (Lubin, Dincer, Kanwal, Wakulchik and Marin, unpublished). These findings advocate an exciting model of hormonally regulated plasticity of the neuronal composition of the MB. If there is such hormonal regulation, however, intracellular factors must transduce the signal to alter expression of genes. Both ecdysone and JH regulate the expression of an early-gene downstream target, the transcription factor *Broad-Complex* (*BR-C*).

Broad-Complex (BR-C)

BR-C activates late-genes involved in programmed cell death of larval tissues for metamorphosis (Ashburner et al., 1974). Specifically, ecdysone induces widespread changes in *BR-C* expression to initiate puparium formation (Fletcher & Thummel, 1995). In addition, *BR-C* mediates the action of the JH hormonal signal in metamorphic reorganization of the CNS, salivary glands, and musculature (Restifo & Wilson, 1998).

Alternative splicing of *BR-C* transcripts links a common 5' BTB/POZ-coding exon to one of four zinc-finger-coding exons (Z1, Z2, Z3, & Z4) (Figure 3) (DiBello et al., 1991; Bayer et al., 1996), generating one of four distinct isoforms. BR-C proteins act as site-specific, DNA-binding transcription factors (Hodgetts et al., 1995). Alternative splicing of the zinc fingers would presumably confer the ability to regulate distinct subsets of genes or mediate protein-protein interactions. For instance, as revealed by mutant alleles disrupting specific isoforms, Z1 mediates salivary gland-specific late gene transcription (*rbp+*), Z2 mediates envagination of imaginal discs (*br+*) and Z3 mediates fusion of imaginal discs (*2Bc+*). There is no known genetic correlation with the Z4 isoform (DiBello, 1991).

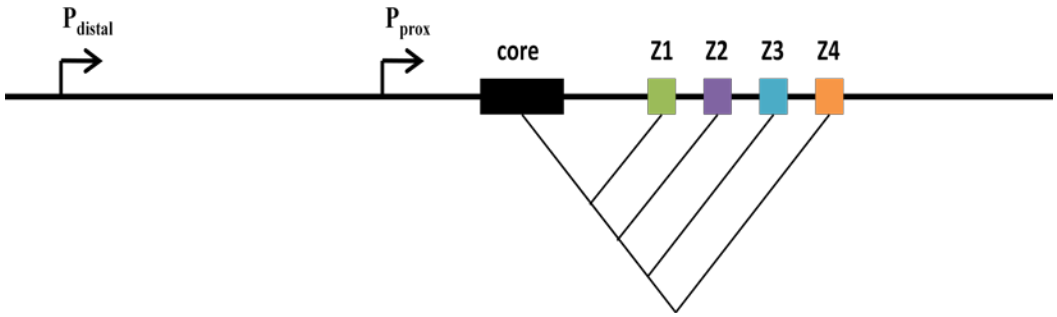

Figure 3 *BR-C* Gene Map. Two promoters, P_{distal} and P_{prox} facilitate the transcription of *BR-C* isoforms. Among all isoforms, a common core 5'BTB/POZ-coding exon is retained. Zinc-finger coding exons are alternatively spliced to form Z1, Z2, Z3, or Z4 isoforms.

Throughout the nervous system, individual *BR-C* isoforms display distinctive temporal and spatial expression patterns. BR-Z3 expression is transiently present in many neuroblasts lineages during embryonic, larval, and pupal stages, with highest expression seen in the earliest born neurons (Zhou et al., 2009). Neurons generated during larval growth express BR-Z3 soon after birth, coinciding with the period when the neuron has finished its outgrowth to initial targets. BR-Z4 is expressed in all neuronal lineages born during a certain period (Zhou et al 2009), and BR-Z1 expression may be limited to glial cells (Spokony & Restifo, 2009); their selective and persistent expression suggest roles in providing molecular time-stamps of birthdates (Zhou et al., 2009). BR-Z2 is expressed in proliferating neuroblasts (Spokony & Restifo, 2009). Importantly, BR-Z3 expression has also been found in the developing MB (Zhou et al., 2009). The differential expression patterns for each isoform suggest that each mediates unique aspects of central nervous system development.

Preliminary studies have uncovered a role for BR-C and specifically BR-Z3 in the MB for neuronal composition, though the mechanism of plasticity is still unknown (Marin, Apenteng, and Truman, unpublished). Initial overexpression of the *broad* isoform BR-Z3 led to a significantly decreased number of γ neurons in the MB accompanied by a similar decrease in total number of neurons (Figure 4b, d). Additionally, knockdown of all four BR-C isoforms through BR-Core RNAi resulted in an increase in all non-γ neurons and total neurons (Figure 4c, d).

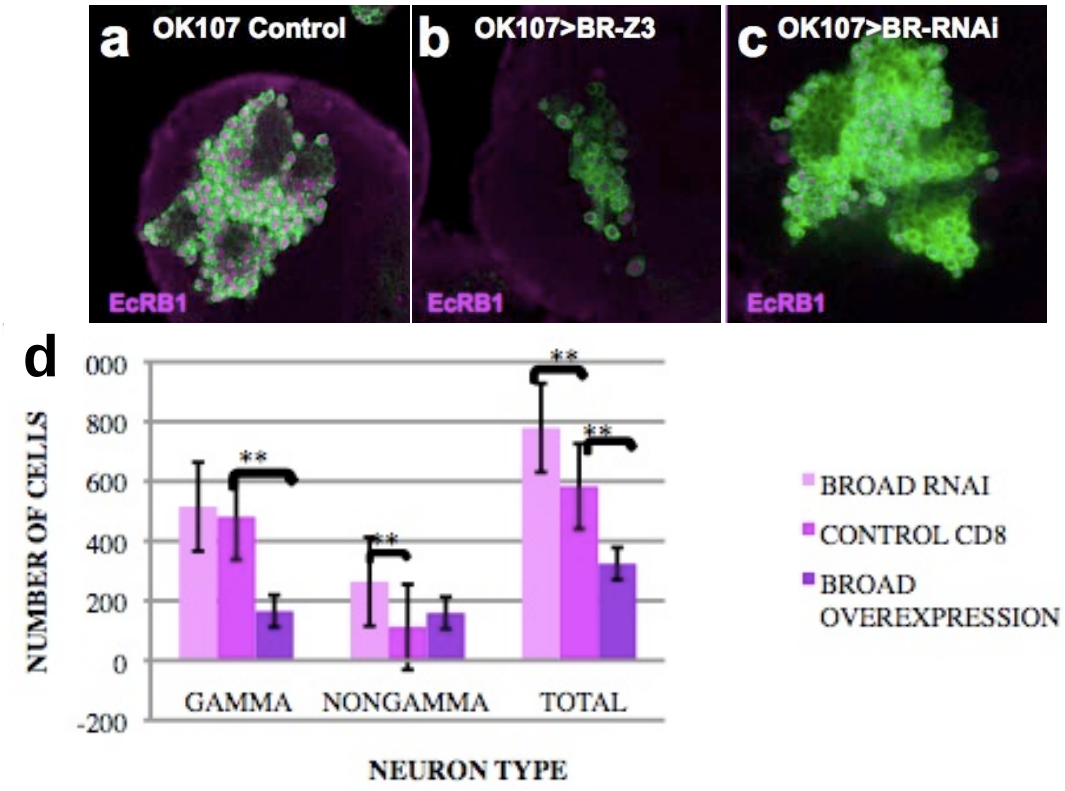

Figure 4 Effects of BR-Core Knockdown and BR-Z3 Overexpression on Neuronal Composition of MB. EcRB1 expression (purple) labels gamma neurons. mCD8 expression (green) labels all MB neurons. (A) OK107 GAL4 driver control. (B) OK107>BRZ3 Overexpression. A dramatic decrease in both γ neurons and total MB neurons is observed. (C) OK107>BR-RNAi knockdown of all *BR-C* isoforms. There is an observable increase in non-γ neurons and total neurons. (D) Numerical analysis and comparison of OK107 control to OK107>BR-Z3 and OK107>BR-RNAi. OK>BR-Z3 animals showed a significant decrease in γ neuron number as well as total number of neurons in the MB. OK107>BR-RNAi animals exhibited a significant increase in non-γ neurons in addition to a significant increase in total MB neurons.

BR-Core RNAi results suggest expression of other isoforms as well. To clarify, if BR-Z3 were the only isoform normally expressed in the MB, knockdown of BR-Core should have resulted in an increase in γ neurons, the opposite result of BR-Z3 overexpression. These results therefore suggest an interesting model (Figure 5) in which Z3 and an unknown isoform have antagonizing roles in mediating neuronal composition

in the MB. Additionally, the differences in total number of neurons imply changes in cell survival or proliferation as a method of isoform-mediated neuronal composition.

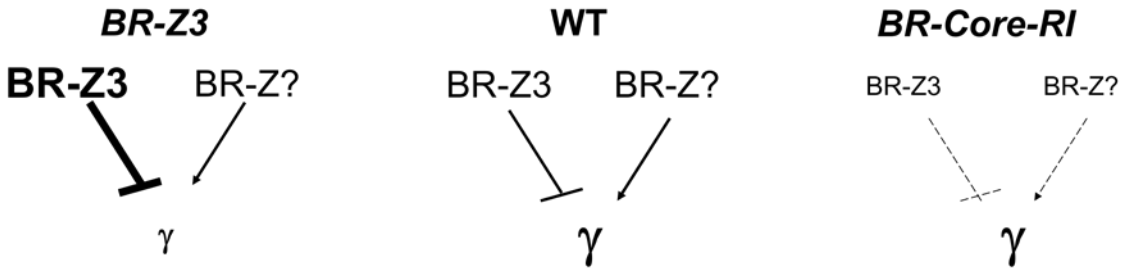

Figure 5 Model of isoform interaction mediating neuronal composition in the MB.

Hypothesis

Based upon prior experiments and results, I hypothesize that BR-C regulates aspects of developmental plasticity in neuronal composition of the MB in an isoform-specific manner. To test this, I pursued three aims for my thesis work. First, I imaged and quantified the γ, non-γ, and total KC's in control, BR-Z1, and BR-3 overexpression *Drosophila* brains that had been previously generated and mounted on slides (A. Barnard and E. Marin, unpublished) in order to analyze the effects of gain of function *BR-C* manipulations on neuronal composition. Second, I built transgenic fly lines with which to drive expression of the RNAi enhancer *dicer-2* as well as each BR-C isoform-specific RNAi construction in MB to perform knockdown experiments and analyze the resulting loss of function phenotypes. Finally, I initiated the creation of pWIZ-based *BR-C*

isoform-specific RNAi constructs to activate tissue-specific RNAi for the aforementioned knockdown of each isoform and analysis of the resulting phenotypes.

The GAL4/UAS System

Developed by Andrea Brand and Norbert Perrimon in 1993, the GAL4/UAS system serves as a molecular genetic method to express transgenes of interest in *Drosophila* (Figure 6) (Brand & Perrimon, 1993). GAL4 is a yeast transcription factor protein that binds to an Upstream Activating Sequence (UAS) to activate gene transcription. Mobilization of an inserted GAL4 to new sites in the genome will allow its expression to be controlled by a nearby tissue-specific enhancer. When GAL4 lines are crossed with strains of flies which have UAS regions cloned upstream of a gene of interest, GAL4 will be expressed, and the GAL4 protein will bind to the UAS region and activate gene transcription, in a specific subset of tissues or cells. Coupled with the RNAi system, tissue-specific disruption of RNA translation of the gene of interest is possible by driving expression of a sequence that forms hairpin dsRNA and activates the fly's endogenous RNAi pathway.

RNA Interference (RNAi)

The development of *Drosophila* transgenic flies for knockdown experiments requires the use of RNAi to silence isoform expression and the GAL4/UAS system to limit RNAi expression specifically to the MB. The pWIZ plasmid, capable of expressing

double-stranded loopless hairpin RNA to activate RNAi, was utilized for the creation of transgenic UAS-RNAi flies.

In many Eukaryotes, an RNAi defense mechanism has evolved against viruses in which the enzyme Dicer prevents translation of RNA to protein thereby eliminating gene products. Dicer cleaves double-stranded RNA into short nucleotides fragments. One of these strands is degraded and the other is used as a template to bind to other RNA and activate the RNAi mechanism. RNAi is a useful tool for research, allowing post-transcriptional modulation of any gene's expression. Coupled with the GAL4/UAS system, RNAi can be specific to tissues or even a small number of cells.

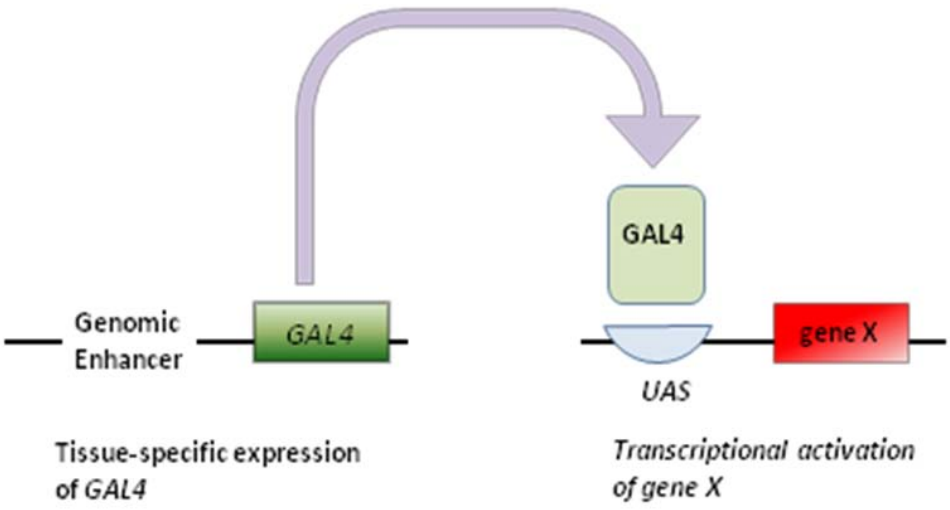

Figure 6 GAL4/UAS System. GAL4 is a yeast transcription factor not normally present in other organisms. A GAL4 gene is placed under the control of a driver gene, while the UAS controls expression of a target gene. GAL4 is then only expressed in cells where the driver gene is usually active. In turn, GAL4 should only activate gene transcription where a UAS has been introduced.

pWIZ Plasmid

The pWIZ (White Intron Zipper) plasmid is a vector capable of expressing loopless hairpin dsRNA (Lee &Carthew, 2003) (Figure 7). Derived from the pUAST plasmid, pWIZ utilizes the GAL4/UAS system, thus providing expression with cell and temporal specificity. Constructing the RNAi-inducing pWIZ plasmid involves sequential insertion of gene fragments upstream and downstream of the 74-base pair (bp) *white* intron in inverse orientation (Figure 8). In the fly, the *white* intron will be spliced out to produce complementary RNA fragments to hybridize with each other, forming a hairpin capable of triggering RNAi. Flanking the intron in pWIZ are restriction sites to insert gene fragments; *AvrII and NheI* exist on the 5' and 3' end, respectively. PCR products therefore must be digested with SpeI, AvrII, NheI, or XbaI to produce ligation-compatible overhangs. For stability purposes, fragments are also required to be 500-700bp in length.

After the insertion of two inverse fragments has been verified, pWIZ will be injected into *Drosophila* embryos. P-elements transposon repeats, or sequences of DNA that are capable of mobility and integration, are present in the plasmid to introduce the transgene into the *Drosophila* genome in the presence of a coinjected transposase. Once stably integrated, this construct will produce BR-C isoform-specific dsRNA in response to transcriptional activation by a tissue-specific GAL4 driver – in this case, in the MB.

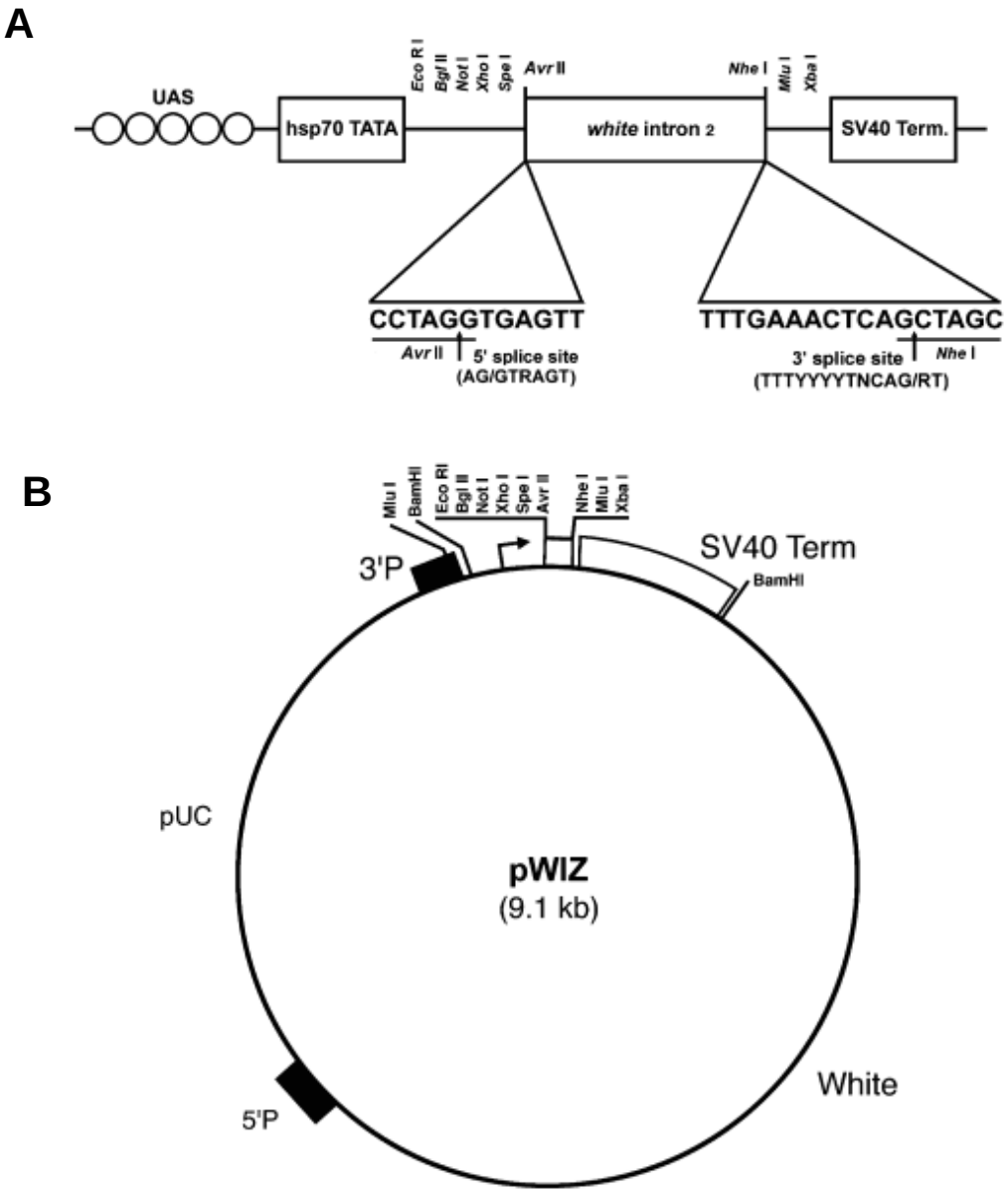

Figure 7 pWIZ Plasmid. A) Restriction sites are shown flanking the *white* intron. B) The pWIZ plasmid includes a *White* gene, a *white* intron, and 3' and 5' P-element sites for transposase-mediated insertion of pWIZ into *Drosophila* genome.

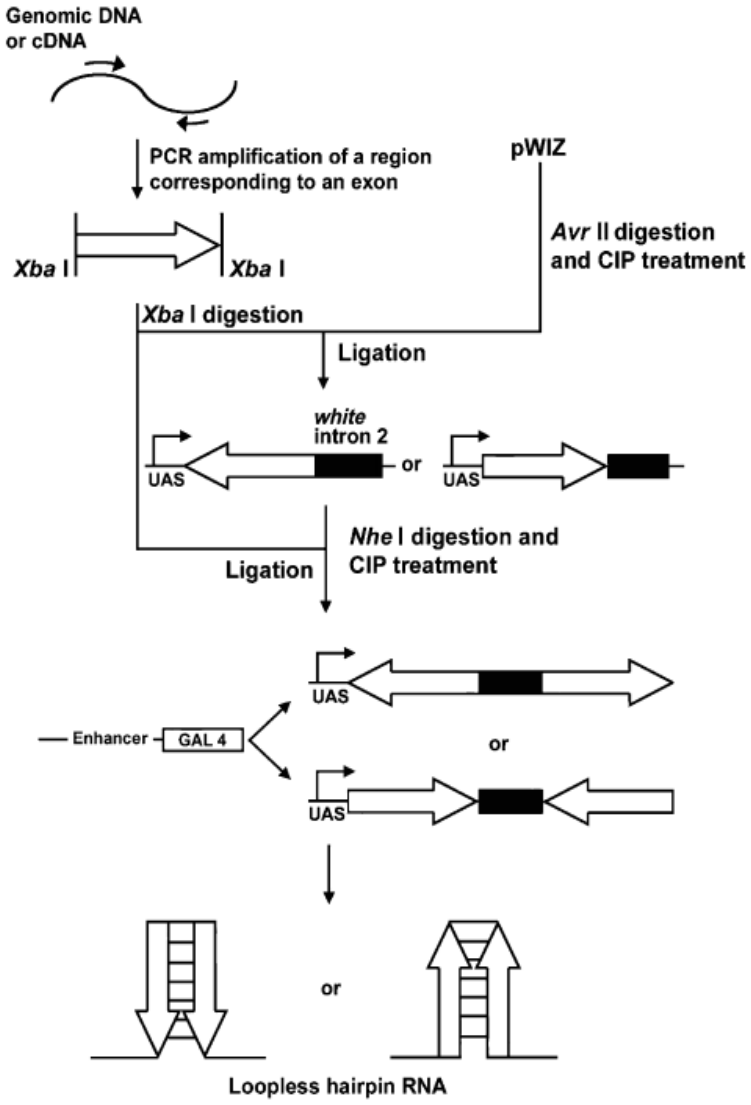

Figure 8 Procedure for making an isoform-specific RNAi construct using the pWIZ vector. A DNA fragment corresponding to a portion of a broad isoform is amplified by PCR. A restriction site compatible with AvrII and NheI is present at the 5' end of each PCR primer. The PCR product is inserted twice, by two ligation steps, into the AvrII and NheI sites of pWIZ. Two ligation steps are required to insert PCR products in an inverse orientation on either side of the white intron. Transformation follows to generate transgenic lines carrying the WIZ gene. Crossing transgenic animals with the WIZ gene with animals carrying MB-specific GAL4 drivers creates F1 progeny, which produce loopless hairpin RNA. This induces RNAi against a specific isoform of *BR-C*.

Materials and Methods

Overexpression and Immunohistochemistry

Overexpression experiments were conducted to observe the effects of increased concentration of *BR-C* isoforms in the MB. BR-C overexpression lines were obtained from X. Zhou and L. M. Riddiford (U. Washington, Seattle). The UAS-dicer-2 line was obtained from M. Rolls (Penn State University). UAS-CD8-GFP, OK107 flies were crossed with w^{1118} (control), UAS-BRZ3 (BR-Z3 overexpression), and UAS-BRZ1 (BR-Z1 overexpression). Progeny were raised on Carolina instant medium at 25°C at the same density.

P_0 brains were dissected, fixed in 3.7% paraformaldehyde, and stained with 1D4 (anti-FasII, Developmental Studies Hybridoma Bank) at 1:20 and rat anti-mCD8 (Caltag/Invitrogen) at 1:100 and then with TxR anti-mouse and FITC anti-rat (Jackson Immunoresearch Labs) at 1:300. The brains were then mounted on slides.

Confocal Microscopy & Image Analysis

Using a Leica SP5 laser scanning confocal microscope, the MB cell bodies were imaged. A stack of images were produced to observe the MB in 3-dimensions, with each slice within a stack representing an increase in the *z-axis.* ImageJ, an image processing and analysis software (http://rsbweb.nih.gov/ij/), was used with a customized Cell Counter Plugin (G.S.X.E Jefferis) to count the number of γ (mCD8+, EcRB1+) and non-γ (mCD8+, EcRB1-) neurons.

.Each cell was manually counted on a single frame and the position was marked +/- 5 frames to avoid recounting the same cell. All frames within a sample were analyzed.

Statistical Analysis of Results

Cell counts were recorded for the MB of each brain and the numbers of γ neurons, non-γ neurons, and total were averaged for at least seven samples. A student's t-test was performed to test for significant differences between the averages of γ neurons, non-γ neurons, and total neurons in control and experimental groups.

Primer Design & PCR

Forward and reverse primers were made for each of the four isoforms. Primer design was based upon creating a PCR product 500-700bp in length with an additional concern for primer stability (Table 1). The necessity of ligand-compatible restriction sites required mismatches in the primers to create novel restriction sites if none existed in the gene fragment; these mismatches were kept at the 5' end to decrease instability caused by a mismatched base. For instance, in the Z1 isoform-specific sequence, there existed a 3' internal SpeI site close with no other compatible site. The sequence was searched for a 6-bp sequence only one bp different from either a NheI, SpeI, Xba, or AvrII site. The sequence 'CCTAGT', similar to the SpeI site 'ACTAGT', was found at an appropriate position within the sequence. The forward primer was made to encompass the SpeI-similar sequence and included a mismatched 'A' base to create the 'ACTAGT' site.

Similarly, the Z3 forward primer required a mismatched base to create a novel restriction site. Compatible restriction sites did not exist in Z2 and Z3 sequences and both forward and reverse primers had mismatched bases substituted to create novel restriction sites. Primers were ordered from Eurofins MWG Operon.

Drosophila DNA was extracted from two w^{1118} flies using DNeasy Blood & Tissue Kit protocol (QIAGEN). DNA was eluted in 100 μL buffer AE, provided by DNeasy kit. Nucleic acid anaylsis was performed with Nandrop1000 (ThermoScientific) to determine concentration and impurities in the sample.

Primers were initially diluted 1:100 in TE buffer (10mM TRIS; 1mM EDTA; pH 8) and further diluted 1:10 in TE buffer for use in PCR reaction. A reaction mix and was made for each isoform and included Platinum *Taq* Polymerase (Invitrogen), Primer Forward and Primer Reverse, and either DNA or water (negative control NC). PCR was performed for 35 cycles of 94°C for 1min, a temperature ranging from 45-55°C depending on primer melting temperatures (T_m) for 30sec, and 74°C for 1min. Samples of the PCR products were electrophoresed on 1% agarose gel at 90V for 1hr.

Cloning into TA Plasmid & Transformation of One Shot® Competent Cells

PCR products were then cloned into the pCRII plasmid with T4 DNA ligase provided by the TA cloning kit, following the manufacturer's protocol (Invitrogen). The appropriate volume of PCR product was used to produce a 1:1 vector:insert molar ratio. Once ligation was completed, the construct was transformed into *One Shot® Competent*

INVαF' cells following the manufacturer's protocol (Invitrogen). To grow the bacterial cells, 40μL of 100mM IPTG and 40mg/mL X-GAL were added to Luria Broth (LB) agar plates containing 100 μg/mL ampicillin. Either 10 or 50μL of each transformation reaction was spread onto LB agar plates. Plates were incubated at 37°C overnight and moved to 4°C for 3hrs to intensify color before counting blue vs. white colonies. For plasmid isolation and restriction analysis, 10 white colonies from each plate were used to inoculate 3mL LB containing 100 μg/mL ampicillin and placed overnight in a 37°C shaking incubator.

Isoform	**Primer Sequence**	**Length**	**% GC**
Z1	Forward: 5' GTCTAGACTAGTTACAATTAGTTGC 3'	25	36
	Reverse: 5' TGTTACGGTGCGCTTAACC 3'	19	52
Z2	Forward: 5' CACCACTAGTATTTCCACCACGG 3'	23	52
	Reverse: 5' TTGGTCTAGAAAATTCATGCGTTCAT 3'	26	34
Z3	Forward: 5' AGGAGCTAGCCATCTACCCAAAGC 3'	24	54
	Reverse: 5' CGTATTCTAGATACTGGTCCATAGGC 3'	26	46
Z4	Forward: 5' CCGTCTAGAGCCTACACAGTCACG3'	24	58
	Reverse: 5' ATACAGACTAGTCGCGGATGGGACA 3'	25	52

Table 1 Forward and Reverse Primers for *BR-C* Isoforms. Underline indicates restriction site. Bases highlighted in red represent a mismatched basepair for the creation of a novel restriction site. Lengths of each forward and reverse primer are shown along with their %GC content.

Miniprep Procedure and Restriction Digests

Following QIAprep Spin Miniprep protocol (QIAGEN), TA plasmid constructs were isolated from each of the bacterial cultures (QIAGEN). After isolation, a restriction digest using appropriate restriction enzymes (New England Biolabs) was performed to cut the fragment from the TA plasmid. Restriction enzymes were chosen based upon the known internal sites or primer-made restriction sites. Reactions were incubated at 37°C for at least 1hr. Digests were then run on 1% agarose gels to confirm successful ligation and isolation of construct. Fragments which corresponded to their predicted size were sent to Genomics Core Facility, University Park for sequencing. Sequencing confirmed the fragments were indeed *BR-C* isoform sequences and free of mutations introduced by DNA replication. For each isoform, the miniprep corresponding to the fragment with the fewest replication errors was chosen for insertion into the pWIZ plasmid.

TA plasmids were digested with the appropriate restriction enzymes (Table 2). The Volume of miniprep DNA required in each reaction was calculated by determining concentration of miniprep and using a total of 5 μg of DNA The total volume of digest DNA was run on a 1% agarose gel at 90V for 45min. The fragment which corresponded to the insert was isolated using QIAEX II isolation kit (QIAGEN) following manufacturer's protocol. Concentration of isolated DNA was determined to verify successful isolation.

Insertion of Fragments into pWIZ: 1st Insert

pWIZ plasmid was transformed into One Shot® Competent Cells following the manufacturer's protocol (Invitrogen). Plasmid was extracted in high volume and concentration using the QIAfilter Plasmid Midi Kit (QIAGEN) following the manufacturer's protocol.

2μg pWIZ plasmid was digested with AvrII in a total reaction volume of 25 μL. Digested pWIZ was treated with alkaline phosphatase (Apex) to prevent religation following the manufacturers protocol (Epicentre). Isolated fragment DNA was then ligated to pWIZ with T4 ligase (Epicentre) using the Fast-Link DNA Ligation Kit. Volumes of pWIZ and fragment DNA were determined by calculating a 5:1 pWIZ:fragment molar ratio (CIP'd pWIZ, fragment DNA, 1.5 μL 10x Fast Link Ligation Buffer, 1.5 μL 10mM ATP, and dH_2O) for a total reaction volume of 15 μL. Procedure followed the manufacturer's protocol (Epicentre).

Ligation products were each transformed into GC10 Competent Cells following manufacturers protocol (Sigma). As pWIZ features ampicillin resistance but not blue/white screening, colonies were selected randomly for verification of insert. Isoform sequence was searched for internal restriction sites for use in a restriction digest to confirm successful insertion. Construct DNA was isolated with miniprep procedure and digested with the appropriate restriction enzyme. Digested miniprep DNA was run on a 1% agarose gel and the appearance of a fragment of the predicted size suggested the construct now included the first insert. DNA from samples believed to contain an insert

was transformed into GC10 Competent Cells following the manufacturers protocol (Sigma) and isolated using a QIAGEN Midiprep Kit.

Insertion of Fragments into pWIZ: 2nd Insert

Midiprep DNA was digested using the NheI restriction enzyme (5 μg midiprep DNA, 2 μL 10X NEB2 reaction buffer, .2 μL 100X BSA, .1 μL NheI, dH_2O) in a total reaction volume of 25 μL. The same procedure for the 1st insert was used to ligate and verify the insertion of the fragment. However, restriction sites were chosen based upon the ability to predict fragment size when digests were run on a gel and infer inverse orientation.

For further verification of the insertion of two fragments, forward and reverse sequencing primers were designed to complement DNA in the *white* intron. Sequencing results were submitted to NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for comparison to the *Drosophila* genome.

Results

BR-C Isoform Overexpression

Alternative splicing of *Broad-Complex* produces for distinct isoforms, Z1-Z4, each with distinct roles in development. I sought to determine if there were differential functions of *BR-C* isoforms in the MB. Overexpression experiments were conducted to observe the effects of increased concentration of *BR-C* isoforms in the MB. UAS-CD8-GFP, OK107 flies were crossed with w^{1118} (control), UAS-BRZ3 (BR-Z3 overexpression), and UAS-BRZ1 (BR-Z1 overexpression).

I first compared animals in which BR-Z1 had been overexpressed in the mushroom body using an OK107 GAL4 driver with matched controls. Qualitiative inspection of the overexpression of Z1 suggested substantially more γ (EcRB1+) neurons at P_0 (Figure 9A). Quantification revealed this increase to be both significant and dramatic (1071 vs 585, $p < .0006$) (Figure 9B). Additionally, the total number of neurons also increased (1225 vs 681, $p < .0001$) along with the number of α'/β' (EcRB1-) neurons (155 vs 97, $p < .01$). The results suggest that BR-Z1 mediates the survival of both γ and α'/β' neurons.

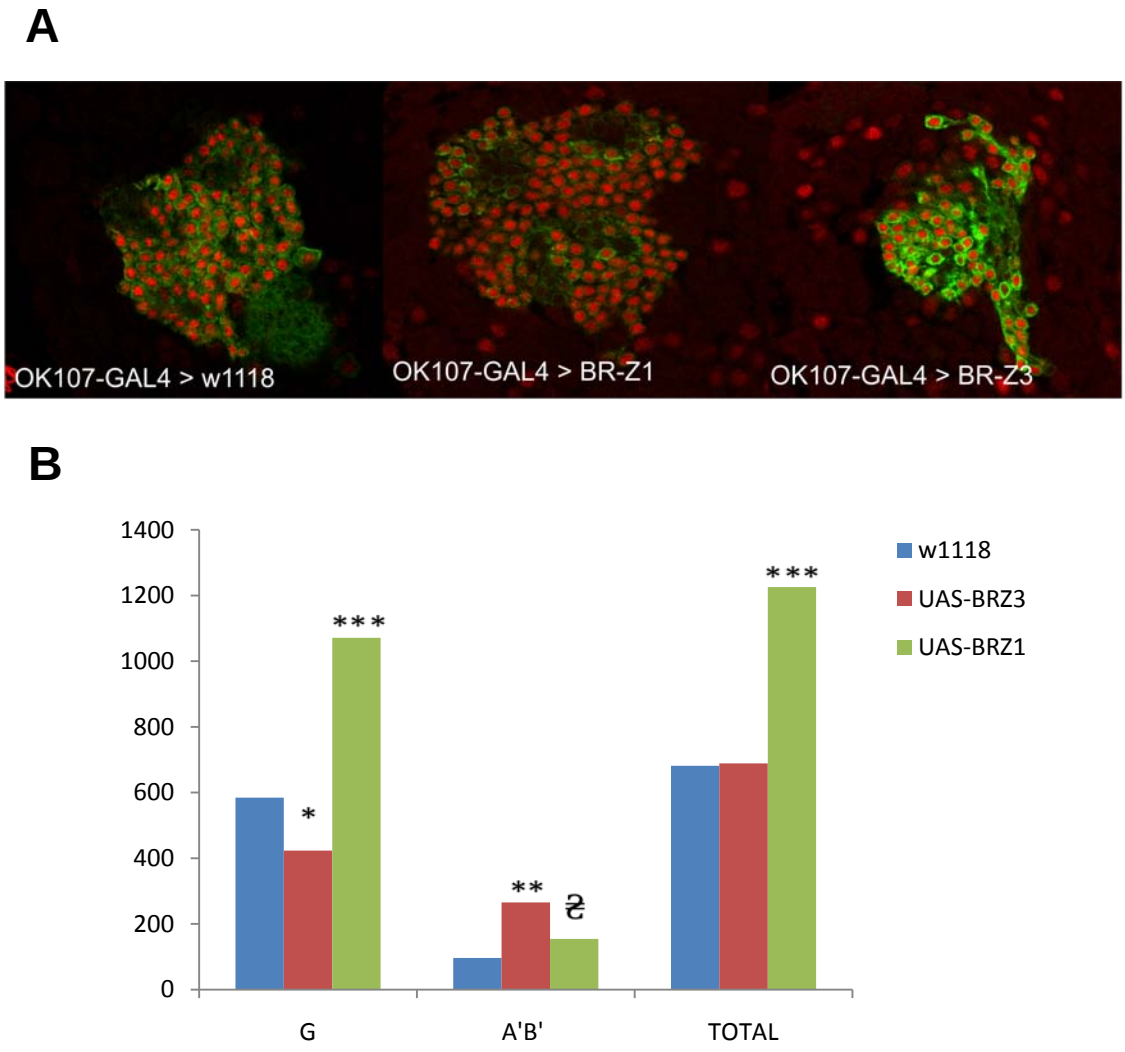

Figure 9 BR-C Isoform Overexpression. A) Confocal imaging of MB for wild type, BR-Z1 overexpression, and BR-Z3 overexpression. EcRB1 expression (red) labels γ neurons and mCD8 expression (green) labels all neurons in the MB. B) Quantification of γ and total neurons in the MB. Overexpression of BR-Z1 results in significantly increased γ neurons (1071 vs 585 $p < .0006$) as well as total MB neurons (1225 vs 681 $p < .0001$). Overexpression of BR-Z3 results in a significant decrease in γ neurons (423 vs 585 $p < .0006$) but total MB neurons remain the same (719 vs 681 $p < .56$).

Animals featuring BR-Z3 overexpression using a MB-specific GAL4 driver were then analyzed. On first inspection of the MB, these animals exhibited a marked decreased in γ neuron number (EcRB1+) (Figure 9A). Numerical analysis supported these findings (Figure 9B). Overexpression of BR-Z3 resulted in a significant decrease in γ neurons (423 vs 585 $p < .0006$), while there was an increase α'/β' neurons (EcRB-) (265 vs 97, $p < .0005$). However, the total number of MB neurons remained the same (719 vs 681 $p < .56$). The results indicate BR-Z1 and BR-Z3 differentially modify neuronal composition in the MB.

Making UAS-RNAi Drosophila Line

A MB-specific GAL4 line is required to selectively initiate RNAi in the MB. The GAL4 enhancer trap, OK107, is an enhancer with high expression in the MB. The OK107, UAS-GFP, and an UAS-*Dicer-2* line will be crossed with the UAS-RNAi line. Progeny from the cross will knockdown expression of *BR-C* isoforms in the MB.

Female virgin *CD8 (UAS-CD8-GFP), yw; Pin/Cyo* stock flies were crossed with *w; UAS-Dicer* males. From the progeny, non-Pin, Cyo males were selected and crossed with CD8, yw; Pin/Cyo virgin females. Non-Pin, cyo flies were selected and crossed to produce a stable *CD8, yw; UAS-Dicer* stock.

Concurrently, another stable stock was being created to cross with CD8, yw; UAS-Dicer. CD8, yw; Pin/Cyo virgin females were crossed with CD8, yw; +/+; OK107 males. CD8, yw; +/cyo; OK107/+ virgin female progeny were crossed with CD8, yw;

Pin/+; OK107/+ male progeny. The progeny of this cross, CD8, yw; Pin/Cyo; OK107?/OK107? were crossed with each other in single sibling pair crosses. After the 2nd generation, vials were selected based on OK107 homozygosity (red eye color due to w+ marker in the OK107 transgene). The crosses produced a *CD8, yw; Pin/Cyo; OK107/OK107* stable stock.

CD8, yw; UAS-Dicer virgin females were crossed with CD8, yw; Pin/Cyo; OK107 males. From the progeny, Cyo, non-Pin virgin females and males were selected and crossed. Single crosses of CD8, UAS-Dicer/Cyo, OK107/OK107? males and CD8, yw; UAS-Dicer/Cyo; OK107/OK107? virgin females were made. After three generations, crosses which produced only red eyes were selected. From these vials, flies were *CD8, yw; UAS-Dicer; OK107*. The *Drosophila* line was verified by dissecting samples from each vial and observing OK107+ expression in the nervous system. This line will be crossed to UAS-RNAi transgenic flies and progeny will be capable of post-transcriptional inactivation of gene expression specifically among tissues expressing OK107, most importantly the mushroom body.

Creation of pWIZ-based Isoform Constructs

Although we had a UAS-RNAi fly line in which all four BR-C isoforms could be knocked down simultaneously by targeting the common core region, UAS-RNAi flies were not available to study individual functions of *BR-C* isoforms. The development of pWIZ-based BR-C isoform-specific RNAi constructs was therefore necessary to

understand the role the isoforms have in neuronal composition in the MB. The vector required the insertion of two fragments from each isoform in inverse orientation such that during transcription, the fragments would complement and form dsRNA (Lee & Carthew, 2003). The fragments were designed to complement sequences of isoform-specific zinc-coding regions in the *BR-C* gene and to amplify a minimum of 500 basepairs (bp) to a maximum of 750 bp (Table 1).

Isoform specific sequences were amplified from genomic DNA isolated from the w^{1118} strain via polymerase chain reaction (PCR). Successful isolation of genomic DNA was verified using the NanoDrop1000, which returned a DNA concentration of 38.9ng/ul and a 260/280 value of 1.73. Using sequences found on FlyBase (http://flybase.org/), novel forward and reverse primers were designed for each isoform. Insertion of fragments into the pWIZ vector requires the use of specific restriction enzymes to create compatible restriction sites, producing complementary overhangs capable of efficient ligation. Given the relatively small size of the four zinc-finger-coding exons, compatible restriction sites sometimes did not exist. Instead, a location within the sequence was chosen that closely matched a restriction site sequence and a primer containing a base mismatch was made to substitute the appropriate nucleotide to form the site. The primers were then used for PCR to amplify each of the isoform-specific regions. The products of each reaction were inserted into a TA plasmid for sequencing. Once insert had been verified, the TA plasmids were digested and run on a gel for fragment extraction. The fragment ultimately will be sequentially inserted twice, in inverse orientation, into the pWIZ vector.

An initial PCR run was conducted using Z1-Z3 primers on *Drosophila* genomic DNA at specific annealing temperatures: 55ºC for Z1, 55ºC for Z2, 59ºC for Z3. Products were run on a 1% agarose gel (Figure 10). The Z1G sample produced a fragment ~500bp in length in lane 2 (Figure 10A). The Z1 PCR product was then ligated to TA plasmids and transformed into OneShot Competent Cells. Minipreps of DNA from white colonies were digested with SpeI and digests were run on 1% agarose gel (Figure 11A).The highest intensity fragments (circled) were chosen for sequencing. Fragments were then sequenced and the results run through BLAST to verify fragment identity and to determine percent mismatch from known isoform sequences. Miniprep Z1_16 matched 99% to the known Z1 sequence (Appendix 1). An 8bp sequence of DNA was unable to be sequenced, most likely due to the presence of repeated thymadine (T) sequence. Miniprep Z1_16 was digested once more and run on a gel for extraction. Gel-extracted DNA was used for ligation into phosphatase-treated pWIZ plasmid digested with AvrII. Successful insertion of the Z1 fragment was verified by digesting pWIZ and running reactions on a 1% agarose gel (Figure 12). Procedures for insertion of the 2nd fragment have been completed but have yet to be verified.

The first PCR amplification of Z2 was unsuccessful. Amplification was reattempted at a lower annealing temperature of 45ºC to facilitate primer binding. The Z2G sample produced a fragment ~1000bp in length (Figure 10C). Because the size was much higher than predicted, the Z2 product was not used for insertion into pWIZ.

PCR successfully amplified a portion of DNA in the Z3G sample and produced a fragment ~350bp in length. PCR products were ligated into the TA plasmid. Miniprep DNA was digested but no fragments were seen when run on a gel (not shown). Repeated cloning procedures of the Z3 isoform produced no visible results (not shown). Methylation of the plasmid was suspected. Therefore, Dcm-/Dam- cells (NEB) were used for cloning to prevent methylation and the cloning and digest procedures redone. Digests were run on a 1% agarose gel (Figure 12B) and two fragments were seen (circled). Miniprep Z3_5 matched 99% with the Z3 sequence with two mismatches. Miniprep Z3_5 was digested once more and run on a gel and the visible fragment was extracted. Gel extraction products were ligated into phosphatase-treated pWIZ plasmid and the pWIZ-construct transformed into Dcm-/Dam- cells. Experiments to verify insertion of the fragment have yet to be conducted.

Initial PCR reaction did not produce any visible fragment for the Z4G reaction mixture. An additional PCR reaction was performed to amplify Z4 isoform sequence at 53ºC and produced a fragment of ~700bp in size (Figure 10B; Lane 4). The PCR product was ligated into the TA plasmid. Miniprep DNA was digested to confirm the presence of the fragment in the plasmid (Figure 11C). The highest intensity fragments (circled) were chosen for sequencing. Of the resulting sequences, the corresponding fragment with the fewest mismatches was chosen for insertion into pWIZ. 99% of miniprep Z4_6 sequence matched the known Z4 sequence, with two mismatches and 1 basepair missing. Miniprep Z4_6 was digested once more and run on a gel for gel extraction. Gel extraction products

were ligated into phosphatase-treated pWIZ. The pWIZ-construct was then transformed and the miniprep DNA was digested to confirm successful ligation (Figure 12).

Isoform	Restriction Enzymes
Z1	SpeI
Z2	SpeI & XbaI
Z3	NheI & XbaI
Z4	XbaI & SpeI

Table 2 Restriction Enzymes for Digestion of Isoform Fragment from TA Plasmid. The Z1 isoform had only one restriction enzyme for digestion. One SpeI restriction site was created by the primer and the other was an internal site within the fragment. Z2, Z3, and Z4 all required double digestion. Z2 required SpeI and Xba, both of which were created by the forward and reverse primers. Z3 required NheI and XbaI, created by the forward and reverse primers. Z4 required XbaI and SpeI; XbaI was created by the forward primer and SpeI was an internal site within the fragment.

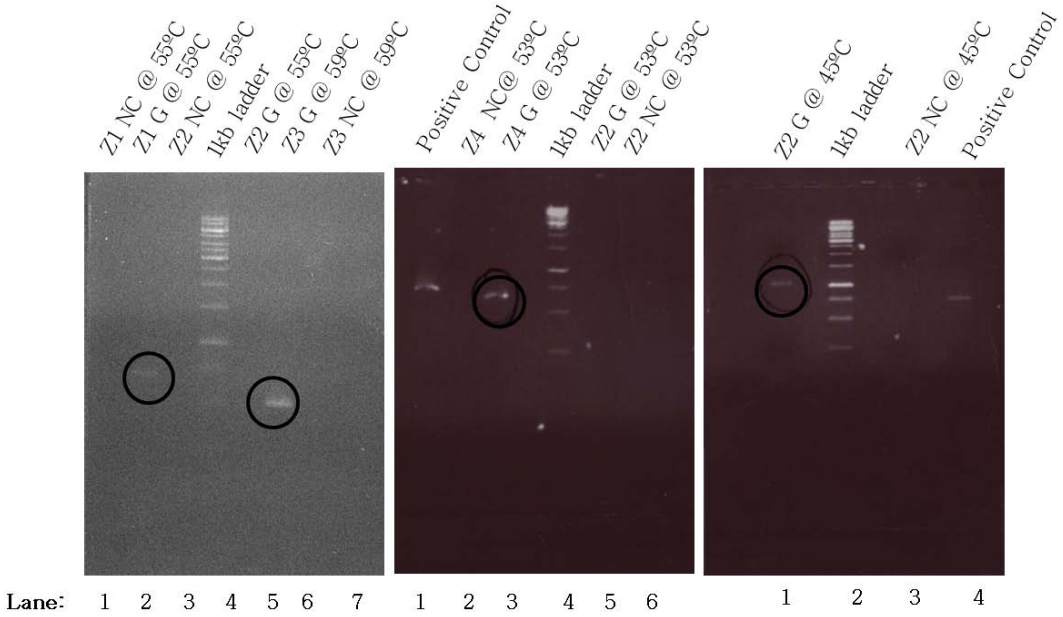

Figure 10 Amplification of Isoform DNA. Circled bands indicate fragments seen under UV light.

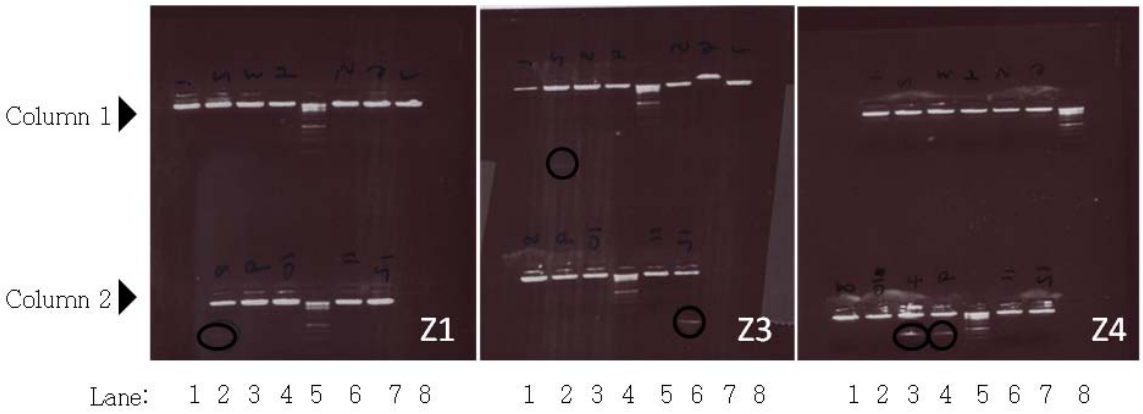

Figure 11 Verification of Cloning in TA plasmid. Circled bands indicate fragments seen under UV light.

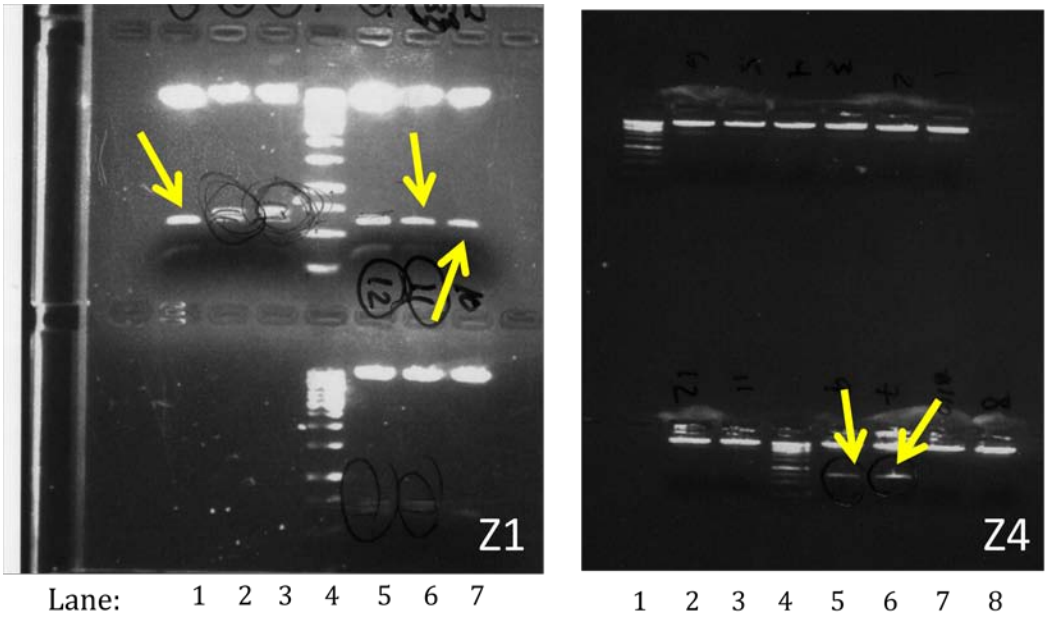

Figure 12 Verification of 1st BR-C isoform fragment in pWIZ. Yellow arrows indicate bands that have been sequenced and verified to be an insert of expected fragment.

Discussion

BR-C Overexpression

The overexpression and knockdown experiments allows us to quantify the effects of loss- or gain-of-function for each *broad* isoforms in the MB. In the present study, we demonstrate *BR-C* isoform-specific roles in mediating neuronal composition in the MB.

Overexpression of *BR-C* isoforms in the MB suggested a role for both BR-Z3 and BR-Z1 in determining neuronal composition. In support of previous results, overexpression of BR-Z3 reduced γ neuron number (Figure 5; Figure 9). However, total neuron number for BR-Z3 overexpression remained the same whereas results of previous studies suggested a reduction in total neuron number (Figure 9) (Marin, Apenteng, and Truman, unpublished). The difference between the results of the studies might be due to several factors. Selection of animals for dissection in this study was limited to those with normal eye size due to ease of dissection. The previous study focused on dissected animals with smaller eyes, suggesting a reduction of cell proliferation and a higher level of OK107 expression. Also, animals were raised on different media in comparison to this study, possibly resulting in altered growth and development. There is additional concern regarding the possibility that different genotypes were used in these two studies since multiple insertion lines exist for each isoform.

Conversely, overexpression of BR-Z1 in the MB resulted in a significant increase in γ-neurons and total number of neurons at P_0 (Figure 9). Taken together, the findings suggest that BR-Z1 increases γ-neuron number while BR-Z3 antagonizes this action.

The means by which the isoforms might direct neuronal composition is still unknown. In *Drosophila, BR-C* regulates physiological and anatomical changes throughout the organism by cell death or proliferation. A reduction in γ neurons without a change in total neurons suggests a role for BR-Z3 in regulating the numbers of both γ and non-γ neurons. For example, BR-Z3-mediated programmed cell death in either/both γ or α'/β' neurons should result in a decrease in total neuron number; this effect was not seen with BR-Z3 overexpression. The findings suggest the possibility that BR-Z3 normally alters neuroblast proliferation rates to create more γ neurons at the expense of non-γneurons. BR-Z3 overexpression could also transform γ neurons to other, EcRB1-, subtypes. However, this effect could also simply result from down-regulated EcRB1 expression in γ neurons due to BR-Z3 overexpression.

In contrast, overexpression of BR-Z1 did alter total neuron number, indicating the isoforms might work via independent pathways to regulate neuronal composition, possibly through cell death or proliferation rates. To test this hypothesis, an antibody for activated caspase proteins, cysteine proteases crucial to cell apoptosis, could be used to compare cell death levels in the MB between overexpression animals and controls. To determine if proliferation rates are affected, BrdU, a synthetic analog of thymadine, can be fed to *Drosophila* larvae. BrdU can be incorporated into synthesized DNA of replicating cells and, using a BrdU antibody, indicate proliferating cells.

Overexpression data also exist for BR-Z2 and BR-Z4. However, the experiments were conducted at 29°C instead of 25°C. The temperature difference does not permit

direct comparison of results of isoform overexpression with control animals. The temperature at which animals are raised has been observed to affect MB neuronal composition (B.A. Apenteng and E.C. Marin, unpublished). The results from Z2 and Z4 overexpression may still be used if control animals are made for 29°C.

Unfortunately, the tools used for genetic manipulation were later found to affect the insulin-secreting cells; OK107 expression was not limited to the MB as previously thought. Insulin has been found to control size and growth of *Drosophila* throughout development (Oldham et al., 20002) and could likely alter MB neuronal composition as well. Therefore, we cannot be certain that the phenotypes were due solely to a cell-autonomous role for *BR-C* in the mushroom body. Further experiments will be conducted in the future to separate the effects of insulin and *BR-C*. New tools are being developed using the MB247 GAL4 driver, known to have expression limited to the MB. The results from OK107 lines still could be used by combining the results with MB247 lines, allowing the possibility of distinguishing the role of insulin and cell-autonomous mechanisms in the MB.

The limitation of overexpression experiments is ectopic, or abnormal, expression of a molecule; a NB that expresses an isoform it normally does not express is not necessarily generating a phenotype indicative of the isoform's normal role during development. For this reason, knockdown experiments will be conducted to provide further evidence and reveal the roles of specific isoforms in the MB.

Creation of pWIZ-based Isoform Construct

PCR amplification of isoform-specific zinc-fingers from genomic DNA resulted in the successful isolation of Z1, Z3, and Z4 fragments. Fragments were then ligated into the TA plasmid to provide stocks for each isoform to confirm successful ligation via restriction digestion and sequencing. Of the 12 white colonies used for each miniprep procedure, 1-2 colonies produced a fragment of the correct size after digestion. Corresponding DNA minipreps were then sequenced. Sequencing results suggested novel mutagenesis had occurred during DNA replication. Because mismatches will decrease the efficacy of RNAi, sequences with the smallest amount of error were chosen.

After fragments were extracted from the agarose gel, they were inserted into the pWIZ vector. The frequency of a successful insert for Z1 and Z4 was 1:12. Currently, the 1st insert for Z1 and Z4 have been verified and procedures are being conducted for the 2nd insert. Digestion of pWIZ-construct after 1st ligation of Z3 did not produce fragments when run on a gel. The restriction enzyme XbaI is blocked or impaired by Dam, a methylation enzyme, resulting in the inability to cleave pWIZ if methylation sequences exist in the Z3 sequence. To prevent methylation, Dam-/Dcm- competent cells were used for Z3. The 1st insert for Z3 into pWIZ has yet to be verified.

PCR was unsuccessful for Z2. Although a PCR product was seen, it was not of the appropriate size. The annealing temperature used in the PCR reaction might have facilitated non-specific binding. Additional attempts to amplify this isoform under varying PCR conditions were also unsuccessful. New primers were designed for PCR but

were also unsuccessful in BR-Z2 amplification. The Z2 zinc-finger sequence is smaller in comparison to other *broad* zinc-finger sequences and the limitation for amplifying fragments of >500bp in addition to having AvrII and NheI compatible restriction sites severely restricted primer design. The sequence will need to be further analyzed and new primers designed that are stable and specific to allow for PCR amplification.

Future Work

Future procedures will continue to complete the pWIZ-based BR-C isoform constructs. Procedures for fragment insertion into the 2nd pWIZ site for Z1 and Z4 are currently being conducted.

Once completed, the pWIZ constructs will be injected into *Drosophila* embryos, creating transgenic flies via p-element-mediated transformation. Flies bearing the RNAi transgene will be crossed to flies bearing a mushroom-body specific GAL4 driver (OK107), a membrane-bound GFP reporter gene, and a copy of *Dicer*-2. *Dicer*-2 is included because it has been found to enhance RNAi effects in neurons.

Once transformed, the efficiency of gene silencing via RNAi will depend on complementary of the UAS-produced dsRNA and genomic-transcribed ssRNA. Using genomic DNA for the UAS-dsRNA raises concerns for post-transcriptional complementarity. RNA undergoes posttranscriptional modification and, because UAS-dsRNA is derived from genomic DNA, there is a possibility that the dsRNA will not be capable of hybridizing with ssRNA. Therefore, the use of cDNA, or DNA reverse-

transcribed from RNA, for the UAS-RNAi lines will likely be the most efficient method of activating RNAi.

Significance of Project

The crucial role of hormones in development suggests a possibility of a likewise fundamental role in developmental plasticity. Indeed, Juvenile Hormone was found to mediate neuronal composition in the MB (Lubin, Dincer, Kanwal, Wakulchik and Marin, unpublished). Downstream of JH is the transcription factor *BR-C*, known to be involved in the transition of *Drosophila* into metamorphosis (Fletcher & Thummel, 1995), but no evidence suggests a role for it in developmental plasticity. Our results indicate at least BR-Z1 and BR-Z3 mediate neuronal composition in the MB, possibly via a JH-regulated mechanism. The responsiveness of hormones to environmental factors presents a likelihood of an environmentally-responsive *BR-C* as well. Determining the effects of JH on *BR-C* in the MB are not yet known but could provide an interesting model in which *BR-C* ultimately transduce environmental cues to either increase or decrease subtypes in the MB and thus appropriately modulate behavior and learning and memory.

Although the genetic basis of conserved developmental programs is widely studied, the molecular mechanisms underlying plasticity, particularly neuronal composition, remains an area largely unexplored. Understanding this process at the molecular level will not only increase our general understanding of plasticity, but also inform efforts in the area of stem cell technology.

The *BR-C* knockdown project will create novel RNAi tools that are capable of depressing isoform-specific *BR-C* expression in any tissue of *Drosophila*. Thus, any researcher interested in the functions of BR-C may use these tools to manipulate expression of the isoforms and elucidate their roles in development. Additionally, the MB has been implicated to have a common ancestral structure to that of the cortex found in vertebrates and invertebrates (Sweeney and Luo, 2010). Increasing our understanding of this brain region found in most insects and arthropods increases our potential to understand more of the mammalian and human brain development. Moreover, elucidating the role of BR-C in the MB has important implications in the roles of hormones in neuronal composition, a field vastly understudied.

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Appendix 1:

BLAST RESULT: Z1_16 Miniprep from TA plasmid

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Query:   281       TGTCCAGGTTCACTGTTTGTCGTCTTAATAAGGAGCTAACAGAGGTGCCCGTAGTGAATT   340
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1538325   TGTCCAGGTTCACTGTTTGTCGTCTTAATAAGGAGCTAACAGAGGTGCCCGTAGTGAATT   1538384

Query:   341       CTTCGAAATGGCTTTAACTAAATACAATTTATCCGATGGACAGCATATTATTCTGTATAC   400
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1538385   CTTCGAAATGGCTTTAACTAAATACAATTTATCCGATGGACAGCATATTATTCTGTATAC   1538444

Query:   401       ACCTCATGATAGGCGGCAATCGCGCACATCGATTAGAAGAGGGAAATGTGAAGCGTTAGA   460
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1538445   ACCTCATGATAGGCGGCAATCGCGCACATCGATTAGAAGAGGGAAATGTGAAGCGTTAGA   1538504

Query:   461       CGTATGAATAATCTCTCTCTTAAAGTTAGACTAAACTAAAGAACGAACTGCACNNNNNNN   520
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1538505   CGTATGAATAATCTCTCTCTTAAAGTTAGACTAAACTAAAGAACGAACTGCACTTTTTTT   1538564

Query:   521       NATTACCATTACGAAGTATATGCATAATGTTAAAAAGTTACGATTTATAGCTAGTTGTAG   580
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1538565   TATTACCATTACGAAGTATATGCATAATGTTAAAAAGTTACGATTTATAGCTAGTTGTAG   1538624

Query:   581       GCGAAGAAACACATGTTTGGAAGCTTAGGAGTTTACAAGAAAAATGAACCGACGAGCAGG   640
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query:   641       GGAGCGACCGACCGAACGACCGACCAATTGCAAAGATCTTTTCTTTAGTACTTTAAACTA   700
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1538685   GGAGCGACCGACCGAACGACCGACCAATTGCAAAGATCTTTTCTTTAGTACTTTAAACTA   1538744

Query:   701       GTTATCAGATTTTGAATAGTTAGGGTTAAGCGCACCGTAACA   742
                  |||||||||||||||||||||||||||||||||||||||||||
Subject: 1538745   GTTATCAGATTTTGAATAGTTAGGGTTAAGCGCACCGTAACA   1538786
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Appendix 2:

BLAST RESULT: Z3_5 Miniprep from TA plasmid

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Query:   60          CGTATTCTAGATACTGGTCCATAGGCGTGAGTTGTGAAAAAGTCAGCAAGTCGATGTATA   119
                     ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1549371     CGTATTCTATATACTGGTCCATAGGCGTGAGTTGTGAAAAAGTCAGCAAGTCGATGTATA   1549312

Query:   120         GATTGATCCGACTAATCTAGGTCTAATCTAAAAGTGTTTCAAATGTCCCCATATAACTAT   179
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1549311     GATTGATCCGACTAATCTAGGTCTAATCTAAAAGTGTTTCAAATGTCCCCATATAACTAT   1549252

Query:   180         CTTACATCTAATGTCTTTAGTTATAATACGCTATTAATTGTATTGTTTGTAATCGTCACC   239
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Subject: 1549251     CTTACATCTAATGTCTTTAGTTATAATACGCTATTAATTGTATTGTTTGTAATCGTCGCC   1549192

Query:   240         ACCTCGTAATAATAAAGATGGGGCCTAAACATTTTTTGTTCGTCGTGTATAATAGATTGG   299
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1549191     ACCTCGTAATAATAAAGATGGGGCCTAAACATTTTTTGTTCGTCGTGTATAATAGATTGG   1549132

Query:   300         TAGGTATGTATATATATCACTACATAATGGCATTGCTTTAAGTTTGAGCTTAGTTCTAGC   359
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1549131     TAGGTATGTATATATATCACTACATAATGGCATTGCTTTAAGTTTGAGCTTAGTTCTAGC   1549072

Query:   360         CGATATCTAGTTGCTCGTGGTGGCAATTGAAG   391
                     ||||||||||||||||||||||||||||||||
Subject: 1549071     CGATATCTAGTTGCTCGTGGTGGCAATTGAAG   1549040
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Appendix 3:

BLAST RESULT: Z4_6 Miniprep from TA plasmid

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Query:   280          CGAGTCCTGGCAGCCAGGCGACGGCAGCGGGAGGAACCGTGACCCAGGCGGGCACGCCTC   339
                      ||||||||||||||||||||||||||||| |||||||||||||||| ||||||||||||||
Subject: 1553175      CGAGTCCTGGCAGCCAGGCGACGGCAGCAGGAGGAACCGTGACCCAAGCGGGCACGCCTC   1553234

Query:   340          CGCTACCGCTCCGCATGCCACCGCCCACCAGCGGCGGCATAAACGAGCCGCAGGAGTGCC   399
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1553235      CGCTACCGCTCCGCATGCCACCGCCCACCAGCGGCGGCATAAACGAGCCGCAGGAGTGCC   1553294

Query:   400          CCTACTGCCGGAGGACCTTCTCGTGCTACTACTCGCTGAAGCGGCACTTCCAGGACAAGC   459
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1553295      CCTACTGCCGGAGGACCTTCTCGTGCTACTACTCGCTGAAGCGGCACTTCCAGGACAAGC   1553354

Query:   460          ACGAGCAGTCGGACACGCTGTATGTGTGCGAGTTTTGCCACCGGCGGTATCGCACCAAGA   519
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1553355      ACGAGCAGTCGGACACGCTGTATGTGTGCGAGTTTTGCCACCGGCGGTATCGCACCAAGA   1553414

Query:   520          ACTCGCTGACCACGCACAAGAGCCTGCAGCATCGCGGCTCTAGCGGCATGCTGAAGCGGC   579
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1553415      ACTCGCTGACCACGCACAAGAGCCTGCAGCATCGCGGCTCTAGCGGCATGCTGAAGCGGC   1553474

Query:   580          TGCTTAAGACGACCGCCATCAAGCACGGACTGGTGGGCCACGGACATGGGCACGGACATG   639
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Subject: 1553475      TGCTTAAGACGACCGCCATCAAGCACGGACTGGTGGGCCACGGACATGGGCACGGACATG   1553534

Query:   640          TCCATCACCCGCATGCGCACCACCACGCCCTGTCCCATCCGCG-ACTAGTCTGTAT   694
                      |||||||||||||||||||||||||||||||||||||||||| ||||||||||||
Subject: 1553535      TCCATCACCCGCATGCGCACCACCACGCCCTGTCCCATCCGCGCACTAGTCTGTAT   1553590
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