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Environmental Influence on Brain, Behavior, and Gene Expression in Drosophila

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ENVIRONMENTAL INFLUENCE ON BRAIN, BEHAVIOR, AND GENE EXPRESSION IN *DROSOPHILA*

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ABSTRACT

Environmental Influence on Brain, Behavior, and Gene Expression in *Drosophila*

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Brain development and behavior are sensitive to environmental stimuli. To gain an understanding of how and to what extent environmental variations, particularly with regard to thermal stress and sensory input, affect brain development, function, and genomic activity, in this dissertation, three interrelated studies were conducted in *Drosophila melanogaster*.

The first study examined the effects of ecologically-relevant hyperthermia stress on development of the *Drosophila* mushroom body (MB), a conserved sensory integration and associative center in the insect brain. A daily hyperthermic episode throughout larval and pupal development was shown to severely disrupt MB anatomy by reducing intrinsic Kenyon cell neuron numbers, but had little effect on other brain structures or general anatomy. This heat stress also greatly impaired associative odor learning in adults, despite having little effect on memory or sensory acuity.

In the second study, individual and combined effects of sub-adulthood hyperthermia stress, larval density, and early-adulthood living space enrichment on brain anatomy and olfactory learning in adult flies were investigated. Both larval crowding and early-adulthood space enrichment did not significantly

increase brain structure volumes or improved odor learning capacities, and did not mitigate heat stress induced MB or learning reductions.

In the third study, a mild thermal pretreatment was applied to *Drosophila* before the acute thermal stress treatment. The heat pretreatment moderately mitigated the hyperthermia-induced MB volume reduction and fluctuating asymmetry increment, but did not protect flies from odor learning defects or male specific early-stage sterility. Moreover, genome-wide transcript analyses revealed that the variation of gene expression pattern in flies exposed to both heat pretreatment and heat stress was much smaller than that in flies exposed to only heat stress. A set of heat stress long-term down regulated genes were tested through mutant analysis and *CG32444* was found to significantly affect MB anatomy.

By establishing empirical linkages between environmental factors, brain structures, and behavior, this research demonstrates that brain's plasticity is reflected not only by its ability to change, but also its adaptability to retain developing and functioning authenticity in response to environmental variations.

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ABBREVIATIONS

AcCoAS	*Acetyl Coenzyme A synthase*
Act5C	*Actin 5C*
AL	Antennal Lobe
ANOVA	Analysis of Variance
CCX	Central Complex
CNS	Central Nervous System
CS^-	Conditioned Stimulus, odor unpaired with electric shock
CS^+	Conditioned Stimulus, odor paired with electric shock
CT	Control
FA	Fluctuating Asymmetry
Gal4	Yeast Transcription Factor
GFP	Green Fluorescent Protein
Got2	*Glutamate oxaloacetate transaminase 2*
HP	Heat Pretreatment
HPHS	Heat Pretreatment and Heat Stress
HS	Heat Stress
HSP	Heat Shock Protein
KC	Kenyon Cell
LPV	Larvae Per Vial
MB	Mushroom Body
MCH	4-Methylcyclohexanol
OCT	3-Octanol
OL	Optic Lobe
PC	Principal Component
Pepck	*Phosphoenolpyruvate carboxykinase*
PI	Performance Iindex
proPO-A1	*prophenol oxidase A1*
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
SE	Standard Error
SNK	Student-Newman-Keuls test
UAS	Upstream activating sequence
w^{1118}; *CS*	White Canton Special

CHAPTER 1

GENERAL INTRODUCTION

One of the most dramatic discoveries in neural and behavioral biology over the past decades is the revealing that brain development is determined by the interplay between inherent genetic programs and a wide range of environmental exposures and experiences (Rutter et al., 2006, Tau & Peterson, 2009). While the DNA code points the direction for the brain to develop, environmental factors play important roles in influencing gene regulation, sculpting neural circuitry, and shaping the consequent behavior (Eisenberg, 1999, Rutter et al., 2006). Although the current scientific view no longer debates "nature vs. nurture," the understanding of how and to what extent environmental stimuli, particularly with stress and enrichment, affect brain development, behavior, and genome activity still rages on (McCain et al., 2007).

Stress

Brain development is vulnerable to environmental stress because its growing processes, including proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis are temporal and regional critical (Rice & Barone, 2000). Noxious experiences during sensitive developmental periods have been observed to damage the brain structure and function in many different animals as well as in humans (Weinstock, 2001, Welberg & Seckl, 2001). One example is hyperthermia being a teratogen to cause both physical and behavioral birth defects in offspring (Edwards, 1986). Hyperthermia is the

first teratogen that has been studied in animals and subsequently proven to be teratogenic in humans (Graham et al., 1998). Experimentally hyperthermia induced malformations involve many organs and structures (Edwards et al., 1995). Among these, central nervous system (CNS) defects are the most common consequence, displayed as anencephaly, micrencephaly, exencephaly, encephalocele, microphthalmia, and other neuroanatomical deficits in a variety of mammals, such as rats, mice, rabbits, sheep, pigs, and monkeys (Graham, 2005). Those animals exhibited associated neurobehavioral abnormalities, particularly reduced learning capacities. In humans, maternal hyperthermia (febrile illness, sauna use, and hot tub use) has been related to neural tube defects (Graham et al., 1998, Miller et al., 1978, Milunsky et al., 1992). For example, anencephaly was reported in infants whose mothers had a high fever during the neural tube closure critical period in pregnancy (Chambers et al., 1998). An analysis of 28 dysmorphic children who experienced maternal hyperthermia in the first trimester showed that all survivors had mental deficiency (Pleet et al., 1981). It has been suggested that cell death and disruption of gene induction of neuroblasts proliferation might be the major hyperthermia damages in CNS that lead to pathogenic defects (Edwards et al., 1974, Li & Shiota, 1999, Upfold, 1989, Wanner et al., 1976). Additionally, the inability to compensate the loss of prospective neurons by additional cell divisions probably explains the reason that CNS is at most risk from hyperthermia (Edwards, 2006, Edwards et al., 1976).

Enrichment

Throughout brain development there are sensitive periods during which particular experiences are essential and important to instruct and refine brain maturation (Bornstein, 1989, Knudsen, 2004). On one hand, the development, organization, and function of particular neural circuits must rely on typical environmental sensory inputs. One of the classic findings indicates that during early developmental periods of kittens, visual deprivation of one eye dramatically reduced the visual cortical cell number responding to the covered eye, and increased the number neurons in the open eye (Wiesel & Hubel, 1963). Other examples include filial imprinting in the forebrain of chicks (Bolhuis & Honey, 1998, Ramsay & Hess, 1954, Scheich, 1987), song learning in the forebrain of songbirds (Bottjer *et al.*, 1984, Marler, 1970), and auditory space processing in the midbrain of barn owls (Brainard & Knudsen, 1998, Knudsen & Knudsen, 1989). In humans, refinement of visual and auditory pathways in the brain also requires optical and acoustic stimulations (Sharma *et al.*, 2007, Vaegan & Taylor, 1979). On the other hand, environmental enrichment can enhance the development and capacity of the brain. Rodents raised in enriched environments have showed significant increases in brain weight and size, survival of newborn neurons, and spatial learning and memory relative to their impoverished siblings (Diamond *et al.*, 1964, Diamond *et al.*, 1966, Fordyce & Farrar, 1991, Kempermann *et al.*, 1997, Rosenzweig & Bennett, 1969, Wainwright *et al.*, 1993). It has been suggested that infants and toddlers require safety, love, conversation, and a stimulating environment to complete brain development that

is essential for subsequent success in curiosity, creativity, and self-confidence (Gable, 2008). Previous studies indicate that a developing brain tends to overproduce synapses between neurons at the early postnatal stage. However, not all synaptic connections will survive. The synapses infrequently used will be eliminated; whereas those frequently used through environmental associated experiences will become a permanent part of the brain and continue to generate new connections (Glaser, 2000, Singer, 1995).

Model System

Whereas the effects of environmental stress and enrichment on developing nervous system are abundantly documented, the causative influences on specific brain targets, consequent behavior, and fundamental mechanism are still not very well understood (Loebrich & Nedivi, 2009, Sale *et al.*, 2009). The revealing of how nature and nurture interact on brain construction and maintenance in anatomy, behavior and gene activity requires the study of model organisms that have (1) well understood CNS development, structure and function, (2) demonstrated CNS plasticity in response to environmental variations, and (3) established genetic and molecular tools and sequenced genomes. One exceptional model system that meets all the requirements is the fruit fly *Drosophila melanogaster*.

In *Drosophila*, the CNS originates from a bilaterally symmetrical sheet of neuroectodermal cells on the ventral side of the embryo, which develops into the ventral nerve cord and the brain (Urbach & Technau, 2008). During embryonic,

larval, and pupal developmental phases, neuroblasts undergo discontinuous proliferation and differentiation to shape and form the adult brain (Hartenstein *et al.*, 2008). The mature *Drosophila* brain consists of an outer layer (cortex) with cell bodies of neurons and glial cells and an inner neuropile with highly branched axons, dendrites and synapses, which are assembled into distinct compartments (Ito & Awasaki, 2008). The mushroom bodies (MBs) are pairs of neuropils implicated in the integration, association, and comparison of olfactory conditionings (Davis, 2005, Heisenberg, 1998). Each MB consists of ~2500 intrinsic neurons called Kenyon Cells (KCs) (Technau & Heisenberg, 1982). The cell bodies of KCs are located posterior dorsally in the protocerebrum. Just anterior and ventral to the cell bodies, KCs give rise to a dendritic field known as the calyx. The axons (fibers) of KCs project to the anterior portion of the brain via a dense structure known as the peduncle, where they branch dorsally and medially and give rise to the lobes of the MBs (Heisenberg, 1980, Ito & Hotta, 1992). Anterior to the MB calyxes, the central complex lies at the centre of the cerebrum and is important for motor coordination control and visual memory (Hanesch *et al.*, 1989, Liu *et al.*, 2006, Strauss & Heisenberg, 1993). The antennal lobes are situated in the anterior ventral part of the brain, with the role of receiving odorous chemical signals and translating them into appropriate attraction or avoidance behaviors (Laissue & Vosshall, 2008, Stocker *et al.*, 1990). More peripherally and on each side of the central brain are the optic lobes, which perceive visual input from the compound eyes and process the

information for higher order motion detection and color vision functions (Fischbach & Dittrich, 1989, Fischbach & Hiesinger, 2008).

Drosophila brain size has been shown to be highly variable and sensitive to environmental influences (Heisenberg *et al.*, 1995, Technau, 1984). Heisenberg and colleagues (1984 and 1995) reported that limited social context reduced MB development in flies reared in isolation or in very small populations; whereas an enriched environment improved MB development in flies reared in groups in large flight cages with various odor and color sources. Their data imply that most neuropil regions in the *Drosophila* brain are continuously reorganized throughout life in response to specific living conditions (Heisenberg *et al.*, 1995). Interestingly, short-term memory mutants dnc^{1} and rut^{1} did not show the experience dependent MB structural plasticity (Balling *et al.*, 1987), which indicates that neuronal and behavioral plasticity may share common genetic pathways in flies.

Drosophila has been used for genetic research since circa 1910 (Morgan, 1910); and the continuous development and application of genetic and molecular tools in fly studies have made it one of the most thoroughly understood metazoan species (Griffiths, 2000). Based on MB structure and cognition defects in mutants, an abundance genes have been isolated that are involved in MB development and associative odor learning and memory (de Belle & Heisenberg, 1996, Dubnau & Tully, 1998, Tully, 1996). Using the GAL4 enhancer trap system (Brand & Perrimon, 1993), MB-targeted expression of transgenic genes (Figure 1-1) has revealed spatial and temporal aspects of MB growth and function

(Connolly *et al.*, 1996, Dubnau *et al.*, 2001, Ito *et al.*, 1998, Lee *et al.*, 1999). Furthermore, in 2000, nearly 120 megabases (Mb) of euchromatic portion of the *Drosophila* genome (~180 Mb) were sequenced (Release 1) and 13,601 genes were annotated and interpreted (Adams *et al.*, 2000); in 2007, another 24 Mb of heterochromatin with 230-254 annotated genes were added to the *Drosophila* genome sequence Release 5 (Smith *et al.*, 2007). These findings established *Drosophila* as an excellent model for unraveling the molecular mechanisms underlying development, behavior, and many other processes.

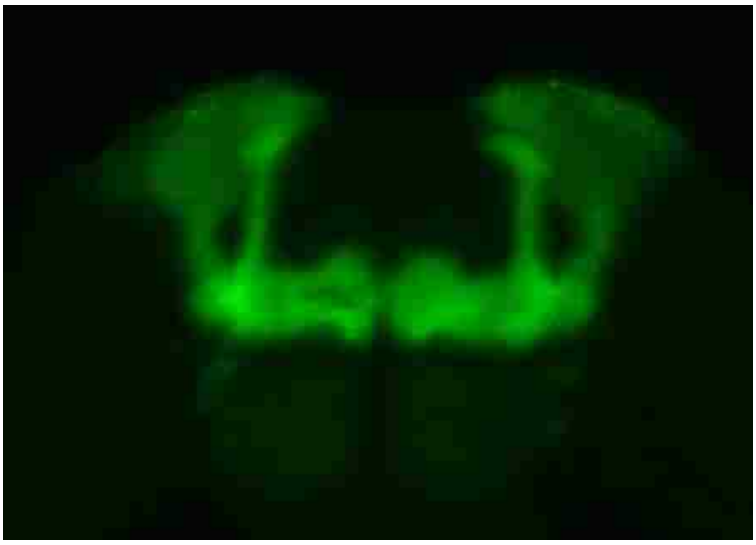

Figure 1-1. *Drosophila* MBs expressing green florescent protein.
Cytoplasm-targeted green florescent protein expression pattern driven by a MB GAL4-expressing element in a whole mount fly brain viewed with a laser scanning confocal microscope.

Scope of the Study

The goals of this research are to identify and quantify the singular and interactive effects of environmental stress and enrichment on brain development,

brain function, and correlated genomic activity through the study of *Drosophila melanogaster*.

Chapter 2 demonstrates empirical influences of an ecologically-relevant thermal stress on MB development and learning potential in *Drosophila*. I show that a daily hyperthermic episode throughout larval and pupal development dramatically reduced MB volume by decreasing intrinsic KC neuron numbers, but had little effect on other brain structures; and considerably damaged associative odor learning in adults, despite having little effect on memory or sensory acuity.

In Chapter 3, I study the individual and combined effects of environmental enrichment and stress on fly brain anatomy and cognitive functions. My data show that the previously suggested enrichment in rearing conditions, such as enhanced social contact in larval crowding and enlarged living space flight cages (Heisenberg et al., 1995) (Technau, 1984), did not increase MB volume and learning ability, nor mitigated the MB development deficiency induced by heat stress.

Chapter 4 illustrates the role of a mild thermal pretreatment in protecting flies from the acute hyperthermia stress. The heat pretreatment moderately alleviated the heat stress caused gene expression variation and MB volume reduction, but not the learning deficiency. By using DNA microarray analysis, I identified various heat stress-related long-term affected genes, which may have important functions in mediating neuroanatomical and behavioral plasticity.

My investigation of stress/enrichment mediated affects on MB development, function, and correlated gene activity reveals a novel and

unprecedented linkage of developmental biology, neurobiology, and gene expression with environmental, behavioral, and social sciences.

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CHAPTER 2

THERAML DISRUPTION OF MUSHROOM BODY DEVELOPMENT AND ODOR LEARNING IN *DROSOPHILA*

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I made the following contribution to this paper: designed and performed the experiment, analyzed the data, and wrote the manuscript.

Abstract

Environmental stress (nutritive, chemical, electromagnetic and thermal) has been shown to disrupt central nervous system (CNS) development in every model system studied to date. However, empirical linkages between stress, specific targets in the brain, and consequences for behavior have rarely been established. The present study experimentally demonstrates one such linkage by examining the effects of ecologically-relevant thermal stress on development of the *Drosophila melanogaster* mushroom body (MB), a conserved sensory integration and associative center in the insect brain. We show that a daily hyperthermic episode throughout larval and pupal development (1) severely disrupts MB anatomy by reducing intrinsic Kenyon cell (KC) neuron numbers but has little effect on other brain structures or general anatomy, and (2) greatly impairs associative odor learning in adults, despite having little effect on memory or sensory acuity. Hence, heat stress of ecologically relevant duration and intensity can impair brain development and learning potential.

Introduction

Whereas the effects of environmental stress on developing nervous systems are well documented (Rice & Barone, 2000, Weinstock, 2001, Welberg & Seckl, 2001), few studies demonstrate causative influences on specific targets in the brain and their consequences for behavior. One familiar exception is the volumetric reduction of basal ganglia, cerebellum and corpus callosum due to in utero ethanol exposure in mammals (Mattson & Riley, 1998). These effects on the developing brain are associated with symptoms of fetal alcohol syndrome in humans, such as impaired verbal and visual-spatial learning, attention, reaction time, and executive functions (Roebuck et al., 1998). Thermal stress is a more common and potentially hazardous feature of the natural environment for developing animals. Indeed, hyperthermia is also an especially powerful CNS teratogen in the laboratory (Milunsky et al., 1992, Suarez et al., 2004). Adult male rats exposed to in utero hyperthermia display aberrant sexual behavior associated with disruptions of the sexually dimorphic nucleus of the preoptic area and the anteroventral periventricular nucleus (Rhees et al., 1999). However, the consequences of natural or ecologically-relevant heat stress for CNS development and function in organisms that normally experience extreme thermal heterogeneity are unknown. *Drosophila melanogaster* developing in necrotic fruit are subject to daily episodes of intense hyperthermia capable of causing significant mortality and disruption of external morphology (Feder, 1997, Roberts & Feder, 1999). Here we show that the anatomy and function of *Drosophila* MBs, structures associated with sensory integration and higher

processing in insects (de Belle & Kanzaki, 1999, Heisenberg, 2003, Zars et al., 2000), are acutely sensitive to ecologically-relevant heat stress experienced during sub-adult stages.

Surprisingly little is known about invertebrate CNS and behavioral responses to thermal stress. In recent studies with honeybees, workers exposed to low temperatures within the range of normal experience showed reduced behavioral performance relative to their siblings raised at higher temperatures (Tautz et al., 2003). Deviations of only one degree from optimum induced striking developmental reductions in sensory mode-specific zones of the calyx, the dendritic input of the MBs (Groh et al., 2006, Groh et al., 2004). These findings imply that temperature-mediated MB plasticity may be important for regulating complex behavioral tasks. MBs are also remarkably responsive to sensory experience, with exposure to either enriched or deprived artificial environments inducing dramatic structural plasticity (Balling et al., 1987, Barth & Heisenberg, 1997, Heisenberg et al., 1995, Technau, 1984). The current study expands our understanding of the acute sensitivity of the MB to stress and to thermal variation in particular. The implications of environment and experience for brain development and adult behavior are discussed.

Results

Heat Stress Influence on Development

D. melanogaster from a large orchard population reared at 23℃ were exposed daily to a brief heat stress (39.5℃ for 35 min) throughout larval and

pupal development. This laboratory treatment mimics documented profiles of thermal oscillation experienced by developing flies in nature (Feder, 1997, Roberts & Feder, 1999), and like such intense natural hyperthermic episodes, yielded approximately 60% increases for both mortality and developmental time (data not shown). Eclosing heat-stressed (HS) adults nonetheless appeared entirely normal, with wild-type walking, flight, activity levels and reproductive capacity. However, the brains of these flies showed striking reductions in MB neuropil when viewed in paraffin sections under a fluorescence microscope (Figure 2-1A). Using planimetric measurements to quantify this observation, we found that MB calyx volume (dendritic elements; Figure 2-1B) and pedunculus cross section area (axonal elements; Figure 2-1C) were both reduced by approximately 30% in HS flies relative to controls (CT) reared at a constant 23°C. In considering more peripheral brain structures associated with sensory input, antennal lobe (AL) volume was reduced by about 15% (Figure 2-1D), while the much larger optic lobes appeared to be unaffected by heat stress treatment (Figure 2-1E). The central complex, controlling aspects of motor output in flies and other insects (Strauss, 2002), was 9% smaller in heat stressed males only (Figure 2-1F). Except for a 6% wing area reduction in females, differences in external anatomical features, such as leg length, were indistinguishable between HS and CT flies (Figure 2-1G and H).

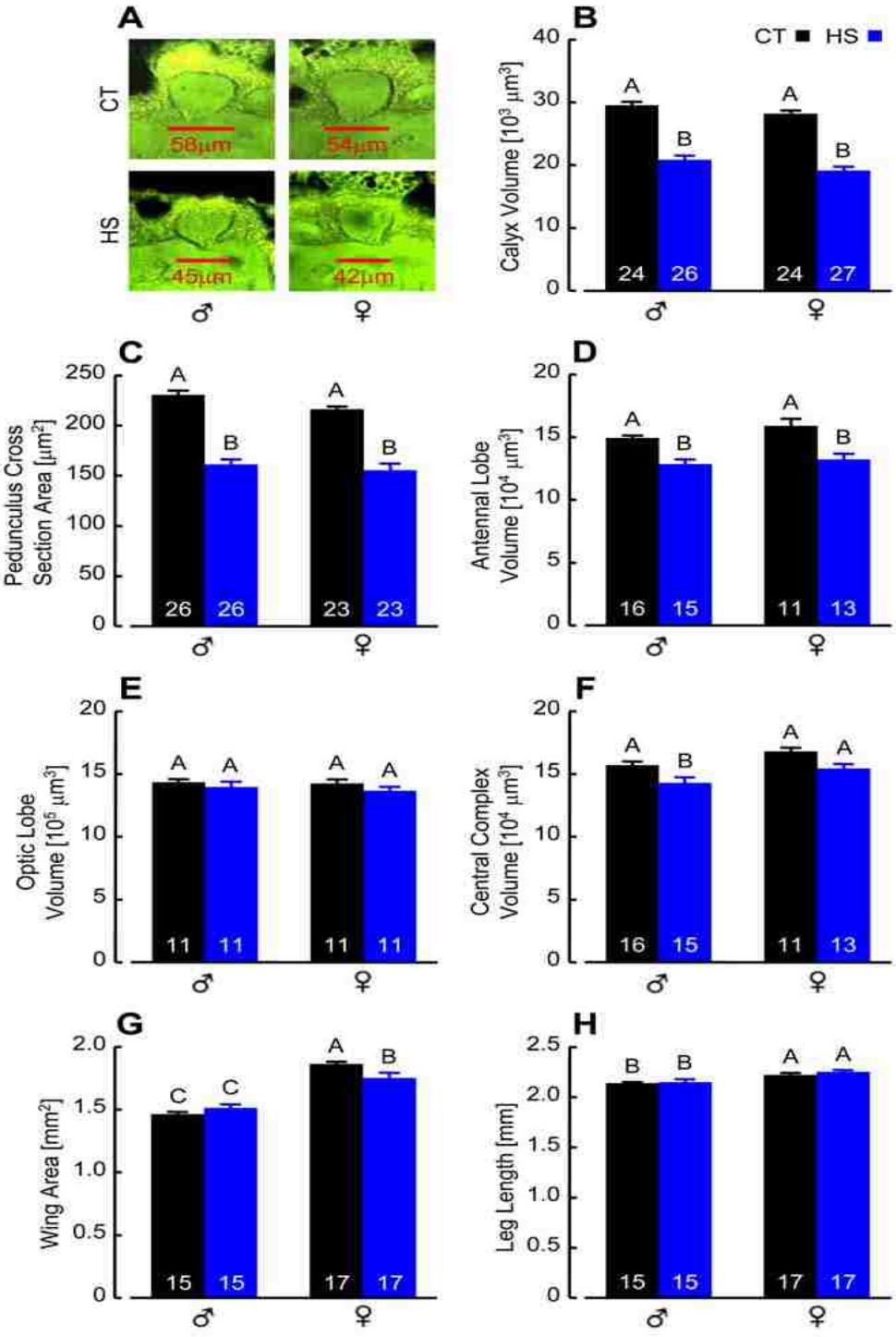

Figure 2-1. Thermal stress disrupts brain development.
(A) Frontal 7 µm paraffin sections of MB calyces at their broadest point, viewed with a fluorescence photo microscope. MBs are smaller in HS flies than in the CT

group. (B) Heat stress induced a significant 31% reduction in MB calyx volume ($F_{[1,97]}$ = 188.39, P < 0.0001), estimated from planimetric measurements of serial sections of HS and CT flies shown in (A). (C) MB pedunculus cross-section area (the means of measurements from three serial caudal sections) was reduced by 29% in HS flies ($F_{[1,97]}$ = 123.43, P < 0.0001). (D) AL volume [derived as in (B)] was reduced by 15% in HS flies ($F_{[1,51]}$ = 26.04, P < 0.0001). (E) Optic lobe volume [medulla + lobula, derived as in (B)] was not significantly influenced by heat stress ($F_{[1,40]}$ = 1.59, P = 0.22). (F) Central complex volume [fan shaped body + ellipsoid body, derived as in (B)] was reduced by 9% in HS male flies only ($F_{[1,51]}$ = 10.78, P = 0.002). (G) Wing area was reduced by 6% in HS female flies only ($F_{[1,60]}$ = 7.04, P = 0.01). (H) Forelimb length was not significantly affected in HS flies ($F_{[1,60]}$ = 1.21, P = 0.28). (B–H) Bars are mean ± standard error (SE); n indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$). doi:10.1371/journal.pone.0001125.g001

In *D. melanogaster* adults, MBs are paired neuropil structures each consisting of about 2500 intrinsic KC neurons (Heisenberg, 2003, Technau & Heisenberg, 1982). Four equivalent neuroblasts in each hemisphere of the developing brain generate three morphologically and spatially distinct classes of KCs in a specific temporal order (Armstrong et al., 1998, Ito et al., 1997a, Lee et al., 1999). Gamma neurons appear until the mid-3rd instar larval stage, followed by α′β′ neurons until puparium formation, with αβ neurons proliferating until adult eclosion. To address whether MB hypersensitivity to heat stress might be limited to any of these classes of neurons, we examined the brains of flies that were heat stressed according to the sequential pattern of KC generation (Figure 2-2A). Adult MBs were reduced following heat treatment during all stages of larval and pupal development, and corresponding temporal windows of KC proliferation (Figure 2-2B). MB calyx reductions induced during γ, α′β′, and αβ neuron

proliferation periods were not significantly different, suggesting that all KC classes have equivalent heat stress sensitivity.

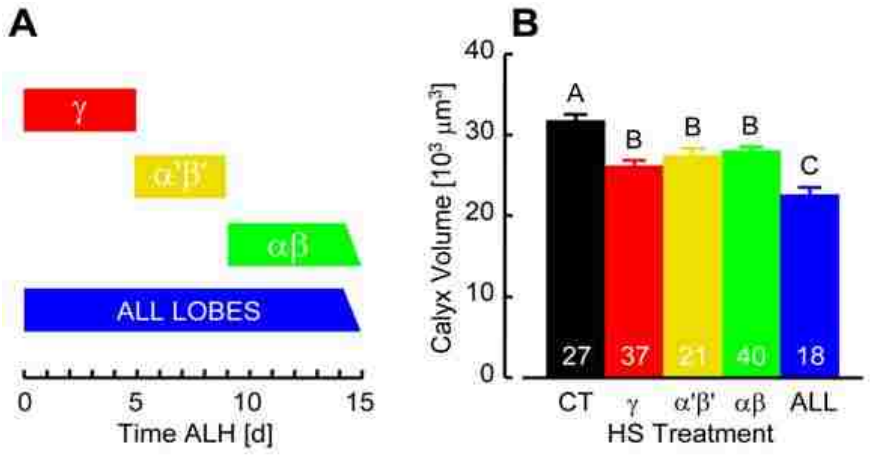

Figure 2-2. All classes of Intrinsic MB neurons are sensitive to thermal stress.
(A) Schematic illustration of heat stress treatment administered 35 min/day throughout larval and pupal development, or restricted to specific developmental stages that correspond with the birth of MB neurons projecting to γ, α′β′, or αβ-lobes. (B) MB calyx volume measurements (derived as in figure 1B). All three classes of MB neurons are sensitive to heat stress ($F_{[4,138]}$ = 17.92, P < 0.0001). Calyx volume in flies receiving daily episodes of heat stress treatment throughout development reflected additive reductions of each of the three neuron classes exposed to heat stress as shown in (A). Bars are mean ± SE; n indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$).
doi:10.1371/journal.pone.0001125.g002

To determine whether MB reduction in HS flies was due to either smaller or fewer KCs, we used the GAL4/UAS reporter gene system (Brand & Perrimon, 1993, Yang *et al.*, 1995) to visualize MB architecture (Ito *et al.*, 1997b, Yang *et al.*, 1995, Zars *et al.*, 2000) and count KC perikarya (Akalal *et al.*, 2006, Mader, 2004). In these experiments, cytoplasm-targeted green fluorescent protein (GFP) expressed by the *T10* element (Ahmad & Henikoff, 2001) was used to label KC projection patterns, and nuclear-localized GFP expressed by the *nls14* element (Robertson *et al.*, 2003) was used to label nuclei in KC perikarya. MBs in HS flies bearing *T10* driven by one of three different *P[GAL4]* drivers (*247* (Schulz *et al.*, 1996), *201Y* (Yang *et al.*, 1995), or *c739* (Yang *et al.*, 1995)) appeared slightly smaller, but otherwise normal in all respects. We observed paired neuropiles with wild-type structural features, including KC clusters, calyces, pedunculi, and lobes (Figure 2-3A). In contrast, there were fewer labeled KCs counted in HS *P[GAL4]/ nls14* flies than in CT groups (Figure 2-3B). Cell numbers differed by 29% in *247/nls14*, 36% in *201Y/nls14*, and 57% in *c739/nls14* (Figure 2-3C). Initially, heat stress appeared to influence numbers of GFP-expressing cells in some genetic backgrounds more than others, suggesting a possible distinction between KC classes. However, the analysis of variance (ANOVA) genotype × treatment interaction component was not significant ($F_{[1,104]} = 2.69$, $P = 0.07$), indicating that intrinsic MB neurons have similar heat stress responses. Thus, heat stress disrupts MB development by either blocking KC proliferation or triggering abnormal KC death.

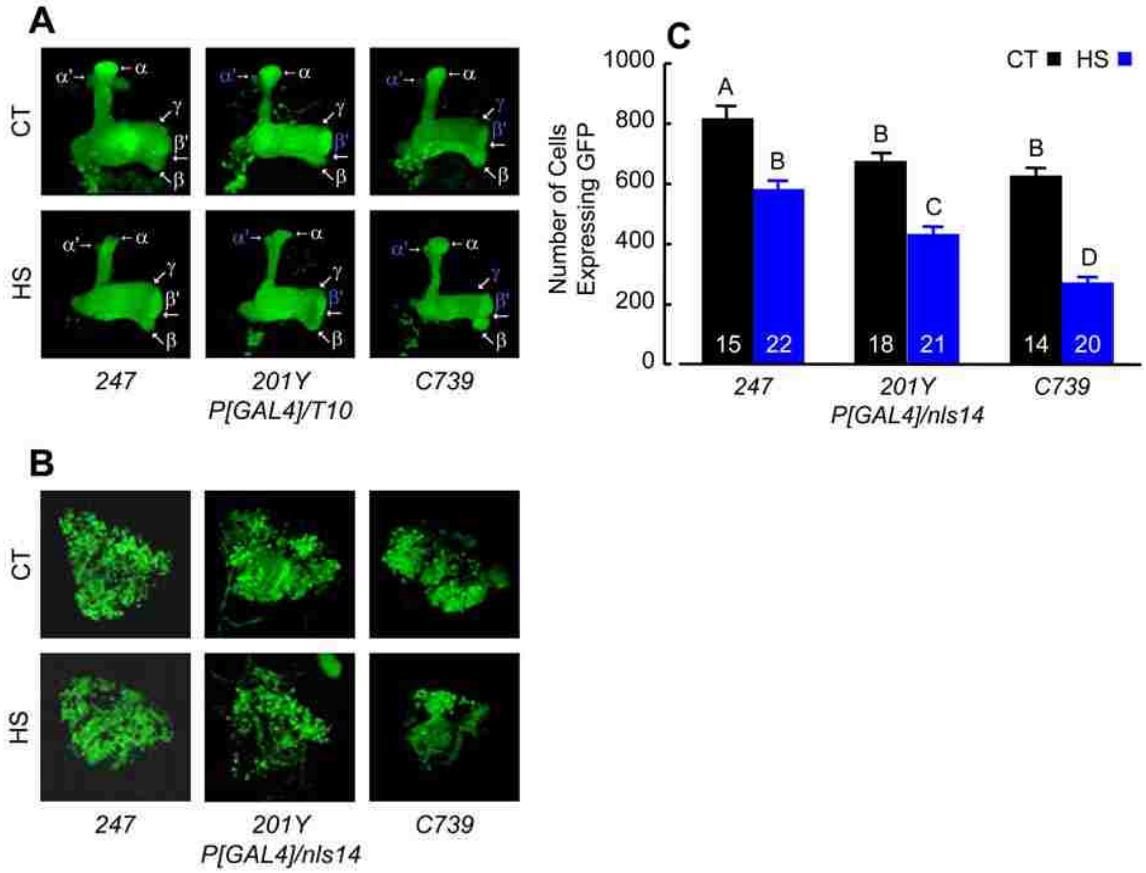

Figure 2-3. Thermal stress disrupts MB development by reducing KC numbers. (A) Cytoplasm-targeted GFP expression patterns driven by different GAL4-expressing elements in whole mount brains of CT (top) and HS (bottom) flies viewed with a laser scanning confocal microscope. All MB structural elements represented in each of three CT *P[GAL4]/T10* genotypes were present (labeled) but clearly diminished in HS flies. We noted that cytoplasm-targeted GFP revealed low-level enhancer activity (labeled in blue) that is often not observed when targeting GFP expression to membranes (Krashes *et al.*, 2007, Pascual & Preat, 2001). (B) Nuclear-targeted GFP expression patterns driven by different GAL4-expressing elements in whole mount brains of CT (top) and HS (bottom) flies viewed with a laser scanning confocal microscope. We observed fewer KCs in the three HS *P[GAL4]/nls14* genotypes compared with CT flies. (C) KCs counted in the brains of flies represented in (B). A two-way ANOVA found highly significant effects of genotype ($F_{[2,104]} = 42.36$, $P < 0.0001$) and treatment ($F_{[1,104]} = 143.00$, $P < 0.0001$), while the interaction component was not significant ($F_{[1,104]} = 2.69$, $P = 0.07$). KC numbers were reduced by 29% in *247/ nls14*, 36% in *201Y/nls14* and 57% in *c739/nls14*. Bars are mean ± SE; n indicated on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$). doi:10.1371/journal.pone.0001125.g003

Heat Stress Influence on Behavior

Since MBs are a secondary olfactory neuropil essential for mediating associative odor learning and memory in *Drosophila* (de Belle & Kanzaki, 1999, Heisenberg, 2003, Zars *et al.*, 2000), we compared the behavior of HS and CT flies using a Pavlovian conditioning assay (de Belle & Heisenberg, 1994, de Belle & Heisenberg, 1996, Tully & Quinn, 1985). Learning of odors paired with electric shock was profoundly reduced (28%) in HS flies relative to CT flies (Figure 2-4A). While memory appears to decay more rapidly in HS flies, this effect is minor since the ANOVA treatment6time interaction component was not significant ($F_{[2,56]}$ = 2.00, P = 0.15). Performance indices averaged over all retention intervals for HS flies were 53% of the CT group. Similar olfactory conditioning defects and rates of memory decay have been described for several *Drosophila* mutants (Margulies *et al.*, 2005, Mcguire *et al.*, 2005), including those with observed reductions in MB anatomy (de Belle & Heisenberg, 1996, de Belle & Kanzaki, 1999, Pinto *et al.*, 1999).

Ablation studies show that *Drosophila* MBs are not required for normal responses to electric shock or noxious odors (de Belle & Heisenberg, 1994). Although heat stress does have a minor influence on the development of other structures (Figure 2-1D, F and G), and lengthens developmental time (Figure 2-2A), HS flies did not have sensory acuity defects in control tests relevant to our conditioning paradigm. They avoided 80 V dc shock pulses normally, and responded to 120 V dc shock with only a slight reduction compared to CT flies (Figure 2-4B). Similarly, HS flies showed normal avoidance of both 4-

methylcyclohexanol (MCH) and 3-octanol (OCT) odorants at the 10×10^{-3} dilutions used in classical conditioning (Figure 2-4C and D). Responses to a 5×10^{-3} dilution of MCH were slightly reduced (Figure 2-4C). Thus, low performance of HS flies in conditioning experiments was not a secondary result of impaired shock reactivity or olfactory capacity as a consequence of AL reduction, but due to weak association of these stimuli paired during training.

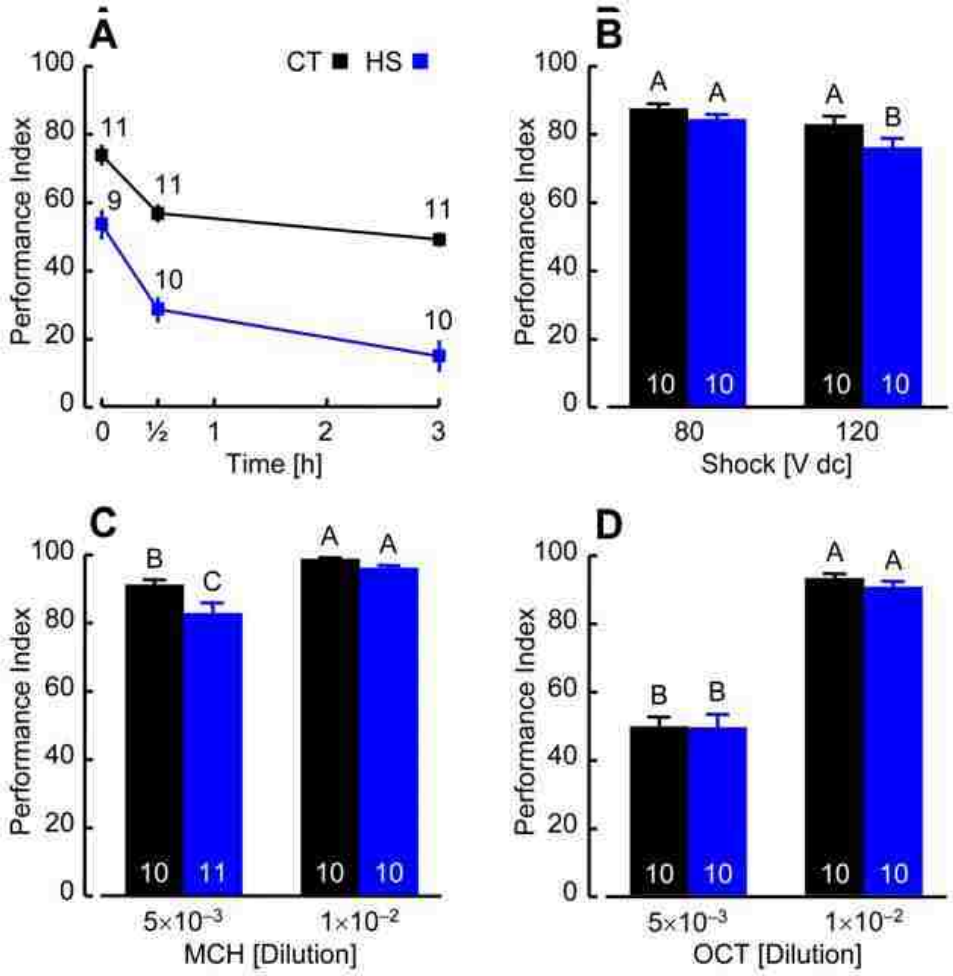

Figure 2-4. Associative odor learning is impaired by thermal stress.
(A) Olfactory learning and memory. The mean performance index calculated for HS flies was lower than CT flies at all time intervals. A two-way ANOVA detected significant effects of treatment ($F_{[1,56]}$ = 101.25, P < 0.0001) and time ($F_{[2,56]}$ =

41.93, $P < 0.0001$), while the interaction component was not significant; $F_{[2,56]} = 2.00$, $P = 0.15$). (B) Shock reactivity. HS flies showed normal avoidance of 80 V dc electric shock used in (A) and a slight reduction in avoidance at 120 V ($F_{[1,36]} = 6.23$, $P = 0.017$). (C) MCH odor avoidance. HS flies demonstrated a normal avoidance of MCH at the 10^{-22} dilution used in (A) and a slight reduction in avoidance at the 10^{-23} dilution ($F_{[1,37]} = 14.72$, $P = 0.0005$). (D) OCT odor avoidance. HS flies demonstrated normal avoidance responses to OCT at both dilutions. (A–D) Symbols or bars are mean ± SE; n indicated above each symbol or on each bar. Different letters designate significant differences (SNK, $P \leq 0.05$).
doi:10.1371/journal.pone.0001125.g004

Discussion

This study demonstrates that adult *Drosophila* brain anatomy and behavior are especially sensitive to acute, ecologically relevant heat stress during development. The effect was most evident in the MBs, which were smaller due to fewer KCs, but otherwise appeared structurally normal. Calyx volume measurements in flies recently derived from a natural population and counts of GFP-labeled KCs in P[GAL4]/nls14 brains suggested equivalent heat stress responses for all three classes of intrinsic neurons and corresponding γ, α′β′, and αβ lobe systems. HS flies were also strongly impaired in associative odor learning, while memory decay, sensory acuity and basic motor behavior remained largely unaffected. Since odor avoidance was essentially normal in HS flies, associative functions that might be attributed to the ALs (Yu et al., 2004) were probably not markedly affected by heat stress. We saw no evidence of necrosis in paraffin sections of HS fly brains (Figure 2-1A), and consequently favor the view that impaired KC proliferation, rather than aberrant KC mortality, was the source of MB and olfactory conditioning reduction. KCs may be

especially sensitive to heat stress because they are derived from only four progenitor cells (of more than 100 in each brain hemisphere (Urbach et al., 2003)) that divide asymmetrically (Campos-Ortega, 1993) and continuously from embryo until adult eclosion (Ito & Hotta, 1992, Lee et al., 1999). AL local and projection interneurons follow a similar temporal course of development (Ito & Hotta, 1992, Stocker et al., 1997) and for this reason might be expected to show a similar sensitivity to heat stress. On the other hand, enhanced structural plasticity may be a fundamental feature of MB neurons, reflecting cellular changes that are particularly responsive to convergent sensory input, and having a profound impact on the behavioral characteristics of adults. The latter explanation may be more likely, since the optic lobes (about half of the brain) were evidently not affected by heat stress occurring throughout their development. The source of these stress response differences in the brain is a focus of our ongoing investigation.

A prevailing neural circuit model for olfactory discrimination and learning proposes that KCs serve as temporal coincidence detectors for odors paired with inherently meaningful or conditioned reinforcement (Gerber et al., 2004, Heisenberg, 2003). KCs might learn and represent odors as memories in their signaling to downstream neurons. In consideration of this model, we expect that training flies to avoid one simple odor will recruit relatively few neurons, whereas the vastly more complex natural olfactory environment should engage large overlapping KC arrays. In HS flies, fewer KCs had a diminished capacity for odor learning, but these remaining neurons had superficially normal projections and

sustained relatively normal representations of odor memory. Correlated reductions of MB structure (Figure 2-1B and C, Figure 2-2B, Figure 2-3C) and learning (Figure 2-4A) by about 30% may reflect a simple relationship between the numbers of KCs capable of representing specific conditioned odors and learning performance, at least for the pure odorants used in our experiments. Moreover, since both MB structure and memory decay were apparently spared in HS flies, we argue that normal KC projection and connectivity are critical for memory storage and retrieval. Several observations support these simple arguments. In MB ablation studies, *Drosophila* larvae fed the cytostatic agent hydroxyurea developed into adults having only a small fraction of the normal KC complement and correlated reductions in odor learning (de Belle & Heisenberg, 1994). A number of these flies had partially ablated MBs that were reduced in size but otherwise appeared anatomically normal. Similarly, mutations that reduce MB neuropil but have no obvious additional structural phenotypes also impair olfactory conditioning but not memory (de Belle & Heisenberg, 1996, Pinto et al., 1999). More recent transgenic studies showed that synaptic transmission from KC terminals in the lobes is required for memory retrieval but not acquisition or storage (Dubnau et al., 2001, Mcguire et al., 2001). In view of these observations, we propose that lower memory scores in HS flies reflects a reduced sum of conditioned KC signals received by extrinsic neurons downstream of the MBs.

Heat stress appears to phenocopy defects described for several *Drosophila* MB anatomy mutants (de Belle & Heisenberg, 1996, de Belle & Kanzaki, 1999,

Heisenberg et al., 1985), providing a practical non-invasive tool for dissecting brain structure-function relationships. The significance of different KC classes, with their discrete temporal and spatial patterns of proliferation and projection to the three lobe systems of the *Drosophila* MB, is largely unknown. Mutant and transgenic studies suggest a possible distinction between them as neural substrates for representations of memories consolidated at different stages of development (Balling et al., 1987), discrete phases of memory, (Akalal et al., 2006, Isabel et al., 2004, Krashes et al., 2007, Margulies et al., 2005, Pascual & Preat, 2001, Zars et al., 2000), or conduits to extrinsic sites downstream of the MBs for memory storage and retrieval (Dubnau et al., 2001, Mcguire et al., 2001). Since temporal windows of heat stress can reliably induce significant and equivalent reductions of each KC class (figure 2, figure 3), this method should distinguish behavioral functions of these neurons and MB structures formed by their projections.

Although the mechanism(s) by which heat stress disrupts neural development and behavior are unknown, the apparent phenocopy of MB mutant defects may provide important clues for understanding how the brain responds to normal environmental variation. Our results suggest that KC proliferation during development is especially sensitive, while KC plasticity in adults may respond with more subtle changes (Balling et al., 1987, Barth & Heisenberg, 1997, Heisenberg et al., 1995, Technau, 1984). Whole genome analyses (e.g., DNA microarrays) should identify potential links between both types of neuronal

plasticity and environmental triggers of gene activity that may either drive or accompany them.

In the wild, flies encounter stress from many sources, but also receive a broad spectrum of complementary enrichment. Stimulating environments augment MB development in a learning mechanism-dependent manner (Balling et al., 1987), while stressful environments disrupt MB anatomy and impair function. Hence, genetic influences and a combination of beneficial and deleterious environmental exposures during development likely have significant roles in determining the neural and behavioral characteristics of adults. Since all nervous systems demonstrate acute sensitivity to environmental stress, our findings have broad implications for brain development and cognitive ability in all animals, including humans.

Materials and Methods

Flies

Wild-type D*. melanogaster* adults were collected from a large orchard population in southern Nevada. The lineage of these flies was used for all paraffin histology and behavior. We generated heterozygous GFP-expressing flies for confocal laser scanning microscopy by crossing either *P[UAS-GFP.S65T]T10* (*T10*; Bloomington Stock Center) (Ahmad & Henikoff, 2001) or *P[UAS-GFP.nls]14* (*nls14*; Bloomington Stock Center) (Robertson *et al.*, 2003) with three different enhancer trap strains in which GAL4 expression was reported in distinct subsets of MB neurons: *P[Mef2-GAL4.247]* (*247*; γ, α′β′, and αβ lobe

neurons; Robert Schulz) (Schulz *et al.*, 1996), *P[GAL4]201Y* (*201Y*; γ and αβ lobe neurons; Douglas Armstrong) (Yang *et al.*, 1995), or *P[GAL4]c739* (*c739*; αβ lobe neurons; Douglas Armstrong) (Yang *et al.*, 1995). Cytoplasm-targeted GFP expression was examined in HS and CT *247/T10*, *201Y/T10* and *c739/T10* heterozygotes. Nuclear-localized GFP expression in HS and *CT 247/nls14*, *201Y/nls14*, and *C739/nls14* heterozygotes was used to count KC nuclei. We cultured flies at equal density in plastic vials with cotton plugs on 8 ml of standard *Drosophila* cornmeal and molasses medium at 23℃ (except for heat stress treatment, below).

Heat Stress

HS treatment consisted of a single daily 39.5℃ pulse for 35 min throughout larval and pupal development. We administered HS by immersing culture vials of flies in a circulating water bath. In staged HS experiments, daily heat pulses were limited to (1) early 1st instar to early 3rd instar, stressing γ-lobe neuron development, (2) late 3rd instar to puparium formation, stressing α′β′-lobe neuron development, and (3) pupal development, stressing αβ-lobe neuron development, respectively.

Histology and Anatomy

We used paraffin mass histology to process flies for neuroanatomical analyses as described previously (de Belle & Heisenberg, 1994, de Belle & Heisenberg, 1996, Heisenberg & Bohl, 1979). Three-4-day-old *Drosophila* adults were cold-anaesthetised and placed in collars. They were then fixed in Carnoy's solution, dehydrated in ethanol, embedded in paraffin, cut in 7 µm serial frontal

sections, and photographed under a fluorescence microscope with an AXIOCAM digital camera (Zeiss). Brain structure volumes were derived from planimetric measurements of serially-sectioned brains (de Belle & Heisenberg, 1994, de Belle & Heisenberg, 1996) using AXIOVISION software (Zeiss). Pedunculus cross section area was derived from the means of measurements taken from three serial sections anterior to the calyx. The means of all paired structures were used for each fly. To examine GFP expression in whole mounted fly brains, heads were dissected in PBS and maintained in FOCUS-CLEAR (Pacgen) for 15 min. They were then mounted and viewed under a fluorescence microscope with a far-blue (FITC) filter. Z-series confocal images were collected (Zeiss LSM510) to cover the whole MB for viewing structure (1.5 µm virtual sections), or perikarya clusters (0.75 µm virtual sections) for counting cells. GFP-labeled KC nuclei in HS and CT brains were counted manually in every 10th section with the assistance of IMAGE-J software (Abramoff, 2004), ensuring that all perikarya (diameters, 6 µm) in each of these sections would each be counted only once.

We measured right wing area and right fore limb length to assess the effects of heat stress on external anatomy. Appendages were removed using micro scissors from cold-anaesthetised flies being processed for paraffin mass histology (above). These were mounted on glass microscope slides with cover slips sealed with nail polish. Images were photographed under a light microscope with an AXIOCAM digital camera and measured using AXIOVISION software (Zeiss).

Behavior

Associative odor learning, memory and sensory acuity controls were assayed using a Pavlovian conditioning T-maze paradigm as described previously (de Belle & Heisenberg, 1994, de Belle & Heisenberg, 1996, Tully & Quinn, 1985). Groups of approximately 100 3-4day-old flies were aspirated into a training tube embedded with an internal double-wound electrifiable copper grid. To assay odor learning and memory, flies were exposed to an air current (750 ml/min) bubbled through one odor [1×10^{-2} dilutions of either MCH (Sigma) or OCT (Sigma) in heavy mineral oil (Sigma)] paired temporally with 1.25 sec pulses of 80V dc electric shock delivered every 5 sec for 1 min. They were then exposed to an air current bubbled through a second odor without electric shock for an additional 1 min. We assessed learning and memory by presenting trained flies with both odors in converging air currents for 2 min. Performance was measured as a function of shock-paired odor avoidance at a variety of time points ranging from 1 min (giving an approximation of learning at the earliest testable time in the T-maze) to 3 hr after training. A second group of flies was trained in a reciprocal manner and tested. Scores from both tests were averaged to account for odor preferences among different populations of flies. In electric shock-avoidance controls, one arm of the T-maze was electrified with 80 or 120 V dc for 2 min. In odor-avoidance controls, flies were exposed to 5×10^{-3} or 1×10^{-2} dilutions of MCH or OCT versus air for 2 min. A performance index represents the average normalized percent avoidance of the shock-paired odor (learning, memory) or individual stimulus (sensory acuity).

Statistical Analysis

The Shapiro-Wilk test (Zar, 1996) showed that all 57 data samples in this report are distributed normally. Comparisons were made using ANOVA followed by the Student-Numan-Keuls (SNK) multiple range test (Zar, 1996) (SAS Institute software).

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Author Contributions

Conceived and designed the experiments: Jd XW SR. Performed the experiments: XW DG. Analyzed the data: Jd XW SR. Contributed reagents/materials/analysis tools: Jd SR. Wrote the paper: Jd XW SR.

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CHAPTER 3

ENVIRONMENTAL EFFECTS ON *DROSOPHILA* BRAIN DEVELOPMENT AND LEARNING

Abstract

Brain development and behavior are sensitive to environmental input. Martin Heisenberg and colleagues observed that a crowded culture density for larvae and an enlarged living space for adults increased the size of mushroom bodies (MBs) in the *Drosophila* brain. The study in Chapter 2 revealed that MB development and associative odor learning were severely impaired by ecologically relevant hyperthermic episodes throughout larval and pupal development. Whereas sensory environment provides a complex experience of both enrichment and stress, little is known about how multiple environmental factors interact to affect the brain and cognitive functions. We addressed these issues by testing the individual and combined effects of sub-adulthood thermal stress, larval density, and early-adulthood living space enrichment on brain anatomy and olfactory learning in adult flies. We found no significant increase in brain structure volumes or odor learning capacities in flies that experienced either larval crowding or early-adulthood space enrichment. Likewise, neither larval culture density nor early-adulthood experience mitigated MB or learning reductions induced by heat stress. These results suggest that brain development and behavior show diverse plasticity in response to environmental conditions. This plasticity also contributes to the brain's resilience in its capacity to adapt to variations.

Introduction

Brain development is tightly regulated by genetic programs, whereas environmental factors play important roles in sculpting and refining the neural circuitry and consequent behavior (Eisenberg, 1999, Rutter et al., 2006, Sale et al., 2009). On the one hand, environmental enrichment has been revealed to have positive effects on the brain and brain function (Rosenzweig & Bennett, 1996, Van Praag et al., 2000). In a series of well-known experiments, rodents raised in enriched environments showed significant increases in neurogenesis, brain weight and size, and learning and memory relative to their impoverished siblings (Fordyce & Farrar, 1991, Kempermann et al., 1997, Rosenzweig & Bennett, 1969). On the other hand, central nervous system development has been found to be disrupted by environmental stress exposure (nutritive, chemical, electromagnetic and thermal) in every model system studied to date, including humans (Ahmed, 2005, Rice & Barone, 2000, Roebuck et al., 1998, Weinstock, 2001). For example, neural tube defects, one of the most common birth defects of the brain and spinal cord in humans, have been associated with maternal early pregnancy hyperemia (Chambers, 2006, Moretti et al., 2005).

The environment-related neuronal and behavioral plasticity phenomenon is not limited to vertebrates. In *Drosophila*, social context was suggested to be an enriched environment that improves brain development, especially in mushroom bodies (MBs), the conserved sensory integration and associative odor learning center. Female flies from high density larval cultures had more MB neuron (Kenyon cell, KC) fibers than flies from low density larval cultures (Heisenberg et

al., 1995). In adult flies, living space was shown to be the most important enrichment parameter. In groups of flies reared in large cages with various odor sources and visual stimulation or with just open food bottles, females had more KC fibers and larger MB calyx volume than their sisters maintained under normal lab rearing conditions in standard food bottles or isolated singly in small plastic vials (Heisenberg et al., 1995, Technau, 1984). In a more recent study, we showed that daily episodes of physiologically relevant hyperthermia throughout larval and pupal development severely reduced MB calyx volume by decreasing the number of KCs (Wang et al., 2007). These flies also had proportional reductions in Pavlovian odor learning abilities.

Given the demonstrated benefits of sensory enrichment and detrimental impacts of stress on CNS development, it is possible that these effects could offset each other in organisms concurrently experiencing variation in sensory enrichment and stress. Indeed, enriched environments aided recovery from cortical and behavioral deficits associated with malnutrition and crowding in rats (Carughi et al., 1989). Remarkably, environmental enrichment has been shown to delay and even recuperate brain disorders such as Huntington's disease, Alzheimer's disease, and Parkinson's disease in rodent models (Nithianantharajah & Hannan, 2006). To further investigate the effects of multiple environmental factors and their interactions on brain development and function, we examined the brain anatomy and learning behavior in flies exposed to sub-adulthood heat stress, larval crowding (larval social enrichment), early-adulthood living space enrichment, and combined rearing conditions. Our data indicate that

neither larval crowding nor early-adulthood space enrichment significantly enhanced brain structure volumes or associative odor learning abilities, nor did they mitigate sub-adulthood daily heat stress-induced deficits in MB development.

Materials and Methods

Flies

Wild-type *Drosophila melanogaster* adults were used to establish populations in the laboratory from a large orchard population collected in southern Nevada in 2002. The lineage of these flies was used for all stress and enrichment studies in which we assessed anatomy and behavior. We cultured flies at 23℃ (except for the heat stress treatment, below). Flies were allowed to oviposit overnight on petri dishes containing 10% molasses and 1% agar. In the larval culture density experiment, we transferred from 1 to 900 1st instar larvae (4-8 h after hatching) into plastic vials (Genesee Scientific) containing 8 ml of standard *Drosophila* cornmeal medium (yeast, soy flour, cornmeal, and corn syrup; recipe from the Bloomington *Drosophila* Stock Center at Indiana University). Adult flies were collected every day after eclosion. Since flies that emerged later in severely crowded densities varied considerably in size, only those emerging in the first 4 days were used in the following experiments to minimize size variation. In the adult deprivation/enrichment experiment, 150 1st instar larvae were transferred to plastic vials with 8 ml of standard medium. After eclosion, single adult flies were isolated in plastic vials with 8 ml of standard medium (deprived environment).

The control group consisted of approximately 100 adult flies that were transferred into each plastic bottle (Genesee Scientific) containing 50 ml of standard medium. For space-enriched treatment, approximately 500 adult flies were released into each of 0.5 m^3 meshed cage containing 5 open food bottles. Food bottles were changed or replaced every 3-4 days.

Thermal Stress

Control (CT) flies were reared at a constant 23°C. Heat stress (HS) treatment consisted of a single daily 39.5°C pulse for 35 min throughout larval and pupal development, administered by immersing culture vials of larvae in a circulating water bath.

Histology and Anatomy

We analyzed the brain neuropil anatomy by using a paraffin mass histology as described previously (de Belle & Heisenberg, 1994, Heisenberg & Bohl, 1979) for 3-4-day-old *Drosophila* adults in the density comparison experiment and 19-21-day-old *Drosophila* adults in the enrichment experiment. Flies were cold-anaesthetized, placed in collars, fixed in Carnoy's solution, dehydrated in ethanol, embedded in paraffin, cut in 7 μm serial frontal sections, and photographed under a fluorescence microscope with an AxioCam digital camera (Zeiss). The volumes of brain neuropil structures were measured planimetrically in serial brain sections using AxioVision software (Zeiss).

Behavior

We analyzed the associative odor learning by using a Pavlovian conditioning T-maze paradigm as described previously (de Belle & Heisenberg, 1994, Tully &

Quinn, 1985) for 3-6-day-old flies in the larval culture density experiment and 19-21-day-old flies in the adult enrichment experiment. Briefly, to assay odor learning, groups of approximately 100 flies were transferred into a training tube embedded with an internal double-wound electrifiable copper coil. Flies were exposed to an air current (750 ml/min) bubbled through one odor [2×10^{-3} dilutions of 4-methyl cyclohexanol (MCH) or 4×10^{-3} dilutions of 3-octanol (OCT)] in heavy mineral oil (CS^{+}) paired temporally with 1.25 s pulses of 90 V dc electric shock delivered every 5 s for 1 min. They were then exposed to fresh air for 1 min, followed by a second odor without electric shock for 1 min (CS^{-}). Immediately after training, flies were transferred to the lower part of the T-maze, where they were exposed to both odors in converging air currents for 2 min, with the binary option to demonstrate a preference for either the CS^{+} or CS^{-} by walking down one of two collection tubes. Flies were then collected from each tube and counted. Learning performance was measured as a function of shock-paired odor avoidance at 1 min (giving an approximation of learning at the earliest testable time in the T-maze). A second group of flies was trained in a reciprocal manner and tested. Scores from both tests were averaged to account for odor preferences among different populations of flies.

Statistical Analyses

All 57 data samples in this report were normally distributed (Shapiro-Wilk normality test, $P > 0.05$). Comparisons were made using ANOVA followed by the Tukey multiple comparisons test with R software (Team, 2008). For multivariate allometry, data were log transformed and calculated using the *prcomp()* function,

or the *pca()* function in the *labdsv* package, in R (Shingleton *et al.*, 2009). The loadings of the first principal component (PC1) multiplying √n (n is the number of variables, here n = 4) gave the bivariate allometric coefficients for each variable against overall brain size (Klingenberg, 1996). We then generated a bootstrap dataset by randomly sampled the original data 10000 times with replacement to calculate 95% confidence intervals of PC1 loadings as described by Shingleton et al. (2009).

Results

High Larval Density and Thermal Stress Influences on Brain Structures

Experiments with *Drosophila* have demonstrated that larval crowding has negative effects in culture, such as decreased adult body weight and size, increased developmental time, increased variability of adult body weight, size and developmental time, and increased larval mortality (Ashburner, 1989). Nonetheless, adult flies derived from high larval culture density have increased longevity and thermal stress resistance (Miller & Thomas, 1958, Sorensen & Loeschcke, 2001). Female flies developing as larvae under crowded rearing conditions had up to 20% more MB KC fibers than their siblings grown with ample space and food supply (Heisenberg *et al.*, 1995). To investigate a broad effect of crowded larval culture density, as well as the interaction of larval density and hyperthermic stress on brain development, we measured brain structure volumes in adult flies reared at constant 23°C (CT) with cultures of 50, 150, 300, and 450 larvae per vial (LPV), and in flies exposed daily to a brief heat shock at

39.5°C for 35 min (HS) throughout larval and pupal development with cultures of 50, 150, 300, 450, and 900 LPV. As in previous studies, we found that larval culture density had a strong impact on development. While 150- and 300-LPV ("crowded") conditions were still endurable densities, 450-LPV ("overcrowded") severely delayed development, increased the variability of developmental time, and decreased larval and pupal viability and adult eclosion rates.

The volumes of adult MB calyx, central complex (CCX), antennal lobe (AL), and optic lobe (OL) were examined using planimetric microscopy measurements of serial paraffin brain sections (Figure 3-1A). In *Drosophila*, KC perikarya are located posterior dorsally in the protocerebrum; just anterior and ventral to the perikarya, KCs give rise to dendritic fields forming the calyx (Heisenberg, 1980, Ito & Hotta, 1992). Anterior to the MB calyxes, the CCX is situated centrally between the two protocerebral hemispheres (Hanesch *et al.*, 1989). The ALs sit in the anterior ventral part of the brain (Stocker *et al.*, 1990). More peripherally and on each side of the central brain are the OLs, which volume approximates half of the brain (Fischbach & Dittrich, 1989). These different brain structures showed variable plasticity in response to high larval density and heat stress. In CT flies, MB calyx volume was not sensitive to most larval culture densities (Figure 3-1B). The volume of CCX was not influenced by crowded (150 and 300 LVP) cultures, but was significantly reduced by overcrowded (450 LVP) culture compared to non-crowded (50 LVP) culture (Figure 3-1C). Overcrowded (450 LVP) culture strongly decreased both AL and OL volumes relative to crowded (150 and 300 LVP) and non-crowded (50 LVP) cultures (Figure 3-1D and E). In

HS flies, both MB calyx volume and CCX volume showed insensitivity to most larval densities (Figure 3-1B and C). The volumes of AL and OL were smaller only in the 900-LPV culture compared to other lower density cultures (Figure 3-1D and E). Overall, in both CT and HS flies, the volumes of MB calyx, CCX, AL, and OL were not enlarged by larval crowding. Instead, particularly at extremely densely populated cultures, all brain structures volumes were inversely related to larval culture densities. Consistent with our previous findings, heat stress dramatically reduced MB calyx volume, but had less or no effect on other brain structures in non-crowded (50 LVP) larval cultures (Figure 3-1B, C, D, and E). However, the volumes of MB calyx in overcrowded (450 LVP) cultured CT and HS flies were not significantly different.

Static allometry reveals the scaling relationship among individuals between one body part and overall body size or between two body parts (Stern & Emlen, 1999). The slope of such scaling relationships, represented by the letter b, is the allometric coefficient. When there is complete proportionality between a body part and overall body size, the relationship is isometric ($b = 1$). A relatively smaller body part is hypoallometric ($b < 1$), while a larger body part is hyperallometric ($b > 1$) (Huxley & Teissier, 1936). Multivariate allometric coefficients for MB calyces, CCX, ALs, and the OLs were studied to address the scaling relationship between each brain structure and overall brain size produced by larval culture density and the combination of larval culture density and heat stress. Brain structures showed diverse allometry to different environmental factors (Figure 3-1F). In thermally-benign conditions, although there are slight

differences, all structures were virtually isometric to overall brain size. That is, they scaled quite similarly along with each other in response to larval culture density. The combination of heat stress and larval culture density dramatically changed the brain allometry pattern. The MB calyx was considerably hyperallometric to overall brain size, indicating that the decrease in volume in MB calyx is much sharper than that in overall brain as the larval culture density increased. Consequently, smaller flies have proportionally smaller MB calyx than larger flies. On the contrary, the CCX were hypoallometric to overall brain size, namely smaller flies have relatively larger CCX. The ALs and OLs are rather close to isometry.

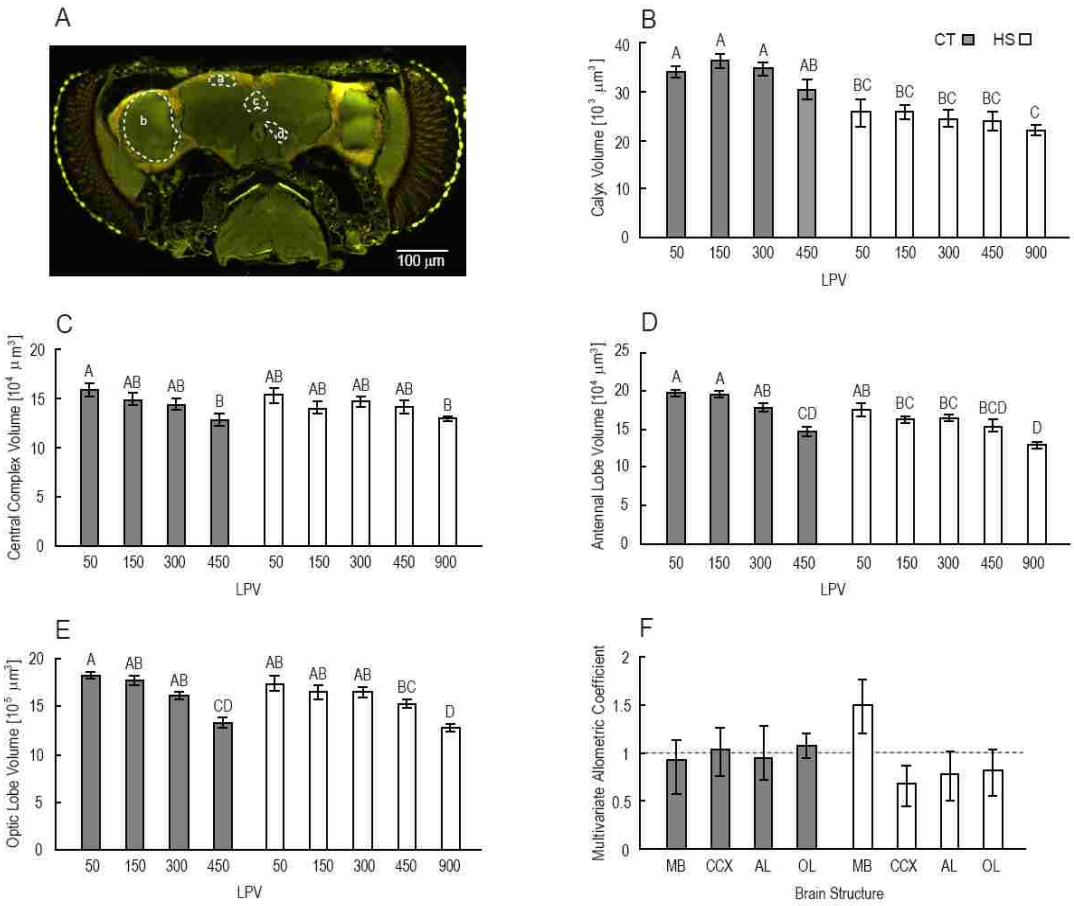

Figure 3-1. Brain development was effected by high larval rearing densities.

Figure 3-1. (Continued)
(A) A frontal paraffin section of the fly brain viewed with a fluorescent microscope. a, MB calyx; b, OL; c, CCX; d, AL. Volume of each structure was estimated from planimetric measurements of serial sections. (B) The MB calyx volume was not significantly affected by larval culture densities, but significantly reduced by heat stress ($F_{[8,72]}$ = 9.37, P < 0.0001). (C) The CCX (ellipsoid body and fan-ship body) was significantly reduced in 450-LPV culture CT flies compared to that in 50-LPV cultured CT flies, but not in HS flies ($F_{[8,72]}$ = 3.81, P = 0.0009). (D) The AL volumes in 450-LPV cultured CT flies and 900- LPV cultured HS flies were significantly decreased relative to 50-,150-, and 300- LPV cultured flies ($F_{[8,72]}$ = 17.62, P < 0.0001). (E) The 450- LPV culture significantly reduced OL volume (medulla, lobula, and lobula plate) in CT flies, and the 900-LPV culture significantly reduced OL volume in HS flies ($F^{[8,72]}$ = 12.85, P < 0.0001). (F) Multivariate allometric coefficients for MB, CCX, AL and OL. Allometric coefficients equal to 1 indicating isometry, larger than 1 indicating hyperallometry, smaller than 1 indicating hypoallometry. Error bars in (B-E) are standard error (SE) and in (F) are 95% confidence intervals. n = 10 / bar in (B-E), n = 40 / bar in CT treatment and n = 50 / bar in HS treatment in (F). Different letters designate significant differences (Tukey, P < 0.05).

Low Larval Density and Thermal Stress Influences on MBs

It has been reported that low larval density increased adult body weight and size, though it also increased developmental time and larval mortality (Ashburner, 1989). Little is known about the influence of sparse larval density on brain development. To address the effects of low larval culture density and interaction of thermal stress and low larval culture density on MB development, we examined MB calyx volume in adult CT flies reared with cultures of 1, 5, 15, and 50 LVP, as well as HS flies reared with cultures of 1, 5, 15, 50, and 150 LVP. There was no statistic difference in MB calyx volumes of any low larval density cultures, in either CT or HS flies (Figure 3-2).

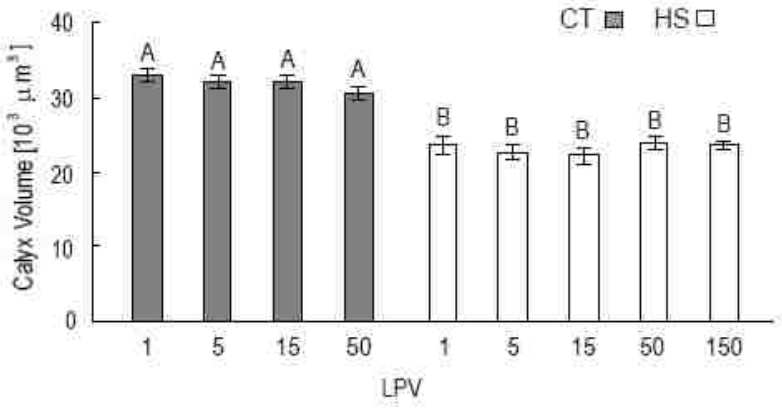

Figure 3-2. MB development was not affected by low larval rearing densities.
MB calyx volume was not influenced by low larval rearing densities, but was significantly reduced by heat stress ($F_{[8,213]}$ = 26.11, P < 0.0001). Bars are mean ± SE, 25 ≤ n ≤ 27 / bar. Different letters designate significant differences (Tukey, P < 0.05).

Adult Living Space and Preadult Thermal Stress Influences on MBs

In addition to the sub-adulthood enrichment and stress stimuli, we studied the influences of adult living experiences, and its combination with sub-adulthood hyperthermic stress on MB anatomy. Previous studies have observed that flies reared in large flight cages have about 15% more KC fibers than their “deprived” siblings reared singly in small plastic vials (Balling *et al.*, 1987, Heisenberg *et al.*, 1995). Here we used volumetric analysis to assess the influence of different rearing conditions on MB anatomy. In the enriched treatment, adult flies were kept in large populations (~500) in big cages (50 cm^3) with open food bottles where they could have social contact and space to fly. In the deprived treatment, adult flies were isolated individually in small vials after eclosion. As a control, ~100 flies were reared as a group in regular food bottles. MB calyx volumes in

these flies with completely different adult experiences were indistinguishable, in spite of their non-heat stressed or heat stressed sub-adult experiences (Figure 3-3). Despite the adult experiences, consistently, sub-adult heat stress severally reduced MB calyx volume.

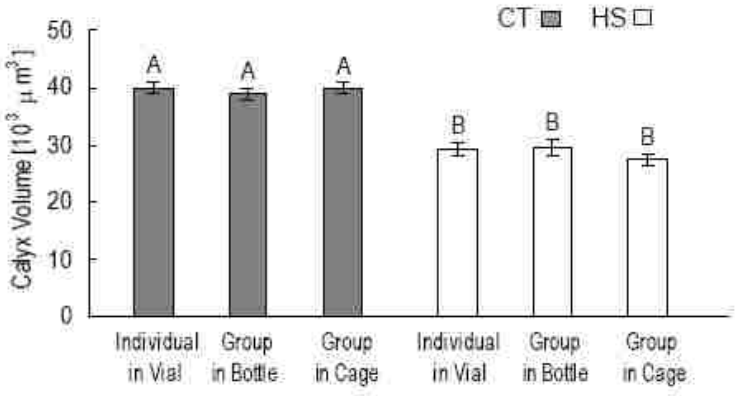

Figure 3-3. MB was not affected by adult living experience.
MB calyx volume was not influenced by either adulthood deprived living condition (single flies isolated in vials) or enriched living condition (flies kept in group in flight cages), but was significantly reduced by daily bouts of thermal stress during development ($F_{[5,185]}$ = 34.64, P < 0.0001). Bars are mean ± SE, 28 ≤ n ≤ 36 / bar. Different letters designate significant differences (Tukey, P < 0.05).

High Larval Density and Adult Living Space Influences on Learning Ability

In *Drosophila*, associative odor learning and memory are mediated by MBs (de Belle & Kanzaki, 1999, Heisenberg, 2003, Zars *et al.*, 2000). We tested the learning abilities of flies with different sub-adult and adult experiences using the pavlovian condition assay (de Belle & Heisenberg, 1994, de Belle & Heisenberg, 1996, Tully & Quinn, 1985) to further investigate the environmental influence on brain cognitive behavior. Larval crowding did not show significant impact on odor learning. Flies grown in crowded (150 and 300 LVP) and overcrowded (450 LVP)

larval cultures performed normally in the associative odor learning test (Figure 3-4A). The early adulthood enrichment with increased living space in cages did not improve odor learning either. The learning performance of flies reared in large cages was similar to that of their siblings reared in regular bottles (Figure 3-4B).

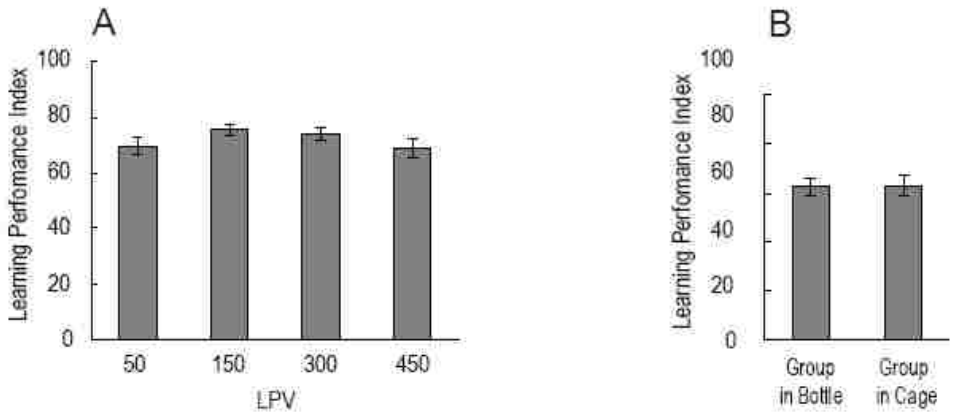

Figure 3-4. Associative odor learning was not affected by larval rearing density or adult living experience.
(A) All flies reared with different larval densities demonstrated similar olfactory learning ($F_{[3,44]}$ = 1.45, P = 0.24). (B) Both fly groups reared in bottles and in flight cages demonstrated similar olfactory learning (P = 0.94). Bars are mean ± SE, n =12 / bar in (A) and n = 10 / bar in (B).

Discussion

Contradictory Findings in MB Studies

In *Drosophila*, increases in brain structure size, especially MBs, have been observed as an enrichment result from densely populated larval culture and enlarged adult living space (Heisenberg *et al.*, 1995, Technau, 1984). This current study, however, does not find any significant enhancement in brain anatomy and cognitive behavior in flies from either crowded larval cultures or flight cages. Additional contradictory findings in MB studies have also been

reported. For example, both Technau (1984) and Heisenberg (1995) showed that MB fiber numbers of the flies from flight cages were larger than those of flies kept isolated in vials. However, Balling et al. (1987) observed that in one of their enrichment/deprivation experiments the difference of MB fiber number was very small and non-significant. In addition, they reported that MB fiber number in newly eclosed flies was remarkably high and it declined during the first week, which was contrary with Technau's (1984) report, indicating that MB fiber number started with a low number and increased during early adulthood.

Given the bizarre architecture of the MBs, early studies (before 1995) counted the numbers of KC fibers from cross sections through the peduncle using electron microscope to represent the size of MBs. In later studies, volumes of MB calyxes were derived from planimetric measurements of serially sectioned brains aided by fluorescence microscope to characterize the MB sizes (Heisenberg *et al.*, 1995). However, the results of MB KC fiber number and calyx volume are not always in complete agreement. Female peduncles contain more KC fibers than male peduncles, though male flies showed larger calyxes than female flies (Heisenberg *et al.*, 1995). Heisenberg et.al (1995) pointed out that KC fiber numbers probably do not reflect MB cell bodies precisely, as the outgrowth and degeneration of fibers might occur independently of cell death in the adult brain. In our previous study we found that MB calyx volume, peduncle cross section area, and KC perikarya number were all reduced by approximately 30% or more in flies experiencing daily thermal stress throughout sub-adult development relative to CT flies reared at a constant benign temperature (Wang *et al.*, 2007).

Here we found no significant increases in the volumes of the MB calyx or other brain structures as consequences of enrichment from either the enhanced larval social contact or enlarged adult living space in flies.

Enrichment Influences on Brain Development and Behavior

Earlier isolation and overcrowding observations have suggested that normal development in brain and behavior requires an optimal environmental stimulation (Rosenzweig & Bennett, 1976). While low larval density may not provide adequate stimulation for the brain to develop, high larval density may be potentially stressful or even harmful because of the excessive utilization and interference competition of food and space (Beebee & Wong, 1992, Roberts, 1998, Rodriguez-Munoz *et al.*, 2003, Walls, 1998). In our study, although it was not significant, MB calyx volume of crowded larval cultures (150 and 300 LVP) was larger than that of uncrowded larval culture (50 LVP), which was larger than that of overcrowded larval culture (450 LPV) (Figure 3-1B). The similar trend also appeared in the odor learning tests. Flies reared from crowded larval cultures (150 and 300 LVP) showed slightly higher (not significant) learning abilities than flies from uncrowded (50 LVP) and overcrowded (450 LVP) larval cultures (Figure 3-4A). Moderate larval crowding might provide a favorable density that improves brain development and cognitive function in *Drosophila*, while it provides enhanced social stimulation, modest competition, and sufficient nutrition.

Environmental enrichment has been shown to enhance neuroblast proliferation, neuronal survival, as well as morphological changes like

synaptogenesis and dendrite branching (Kempermann *et al.*, 1997, Sandeman & Sandeman, 2000, Van Praag *et al.*, 1999b, Volkmar & Greenough, 1972). Crowded larval cultures and space enriched flight cages might induce neuronal re-growth or re-sculpture, but those fine changes may not be discovered by our volume measurement with fluorescence microscope. Application of confocal microscopy and electron microscopy might be required to locate sub cellular changes in the fly brain. In addition, rodents reared in enriched laboratory environments were found to have improved learning and problem-solving abilities (Renner & Rosenzweig, 1987, Van Praag *et al.*, 1999a, Wainwright *et al.*, 1993). However, the results were often short-lived and depended on multiple factors, for example the age at which enrichment was experienced, and the tasks that were learned and measured (Rosenzweig *et al.*, 1972). Rosenzweig (Rosenzweig, 2003) has cautioned against over-interpretation of enrichment experiments on learning ability: “Early enrichment may improve subsequent learning of one task, have no effect on another task and actually impair learning of a third. Perhaps we should not expect much transfer of capacity among entirely different kinds of behavior. Nor should we expect experience in an enriched environment to lead to an increase in ‘general ability.’” In the olfactory aversive Pavlovian conditioning paradigm, we found no significant learning difference among flies reared from variable larval densities (50, 150, 300, and 450 LVP), or among flies with different early-adulthood experiences (space enriched flight cages or regular food bottles). In line with Rosenzweig’s suggestion, alterations in behavior might be stimulated in flies that experienced crowded larval cultures and space enriched

flight cages, but more prominent in just certain neural circuits. More behavior assays (Pitman *et al.*, 2009), such as courtship conditioning (Siegel & Hall, 1979), olfactory appetitive conditioning (Tempel *et al.*, 1983), visual learning (Dill *et al.*, 1993), heat box spatial memory (Putz & Heisenberg, 2002), aversive phototaxic suppression (Le Bourg & Buecher, 2002), might be helpful to uncover the possible difference induced by those environmental enrichment factors.

However, it is possible that neither moderate larval crowding nor increased space in a flight cage constitute enriched environments for flies. Enrichment can be defined as "a combination of complex inanimate and social stimulation" (Rosenzweig *et al.*, 1978); though the so-called experimental enriched environment should be also defined relative to the regular laboratory impoverished settings, rather than enrichment over the natural living conditions. Additionally, studies in rats revealed that enriched environment induced affects were mostly associated with an increase in voluntary motor behavior or exercise (Kempermann *et al.*, 1997, Van Praag *et al.*, 1999a). In crowded cultures, larvae were exposed to increased social interactions, but with few changes in activity. We also noticed that flies were inactive unless disturbed in flight cages as well as in the bottles. Most of the time, we observed flies remaining inside or at the edge of the food bottles. The lack of stimulation of exploratory movement or voluntary exercise might be one of the reasons that our laboratory rearing conditions were not sufficiently enriched to induce significant responses in brain structures and behavior in *Drosophila*.

Combination of environmental Influences on Brain Development

Larval crowding in *Drosophila* has been reported to induce heat shock protein 70 (Hsp70) expression, and lead to increased adult longevity and adult thermal stress resistance as Hsp70 has positive effects on survival to stress (Sorensen & Loeschcke, 2001). We combined larval crowding and heat stress to study their combined effects on brain sizes. In our experiment, the negative effects of heat stress on the volume of MB calyx appeared to be counterbalanced in overcrowded larval rearing density (450 LVP, Figure 3-1B). In 50-LPV cultures, MB calyx volume was reduced in HS flies relative to CT flies (statistic groups A vs. BC). In 450-LPV cultures, MB calyx volume was not significantly different in HS and CT flies (Figure 3- 1B, groups AB vs. BC). The effects of daily hyperthermic stress were so deleterious that they caused more than 60% larvae mortality. That is, the heat stress would decimate a culture density from 450-LPV to about 150-LPV. Therefore, MB calyx volume in HS 450-LPV cultured flies was actually more comparable with that measured in CT 150-LPV cultured flies by the end of development. Indeed, MB calyx volume of HS 450-LPV cultured flies (150-LPV as final density) was smaller than that of CT 150-LPV cultured flies. High larval density did not mitigate the harmful effects of the daily hyperthermic stress. Instead, the heat stress probably alleviated the high larval density induced developmental pressure of malnutrition and competition by increasing larval mortality (i.e. decreasing larval density), although it still disrupted MB development. Thus, our study provides an example of multiple harmful stimuli

combining to give a beneficial effect. The negative impact of one limits the damaging impact of another.

Environmental Influences on Brain Allometry

Static allometry has been used to study the variation in relative sizes in a population or species in response to variant genetic and environmental regulators. In *Drosophila*, one thorough study showed that different parameters, such as larval rearing density, nutrition, and temperature, result in diverse allometries for different body traits (Shingleton *et al.*, 2009). This is also true of our data. High larval rearing density at normal rearing temperature combined with heat stress produced distinct patterns of scaling relationships between individual brain components and overall brain size (Figure 3-1F). In benign thermal conditions, all measured brain structures were nearly isometric to overall brain size, meaning that all brain structures scaled proportionally together in response to larval culture density. This likely accounts for the similar odor learning abilities of flies reared at different larval densities. In severe thermal conditions, the MB calyx was greatly hyperallometric to overall brain size, while the CCX, AL and OL were hypoallometric or near to isometric to overall brain size in relation to larval rearing density. This result is similar to our previous finding where heat stress effects were exclusively studied. Heat stress severely reduced the MB calyx volume but had less effect on other brain structures at non-crowded (50 LVP) larval culture (Wang *et al.*, 2007). Analyzing those data in multivariate allometry revealed that the MB calyx was particularly hyperallometric (b = 1.76) to overall brain size, while the CCX (b = 0.51), AL (b = 0.40), and OL (b = 0.67) were

hypoallometric or slightly isometric to overall brain size as a result of heat stress. The similarity of allometries resulting from combined impacts of different thermal environments and rearing density compared with allometries resulting from different thermal treatment alone suggests that larval density did not have any compensatory effect in the brain. While the MBs were especially sensitive to heat stress (in terms of both absolute and relative size), their response to differences in larval density were comparatively minor.

Conclusions

Our previous study found that a daily episode of hyperthermia throughout sub-adult development dramatically disrupts MB anatomy (with only minor impacts on other brain structures) and odor associated learning ability in *Drosophila*. Here, we showed that neither larval crowding nor early adult rearing space enrichment significantly enhanced brain structures volume or odor learning performance in flies, while all brain structures scaled proportionally at high larval rearing densities. We found that sparse larval density did not impede MB development. These results show that although some brain structures and behaviors are especially vulnerable to some stressful environmental impacts, the brain is also resilient that it tends to retain its authenticity in its genetically determined development and function under a certain range of situations. A recent study reported that laboratory rearing does not reduce the capacity of snails to form memory compared to others reared in their natural environments (Orr *et al.*, 2008). The study suggests that their laboratory rearing conditions might not be impoverished enough to affect brain development and memory

ability, or the behavior they examined might be “unaltered by environment challenges during ontogeny”. In humans, there is an abundance of data showing that stressed situations, such as severe malnutrition at early ages, causes delayed brain development and decreased intelligence (Grantham-Mcgregor & Fernald, 1997, Ivanovic *et al.*, 2000, Winick & Rosso, 1969). On the other hand, it has also been reported that adaptations can be made by the brain itself in response to retarding growth conditions to maintain successful neuronal development and later cognitive performance (Martyn *et al.*, 1996). Thus, brain plasticity should be defined not only by its ability to change, but also by robust maintenance of developmental and behavioral fidelity in response to environmental variations.

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CHAPTER 4

THERMAL PRETREATMENT MITIGATES HYPERTHERMIA INDUCED MUSHROOM BODY DAMAGE AND GENE EXPRESSION

Abstract

Chapter 2 showed that a daily hyperthermic episode throughout larval and pupal development severely disrupts the mushroom bodies, the centers for sensory integration in the insect brain, and hence impairs the associative odor learning in adult *Drosophila*. In the present study, we applied a mild thermal pretreatment before the acute thermal stress. The heat pretreatment moderately mitigated the hyperthermia induced mushroom body calyx volume reduction and fluctuating asymmetry increment, but did not protect flies from the decrease of cognitive ability and male specific early-stage sterility. Moreover, we analyzed genome-wide transcripts alteration associated with thermal pretreatment and stress. The variation of gene expression pattern in flies treated with both heat pretreatment and heat stress was much smaller than that in flies treated with heat stress only. A small set of the differing expressed genes were tested through mutant analysis and one was found to significantly affect mushroom body anatomy. These results suggest that (1) the protection of heat pretreatment against heat stress induced damage on mushroom body development and gene activity in the brain is incomplete, and (2) the long-term hyperthermia disturbed genes may have important functions in mushroom body plasticity.

Introduction

Hyperthermia has been shown to be one of the most deleterious environmental stresses that can disrupt organismal development. In the laboratory, experiments carried on mouse, rat, rabbit, cat, dog, etc. pointed out that thermal stress may cause histological and physiological changes on the central nervous system (CNS), and impede CNS neurogenesis and growth (Ahmed, 2005). *Drosophila melanogaster*, one of the most extensively studied metazoan models outside mammals, is also vulnerable to hyperthermia (Feder et al., 1997, Krebs & Feder, 1997b). More than 10% of eclosing adult flies that had survived natural heat stress were found to exhibit severe developmental anomalies of wing and abdominal morphology (Roberts & Feder, 1999). In a recent study, We observed that a daily episode of ecologically relevant hyperthermia throughout larval and pupal development dramatically disrupts the anatomy of mushroom bodies (MBs), the conserved integrative sensory centers in the brain, but has little effect on other brain structures; and greatly impairs the associative odor learning without affecting memory in adult flies (Wang et al., 2007).

On the other hand, to defend development and enhance fitness, upon heat and other stresses, nearly all organisms express heat shock proteins (Hsps), which help to protect cells by functioning as molecular chaperones (Feder & Hofmann, 1999, Parsell & Lindquist, 1993). In *Drosophila*, it has been demonstrated that thermal pretreatment can induce Hsp70 expression, therefore increasing larval thermotolerance (Krebs & Feder, 1998) and alleviating heat-

induced locomotor impairment (Roberts et al., 2003). However, acute tissue damage was not prevented by thermal pretreatment (Krebs & Feder, 1998). In addition, there have been experimental evidences indicating disadvantages of Hsp expression, such as that overexpression of Hsp70 decreases larval development, growth, and thermotolerance (Krebs & Feder, 1997a). To further investigate whether a heat pretreatment is able to protect against the detrimental hyperthermic influences on brain development and its consequent function, in the present study, we examined the MB calyx volume and learning and memory abilities in flies that experienced daily heat stress, with and without a heat pretreatment, during larval and pupal development.

Full genome gene expression of the heat stress response has been widely studied recently (Furusawa et al., 2009, Sonna et al., 2002, Sonna et al., 2004). In *Drosophila*, 1222 genes, including heat shock genes, have been identified to be up as well as downregulated after the application of heat hardening (Sorensen et al., 2005), which provided tremendous information in understanding cellular injury and self-protect mechanisms. However, little is known about the long-term effects of developmental periodic thermal stress on the adult gene activity. Here, we used DNA microarray to investigate the expression pattern of genes in the brain of flies survived from sub-adulthood hyperthermia, with and without a thermal pretreatment, and to explain the thermal stress induced defects in MB and learning.

Materials and Methods

Flies

The lineage of wild-type *Drosophila melanogaster* collected in southern Nevada (population established in the laboratory in 2002) was used for all the hyperthermic treatment and thermal pretreatment studies (histology, behavior, and microarray). The following mutant lines were obtained from the Bloomington Stock Center (Bloomington, IN, USA): w^{1118}; $Mi\{ET1\}Pde1c^{MB02052}$ $CG31704^{MB02052}$ ($CG31704^{MB02052}$), w^{1118}; $PBac\{WH\}CG32444^{f00963}$ ($CG32444^{f00963}$), y^{1} w^{67c23}; $P\{EPgy2\}AcCoAS^{EY12601}$ ($AcCoAS^{EY12601}$), w^{1118}; $P\{GT1\}BG02569$ ($Pepck^{BG02569}$). We background standardized all the mutants by backcrossing them to a white Canton Special line (w^{1118}; *CS*) for 8 generations.

Heat Stress and Heat Pretreatment

Control (CT) flies were reared at a constant 23°C. The heat stress (HS) treatment consisted of a single daily 39.5°C pulse for 35 min during larval and pupal development as described previously (Wang et al. 2007). The heat pretreatment and heat stress treatment (HPHS) comprised 3 stages, 36°C for 1 hr, 25°C for 1 hr, and 39.5°C for 35 min, every day throughout sub-adulthood development. As another control, heat pretreatment (HP), 36°C for 1 hr, was applied to larvae and pupae on a daily basis. We administered all heat treatments by immersing culture vials of larvae or pupae in circulating water baths.

Histology and Anatomy

Paraffin mass histology was used to analyze fly neuronal anatomy as described previously (de Belle & Heisenberg, 1994, Heisenberg & Bohl, 1979). Three-6-day-old *Drosophila* adults were cold-anaesthetized, placed in collars, fixed in Carnoy's solution, dehydrated in ethanol, embedded in paraffin, cut in 7 μm serial frontal sections, and then photographed under a fluorescence microscope with an AxioCam digital camera (Zeiss). The volumes of brain neuropil structures were derived from planimetric measurements of serial brain sections using AxioVision software (Zeiss).

Behavior Assays

We used the Pavlovian conditioning T-maze paradigm to analyze the associative odor learning, memory, and sensory acuity controls as described previously (de Belle & Heisenberg, 1994, Tully & Quinn, 1985). Groups of approximately 100 3-6-day-old flies were transferred into a training tube embedded with an internal double-wound electrifiable copper grid to undergo training. They were exposed to an air current (750 ml/min) bubbled through one odor (2×10^{-3} dilutions of 4-methyl cyclohexanol [MCH, Sigma] or 4×10^{-3} dilutions of 3-octanol [OCT, Sigma]) in heavy mineral oil (Sigma) paired temporally with 1.25 sec pulses of 90V direct current electric shock delivered every 5 sec for 1 min. Flies were then exposed to fresh air for 1 min, and followed with another air current bubbled through another odor without electric shock for 1 min. To assay learning (immediately after training, indicated as 0 min) and memory (30min, 3h, and 6 hr after training), flies were transported to the lower part of the T-maze to

be exposed to both odors in converging air currents from 2 tubes for 2 min, and then collected from those 2 tubes separately and counted. Learning and memory performance index was measured as a function of shock-paired odor avoidance. A second group of flies was trained in a reciprocal manner and tested. Scores from both tests were averaged to account for odor preferences among different populations of flies. In the sensory acuity tests, a performance index (PI) represents the percent avoidance of the electric shock or odors.

Fertility Assays

Virgin male and female flies were crossed within treatment and to CT flies (with normal fertility) to test the fertility. Seven crosses (♂HS × ♀HS, ♂HS × ♀CT, ♂CT × ♀HS, ♂HPHS × ♀HPHS, ♂HPHS × ♀CT, ♂CT× ♀HPHS, ♂HP × ♀HP, ♂HP × ♀CT, ♂CT × ♀HP, ♂CT × ♀CT) were assayed. Seven vials were set up for each cross, with 2 males and two females in each vial. Flies were transferred to new vials every 2 days. The offspring left in each vial were counted after eclosion.

Microarray Analyses

Three-6-day-old male flies were decapitated on a cold plate, and the heads were immediately frozen in liquid nitrogen. Total RNA (Supplemental Figure 4-1) was extracted by using RNeasy Mini kit (QIAGEN, Valencia, CA, USA). Three replicates of 6 microarrays in 3 dye-swap pairs were performed respectively using the DGRC-2 oligonucleotide transcriptome microarrays (*Drosophila* Genomics Resource Center, Center for Genomics and Bioinformatics, Indiana University, Bloomington, IN, USA) and the Cy3/Cy5 Array350 assay kit

(Genisphere, Hatfield, PA, USA) according to the dendrimer use and hybridization protocol (Cherbas, 2006). Microarray slides (Supplemental Figure 4-2) were scanned using the GenePix 4000B scanner and the signal intensities were quantified with the GenePix Pro microarray analysis software (Axon Instruments, Union City, CA, USA)

Quantitative RT-PCR

We performed quantitative RT-PCR (qRT-PCR) to validate 11 genes from the microarray results using the PerfeCTa SYBR Green FastMix for iQ™ kit (Quanta Biosciences, Gaithersburg, MD, USA) on the iCycler iQ™ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The qRT-PCR data were analyzed with the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Primer sequences were designed via Primer-BLAST online software (Rozen & Skaletsky, 2000) (NCBI webpage) according to the DNA sequence printed on the microarray for *Actin 5C* (*Act5C*), *Acetyl Coenzyme A synthase* (*AcCoAS*), *ade5*, *CG11395*, *CG14075*, *CG1628*, *CG32444*, *CG31704*, *CG8193*, *Glutamate oxaloacetate transaminase 2* (*Got2*), *Phosphoenolpyruvate carboxykinase* (*Pepck*), and *prophenol oxidase A1* (*proPO-A1*) (Table 4-1). All primers were ordered from Integrated DNA Technologies (IDT, San Diego, CA, USA). We also used these primers to measure the transcript levels in the mutant flies.

Statistical Analyses

The R software (Team, 2008) was used for all statistical analysis. In Figure 4-1A and C and Table 2, the Shapiro-Wilk test showed that all 44 data samples are distributed normally. Comparisons were made using ANOVA followed by the

Tukey multiple comparisons test. In Figugure 4-1B, differences in fluctuating asymmetry (FA) of MB calyx volume were analyzed (Palmer & Strobeck, 2003). The distribution of differences between right (R) and left (L) sides of MB calyx volume (R-L) and mean of R-L equals to 0 were tested by Shapiro-Wilk test and t-test in each treatment (CT, HP, HPHS, and HS). In each treatment, the frequency distribution of R-L appeared normal and the mean of R-L equaled to 0, which exhibited ideal FA. A following Levene's test was used to test the heterogeneity of variance among treatments (P = 0.013). Subsequently, means of the absolute value of R-L (|R-L|) were compared to find the differences in FA. The Shapiro-Wilk test showed that 3 out of 4 data samples of |R-L| are not distributed normally (P = 0.0968 for HP treatment). Comparisons were made using Kruskal-Wallis test followed by the nonparametric multiple comparisons with unequal sample size (Zar, 2010). In Figure4-3, the Shapiro-Wilk test showed that 7 out of 8 data samples are distributed normally and 1 datum sample has *p-value* = 0.012. Comparisons were made using t-test between each mutant line and the background wild type line for normally distributed data. The nonparametric Wilcoxon test was used to compare the non-normally distributed datum for the mutant *CG31704* line and the wild type w^{1118}*; CS* line. The *limma* package (Smyth, 2004) in R was used for microarray analysis. The M-values (M = log2R − log2G, i.e. the intensity ratios) were normalized with the loess normalization method for each array, and the A-Values (A = ½ (log2R + log2G), i.e. the average intensities) were then normalized with the quantile normalization method between arrays. The data were fitted in a linear model

according to the direct two-color design matrix, and computed with the empirical Bayes method.

Table 4-1. Sequences for qRT-PCR primers.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
AcCoAS	TTCTCCAAGTTCCCAGGCTA	ACACGACCAGTGATCCACAA
Act5C	GAGCGCGGTTACTCTTTCAC	GCCATCTCCTGCTCAAAGTC
ade5	AACTGGCTGATATTGTGCCC	ATCGACAGCTGGTGGCTATC
CG11395	TCACCAGAATTGAGCACAGC	TTGGGATCCAGGTTGAAGAG
CG14075	GTGGAAATCGTCAGCAAGGT	GTTGGCATCGGTGTAGAGGT
CG1628	CAACCCGCAGTCTAAGAAGAA	CATCCTTTTTATTCACAAGCTCTCT
CG31704	TATTCCAGTACTCCTGCCCG	CTTCTCCACGGTAATGGAGC
CG32444	GACGTCAAGGACACCGTCTT	AGCAGTTGTCGTAGCCCTTG
CG8193	CTAGACGATCCGCACCTGAT	AAGCGGCTCAATAAAGATGC
Got2	TTCAAGAAGGACACCAACCC	CGGCTCACCACTCTCTTCTC
Pepck	GTGCCATCAACCCAGAGAAT	GCCCAACCAGTCAGTGATTT
proPO-A1	ACCGTGGACTACATTGAGGC	GGTGAACGAGGCGAATATGT

Results

Heat Pretreatment and Heat Stress Influences on Development and Behavior

Heat pretreatment partially protected the development of MB in the brain. The average MB calyx volume of HPHS flies was increased by approximately 16% relative to HS flies, but was still reduced by approximately 13% relative to CT flies (Figure 4-1A). Whereas the acute daily heat stress severely disrupted the MB development, the mild heat pretreatment by itself had no effect on the volume of MB calyx. The developmental stability of MB was also perturbed by the heat stress. The MBs of HS flies had larger FA than the MBs of CT flies (Figure

4-1B). The HP and HPHS flies exhibited higher FA in MB relative to CT flies and lower FA in MB relative to HS flies, though the effects were not significant.

However, heat pretreatment did not rescue the learning from the impairment caused by heat stress. Learning (PI at 0 min) of odors paired with electric shock was the same in HPHS and HS flies, which was profoundly reduced by about 24% compared with CT flies (Figure 4-1C). HP flies, experiencing only heat pretreatment, did not show any decline in learning or memory retention relative to CT flies. In HS flies, the associative odor memory decreased to the lowest point after 3h (the 6h memory was not statistically different from the 3h memory). Thus, the memory curves from 0min to 3h were compared between the 4 fly groups. Although the PIs averaged overall retention intervals for HPHS and HS flies were 72% and 62% of the CT flies, the ANOVA treatment × time interaction component was not significant (P = 0.38), indicating that the memories of heat stress treated groups (with and without heat pretreatment) do not decay more rapidly relative to the CT group. Both HPHS and HS flies did not have severe sensory acuity defects. They showed normal avoidance of 2×10^{-3} diluted MCH and 4×10^{-3} diluted OCT odorants and 90 V dc electric shocks used in the classical conditioning (Table 4-2). Hence, the low conditioning performance in HPHS and HS flies was not attributed to the weak olfactory or shock reactivity, but a result of impaired association of these stimuli.

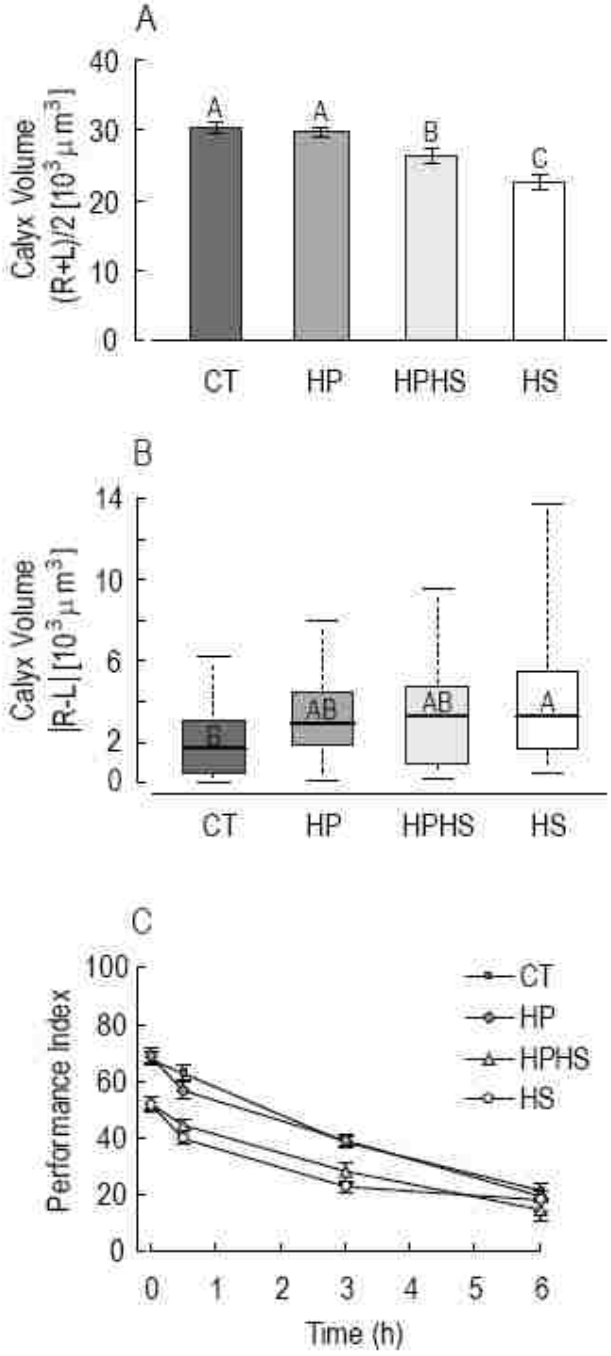

Figure 4-1. Influences of heat pretreatment and heat stress on MB size and associative odor learning and memory.

(A) MB calyx volume (the average of left and right for each fly) for flies with different preadult thermal treatment experiences. 33 ≤ n ≤ 35 in each bar. (B) Box plot of the FA in MB calyx (the absolute value of left and right difference for each fly). Upper and lower edges of each box correspond to the 25% and 75% quantiles; the horizontal line in the box represents the median; the dashed lines

show the 5% and 95% quantiles. 33 ≤ n ≤ 35 in each box. (C) Learning and memory. Performance indexes of learning, 30min and 3h memory of CT and HP flies are significantly higher than those of HPHS and HS flies ($P < 0.001$). n = 12 in each point. (A-C) Each bar, box, or point represents mean ± standard error (SE). Different letters designate significant different groups ($P < 0.05$).

Table 4-2. Aversive olfactory avoidance and shock reactivity.

	Olfactory Avoidance (PI)		Shock Reactivity (PI)
Treatment	MCH	OCT	90 V
CT	74 ± 4	62 ± 3	81 ± 3
HP	67 ± 4	60 ± 5	80 ± 3
HPHS	71 ± 3	55 ± 2	81 ± 3
HS	71 ± 2	57 ± 3	79 ± 4

The odrant MCH was 2×10^{-3} diluted, and OCT was 4×10^{-3} diluted. Each score is expressed as mean PI ± standard error. n= 12 per group for olfactory avoidance, n = 16 per group for shock reactivity.

Moreover, the influence of heat stress and heat pretreatment on flies' fecundity was examined. In the first 6 days, both HS and HPHS males displayed almost complete sterility, although HS and HPHS females were normally fertile (Figure 4-2). Starting from day 7 and 8, the fertility of HS and HPHS males was largely restored. Not surprisingly, the fertility of HP flies was virtually unaffected.

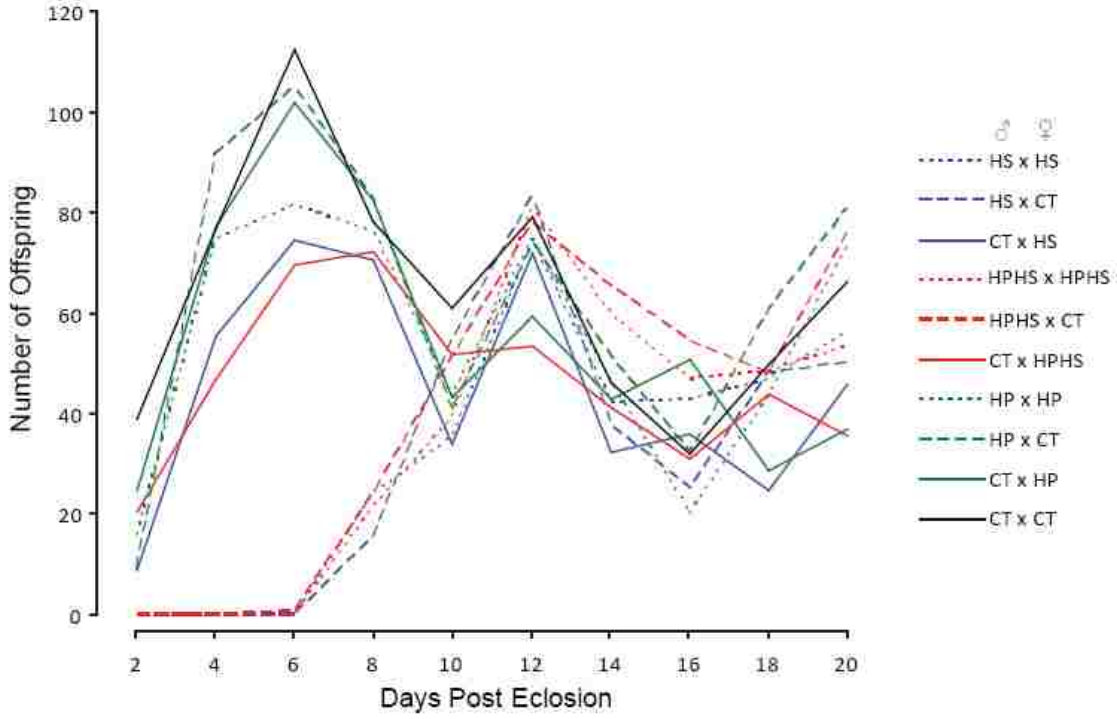

Figure 4-2. Effects of heat pretreatment and heat stress on fertility.
Number of offspring produced by 2 male (♂) and 2 female (♀) flies every 2 days. In the first 6 days, there was no offspring from crosses ♂HS × ♀HS, ♂HS × ♀CT, ♂HPHS × ♀HPHS, and ♂HPHS × ♀CT, but from crosses ♂CT × ♀HS and ♂CT× ♀HPHS, indicating that young HS and HPHS males are sterile, while HS and HPHS females are normally fertile. The fertility of HS and HPHS males was largely restored starting from day 8.

Heat Pretreatment and Heat Stress Influences on Gene Expression

The differences in gene expression in the heads of HS, HPHS, and CT flies were studied to find out the long-term effects of preadult heat stress, with and without heat pretreatment, on the adult. Since HP flies did not show any defect in MB development, or learning or memory abilities, it was not included. A loop dye-swap design was used for the two-color microarray experiment (Smyth, 2004; Yang & Speed, 2002) (Figure 4-3A). Of 14018 transcripts, 8618 transcripts were

identified above the background threshold (signal intensity > 132), of which 7343 transcripts showed non-probe specific dye effects ($P_{\text{dye-effect}} > 0.05$). We then used both statistical ($P < 0.05$) and fold-changing (fold-change > 1.5) criteria to filter the significantly differently expressed transcripts. 4.11%, 0.91%, and 0.15% of the transcripts showed significant different expression pattern between HS/CT, HPHS/CT, and HS/HPHS flies (Figure 4-3B, C, and D). Of those, 61 genes were up regulated and 8 genes were down regulated only in HS flies; 1 gene was up regulated and no genes were down regulated only in HPHS flies; 9 genes were up regulated and 3 genes were down regulated in both HS and HPHS flies relative to CT flies. The annotation from FlyMine (Lyne *et al.*, 2007) revealed that about half of proteins encoded by these genes function in diverse biological processes, including proteolysis, metabolic process, and protein transport among others (Table 4-3). The molecular functions of the other half genes are still unknown.

To validate the microarray results, we used qRT-PCR to assay the expression pattern of 8 HS downregulated genes and 3 HS and HPHS downregulated genes. There were 7 genes that showed similar reduced expression in HS and HPHS flies (fold-change > 1.5, Table 4-4). To evaluate whether these genes are involved in the MB development and odor associated learning, we then chose 4 mutant *Drosophila* lines corresponding to confirmed stress downregulated genes that were publicly available. Further qRT-PCR indicated that the gene expression of *CG31704*, *CG32444*, and *Pepck*, but not *AcCOAS*, was interrupted in the cantonized mutants (data not shown). Mutant $CG32444^{f00963}$ showed a significant

decrease in MB calyx volume (Figure 4-4A, *P* = 0.005). However, none of the mutants displayed any significant difference in learning compared with wild type flies (Figure 4-4B).

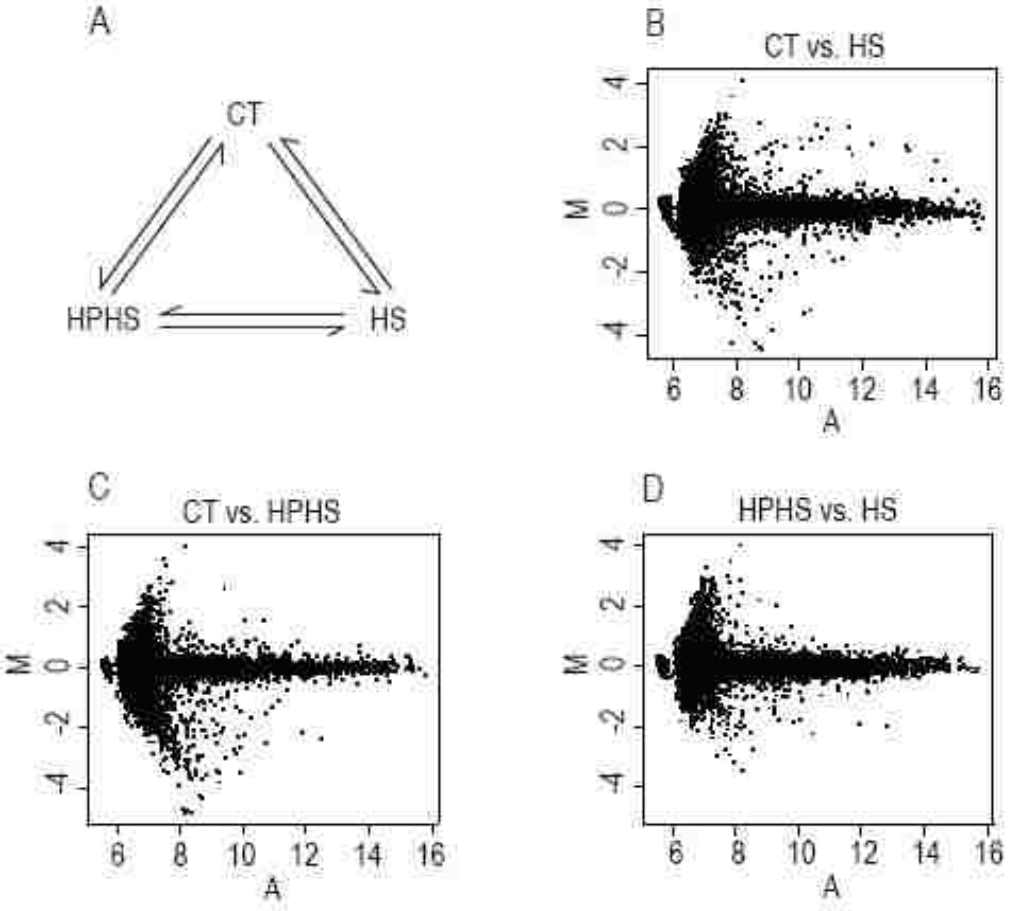

Figure 4-3. Influences of heat pretreatment and heat stress on gene expression. (A) Microarray two-color experimental design. Each microarray chip is represented by one arrow, which points in the Cy3 to Cy5 direction. (B-D) MA plots of microarray data reflect the comparison of gene expression between CT and HS, CT and HPHS, and HPHS and HS. M represents the signal intensity ratio and A represents the average signal intensity for a dot in the plot.

Table 4-3. List of genes showing significant differences in gene expression between HS, HPHS, and CT flies.

Gene	Biological process	Gene	Biological process
HS up-regulation			
alphaTry	proteolysis	Mlc1	mesoderm development, muscle contraction
Bace	proteolysis	Cpn	rhabdomere development
CG12374	proteolysis	CG10910	
CG7542	proteolysis	CG1136	
epsilonTry	proteolysis	CG11672	
Jon25Bii	proteolysis	CG12699	
Jon25Biii	proteolysis	CG13324	
Jon65Aiii	proteolysis	CG14022	
Jon65Aiv	proteolysis	CG15043	
Jon74E	proteolysis	CG1674	
Jon99Ci	proteolysis, digestion	CG16884	
Jon99Ciii	proteolysis, digestion	CG16885	
yip7	proteolysis	CG33346	
Gasp	chitin metabolic process	CG3819	
obst-B	chitin metabolic process	CG3906	
CG14125	chitin metabolic process	CG4000	
CG14645	chitin metabolic process	CG4363	
serp	chitin metabolic process	CG4783	
	open tracheal system development	CG5107	
CG33173	chitin metabolic process, transport	CG5172	
LysP	cell wall macromolecule catabolic process	CG5399	
	antimicrobial humoral response	CG7203	
LysS	cell wall macromolecule catabolic process	CG8927	
	antimicrobial humoral response	Cpr100A	
LvpH	carbohydrate metabolic process	Cpr92F	
CG6295	lipid metabolic process	Cpr97Eb	
Strn-Mlck	protein amino acid phosphorylation	Cry	
KP78b	protein amino acid phosphorylation	dpr13	
CG7214	positive regulation of NFAT protein	Lcp1	
	Import into nucleus	m1	
CG6484	transmembrane transport	TpnC4	
Act88F	cytoskeleton organization, phagocytosis	TpnC41C	
fln	muscle thick filament assembly		
HPHS up-regulation			
CG13305			
HS and HPHS up-regulation			
CG18180	proteolysis	CG13071	
CG30360	carbohydrate metabolic process	CG8736	
Acp1		Cpr47Ea	
CG12998		Cpr62Bc	
CG13056			

Table 4-3. (Continued)

Gene	Biological process	Gene	Biological process
HS down-regulation			
AcCoAS	metabolic process	ade5	de novo' IMP biosynthetic process
CG32444	hexose metabolic process		inter-male aggressive behavior
Pepck	gluconeogenesis	CG1628	transmembrane transport
Got2	glutamate biosynthetic process	CG11395	
	neurotransmitter receptor metabolic process	CG14075	
	synapse assembly		
HS and HPHS down-regulation			
CG31704	proteolysis	proPO-A1	catechol oxidase activity
CG8193	metabolic process		monophenol monooxygenase activity
	oxygen transporter activity		
	monophenol monooxygenase activity		

Table 4-4. Comparison of microarray and qRT-PCR results in gene eaxpression pattern.

Symbol	Treatment	Microarray Down-regulation Fold change	q RT-PCR Down-regulation Fold change
AcCoAS[ab]	HS	1.54	1.53
ade5	HS	1.50	1.23
CG11395[a]	HS	1.62	1.71
CG14075	HS	1.50	1.41
CG1628	HS	1.55	1.14
CG32444[ab]	HS	1.65	1.63
Got2	HS	1.50	1.29
Pepck[ab]	HS	1.78	1.89
CG31704[ab]	HS	1.66	2.24
	HPHS	1.64	1.63
CG8193[a]	HS	2.31	2.74
	HPHS	1.99	1.51
proPO-A1[a]	HS	1.90	2.87
	HPHS	1.73	2.07

[a]Genes show same expression pattern in microarray and qRT-PCR analysis.
[b]Genes have publicly available mutant line

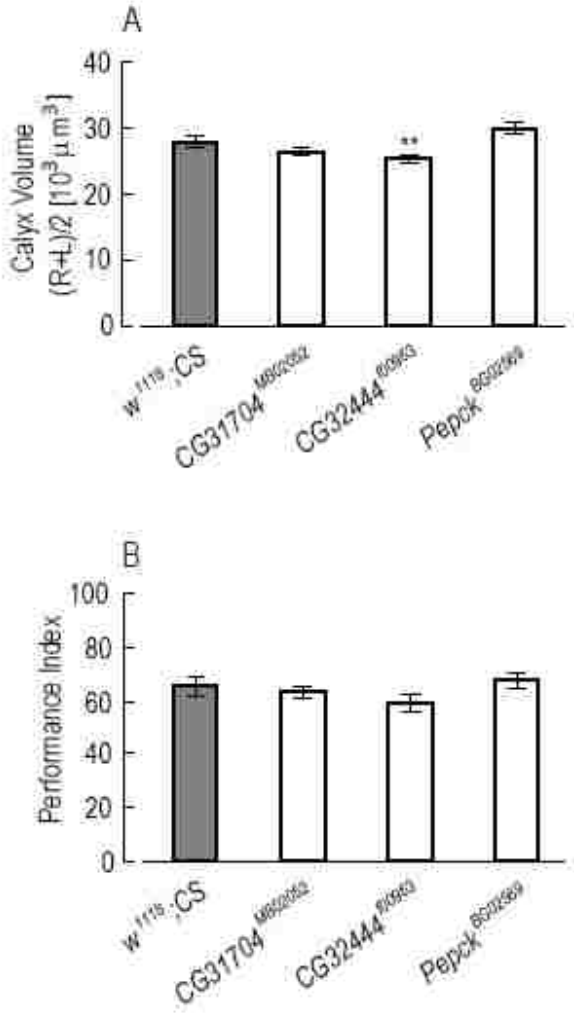

Figure 4-4. Phenotypes of mutants in MB size and associative odor learning. (A) MB calyx volume for wild type and mutant flies representing genes identified in microarray. ***P* < 0.001 for comparison between CG32444f00963 and w1118; CS. 20 ≤ n ≤ 22 in each bar. (B) Learning and memory. None of the mutant lines shows significantly different performance index relative to the wild type line (*P* > 0.05). n = 12 in each point. (A, B) Each bar represents mean ± SE.

Discussion

Here we describe a partial protective effect of heat pretreatment against heat stress at the physiological and molecular levels. The administration of heat pretreatment before heat stress moderately mitigated heat disruption of MB

anatomy, despite having little effect on the impairment of associative odor learning. Our previous work found that the heat stress induced reduction in MB calyx volume was due to fewer intrinsic MB Kenyon cells (KCs) (Wang et al., 2007). It has been also reported that heat pretreatment can abate developmental damages caused by mitotic poisons in *Drosophila* (Isaenko et al., 2002). Heat pretreatment might partly protect KC proliferation from heat stress, thus increased KC numbers and calyx volume. The failure

of heat pretreatment to partly rescue heat stress induced associative odor learning reduction was surprising, given that the heat pretreatment partly increased MB calyx volume. We showed that HS flies with smaller MB calyx had a diminished capacity for odor learning. de Belle and Heisenberg (1994) indicated that hydroxyurea fed *Drosophila* with partially ablated MB showed incomplete loss of odor learning. Both studies suggest a correlation between MB calyx volume and olfactory conditioning, although this cognitive behavior also requires other upstream and downstream extrinsic neurons of the MB. We propose that the changes in MB calyx volume can be reflected by the differences in odor learning, which may be both a continuous and a threshold-like association. Here, the decrease in MB volume in HS flies compared with CT flies reduced the odor learning, whereas the minor increase in MB calyx volume in HPHS flies relative to HS flies might not have been enough to enhance the odor learning. A more supportive observation is that although the KC fiber number slightly decreases in aged flies (Balling et al., 1987, Technau, 1984), the odor

learning remains the same from 10-day-old flies up to 50-day-old flies (Tamura et al., 2003).

Under severe stress conditions, development tends to be disturbed. This developmental instability can be measured as FA, the small and random fluctuation from perfect bilateral symmetry of body traits (Palmer & Strobeck, 1992). FA in MB calyx in HS flies was much larger than that in CT flies, indicating that heat stress is a considerable perturbing MB developmental noise. It has been suggested that heat shock proteins may function as molecular chaperones involved in maintaining developmental stability, as mutations in the Hsp83 gene were found with malformations in various body parts (Rutherford & Lindquist, 1998). Our study shows evidence that heat pretreatment, which induces expression of numerous heat shock proteins, protected MB developmental stability characterized by reduced FA.

Early stage sterility in male, but not in female, HS and HPHS flies was observed. Interestingly, male or female sterility has also been reported in many MB mutations (de Belle & Heisenberg, 1996). The gene mushroom body defect (mud) is such an example that it is involved in MB neuroblast proliferation (Guan et al., 2000) and female meiosis II spindle assembly (Yu et al., 2006). However, the recovery of fertility in older male HS and HPHS flies indicated that their infertility is not strictly an organically developmental defect, but may just be a temporary spermatogenesis halt capable of recovery.

The preadult stage heat stress significantly changed the expression level of 579 transcripts in adult fly heads ($P < 0.05$), among which 92 genes showed fold

changes larger than 1.5 compared with the control. There were 127 transcripts expressed significantly different between HPHS and CT flies ($P < 0.05$), with only 19 genes showing changes larger than 1.5 fold. Remarkably, heat pretreatment alleviated the long-term gene expression alteration caused by heat stress. Nevertheless, this alleviation in gene expression is not complete. Additionally, heat pretreatment did not completely mitigate the heat stress related reduction in MB calyx volume, and it did not affect the odor learning decline or male sterility. Although heat pretreatment induces heat shock responses, which help organisms to be prepared for stresses, it cannot absolutely protect organisms if stresses are extremely harsh and persistent. Here, the changed gene expression in HS and HPHS flies might be both a reflection and an adjustment of the heat stress injury.

The largest overlapping groups of genes that showed long term changes in their expression due to heat stress have been identified in other microarray studies as being involved in immune and stress responses. Of all the overlapping genes, thirteen of them (Act88F, CG4000, CG7542, epsilonTry, fln, Jon25Bii, Jon25Biii, Jon65Aiii, Jon74E, Jon99Ci, Mlc1, TpnC41C, yip7) were found in response to the infection with a sigma virus (Rhabdoviridae) (Carpenter et al., 2009); Ten genes (Acp1, Act88F, alphaTry, CG8736, Cpr47Ea, Cpr62Bc, Cpr92F, fln, Gasp, Strn-Mlck) were involved in the defense triggered by the Pseudomonas aeruginosa (Apidianakis et al., 2005); six genes (CG13324, CG18180, Jon25Bii, Jon25Biii, Jon65Aiv, Jon99Ci) were affected by septic injuries (De Gregorio et al., 2001, De Gregorio et al., 2002); and six genes

(Act88F, CG7203, CG7214, fln, Mlc1, TpnC41C) were related to innate immunity after microbial challenges (Boutros et al., 2002). In addition, seven genes (AcCoAS, CG32444, CG6295, Got2, m1, Pepck, serp) responded to starvation treatment (Fujikawa et al., 2009, Zinke et al., 2002); and six genes (AcCoAS, CG12374, CG32444, fln, Jon65Aiii, Strn-Mlck) exhibited changes in aging or oxidative stress response (Zou et al., 2000). These findings suggest that the most disrupted or adjusted genes in HS and HPHS flies are related to self-protective signaling pathways. There were no genes that were overlapped with the heat shock response genes that are induced shortly after the heat stress. This is not surprising because we examined gene expression profiles in adult flies that were exposed to preadult heat stress.

The long-term heat stress effects on gene activity might correlate more with the developmental and behavioral defects. Such molecular dissection on long-lasting consequences has been studied in MB ablation (Kobayashi et al., 2006) and other behaviors including geotaxis and aggression (Dierick & Greenspan, 2006, Toma et al., 2002). Since the MB calyx volume and the associated odor learning are reduced in HS and HPHS flies, we anticipated that the expression pattern of some MB development and/or odor learning related genes might have also changed. However, all the known MB and/or learning genes showed no significant changes, for example, mushroom body miniature (mbm, CT/HS = 1.05, P = 0.40; CT/HPHS = 1.14, P = 0.28) and latheo (lat, CT/HS = 1.03, P = 0.27; HPHS/CT = 1.12, P = 0.78). One possible explanation might be that many of these genes are not just exclusively expressed in MBs, but also in other brain

structures. These structures are less or not affected by heat stress, but comprise considerable parts of the brain. Further mutant analysis of a set of HS/HPHS disrupted genes detected that only CG32444f00963 is a MB single gene mutant, though none of the mutants showed any significant change in odor learning. It is possible that the interruption of most of these genes is not strong or specific enough to disturb MB development and/or odor learning, since the disruption of MB and/or learning in HS and HPHS is a result of complex changes of many genes. Moreover, our previous finding suggests that the reduced learning in HS flies is due to a lower MB KC number, but not a KC dysfunction (Wang et al., 2007), which might be an alternative explanation for no finding of learning genes and mutants.

The CG32444f00963 mutant showed reduced MB calyx volume, but the reduction in odor learning was not significant. Probably, the decrease in MB calyx volume is not sufficient for a significant reduction in odor learning in the CG32444f00963 mutant, since the association between MB calyx volume and behavior is both continuous and threshold-like, as we suggested above. The annotation of the CG32444 sequence suggests that it has the aldose 1-epimerase activity and may be involved in the hexose metabolic process (FlyBase report). CG32444 has also been identified in other microarray analysis, such as aging and oxidative stress response (Zou et al., 2000), starvation (Fujikawa et al., 2009), copper homoeostasis (Southon et al., 2004), circadian behavior (Ceriani et al., 2002), and aggression (Dierick & Greenspan, 2006) in

Drosophila. These discoveries suggest that CG32444 might play important roles in development and stress response.

It should not be neglected that some of the genes, for example, several of the Jonah gene family (Jon25Bii, Jon25Biii, Jon99Ci, et.al.) that were detected in the fly head by our microarray analysis were reported as only being expressed in the adult fly midgut (Carlson & Hogness, 1985a, Chintapalli et al., 2007). The expression level of Jonah transcripts is very high in midgut; it is actually discovered from an unexpected contamination of midgut in other mass dissected tissue in larvae (Carlson & Hogness, 1985b). We do not suspect that there might be contaminated tissue in the hand-sectioned-adult-head. A possible explanation is that the microarray signal intensities of some of the Jonah genes are close to the background threshold (slighter higher), their sensitivity might not be accurate. In addition, seven out of 11 genes confirmed by the qRT-PCR showed the same expression changes as our microarray results, which indicated a relatively high false positive rate in the microarray data. As a compensation for the common disadvantages of microarray, a new approach, RNA-sequencing (RNA-Seq), is becoming more promising with its low background signal, high quantification accuracy, few RNA sample requirements, and even no requisite for existing genomic sequence (Shendure, 2008, Wang et al., 2009). A genome-wide transcriptome study will be more precise and comprehensive in the future, though the elucidation of the biological processes and networks of all identified genes is still one of the challenges. Here, the analysis of full genome gene expression, especially further studies of the candidate genes, will help us begin

to understand the complex long-term effects of heat pretreatment and heat stress on brain development and behavior.

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CHAPTER 5

CONCLUSIONS

The studies presented in this dissertation used histological, behavioral, genetic, and molecular tools to investigate how and to what extent environmental factors affect brain development and cognitive functions in *Drosophila melanogaster*.

Thermal Stress

The first study demonstrates that adult *Drosophila* MB anatomy, but no other brain structures, is especially susceptible to acute, ecologically relevant heat stress during development. Calyx volume measurements, counts of GFP-labeled KCs, and visualization of GFP-labeled MBs revealed that reduced MB volume is due to fewer KCs, rather than smaller aberrant MB structures. There was no evidence of necrosis in paraffin brain sections of HS flies, which suggests that thermal stress did not induce KC mortality, but impaired KC proliferation. To further determine how thermal stress disrupts the proliferation of MB neuroblasts, but not the migration growth or synaptogenesis of MB neurons, the MARCM (mosaic analysis with a repressible cell marker) system (Lee *et al.*, 1999) can be used to visualize fluorescence labeled single-cell and two-cell clones to trance neurogenesis in a further study.

Associative odor learning, but not memory, was also impaired in HS flies. The approximate 30% learning reduction was correlated with the approximate 30% MB volume and KC number reductions. This relationship indicates that lower memory scores in HS flies were reflections of the reduced sum of conditioned KC

signals received by MB downstream extrinsic neurons. Moreover, since both MB structure and memory decay were not affected in HS flies, it supports the idea that normal KC projection and connectivity are critical for memory storage and retrieval.

Stimulating Enrichment

In *Drosophila*, enhanced social context in densely populated larval culture and enlarged adult living space have been reported to be enriched factors that improve brain development (Heisenberg *et al.*, 1995). However, in the second study, I did not find any significant increases in brain structure volumes in flies reared from either crowed larval cultures or flight cages. These rearing conditions might induce fine neuronal changes, which may not be discovered by the volume measurement with fluorescence microscope. Improvement of microscopy contrast, resolving power, and magnification by use of specific neuronal markers, application of antibodies, and use of confocal microscopy or electron microscopy might be required to locate sub cellular changes in the fly brain.

In addition, the associative odor learning was neither significantly increased by crowded larval cultures nor spatially enlarged flight cages. This finding supports the argument that these laboratory improved settings were possibly not enriched enough. Further research might be necessary to find out the effective enriched laboratory rearing conditions for the flies.

Moreover, numerous studies have reported that larval crowding in *Drosophila* during development actually has negative effects on growth, especially in body

size (Imasheva & Bubliy, 2003, Lefranc & Bundgaard, 2000, Miller & Thomas, 1958). My data showed that the volumes of CCX, AL, and OL were inversely related to larval rearing density. In *Caenorhabditis elegans*, high population density promotes formation of dauer larvae, a non-feeding and non-aging specialized form for stressed environments. The crowded culture is sensed through secreted small-molecule pheromones (Butcher *et al.*, 2007, Golden & Riddle, 1982), and then coupled to endocrine pathways to regulate development and survival (Fielenbach & Antebi, 2008). It is worth mentioning that Heisenberg et al. (1995) showed that the increase in adult fly MB KC fiber numbers in crowded larval culture was mediated by diffusible substances produced by larvae themselves. Rather than depressing MB development, they suggested that this diffusible factor might directly stimulate KC proliferation or fiber growth, or deplete the inhibitory substances in the food. Yet, how rearing density is sensed or coordinated in growth by the *Drosophila* larvae remain poorly understood.

To illustrate the variation in relative sizes in response to heat stress and larval density, I applied static allometry to analyze brain structures. MBs were isometric to overall brain size in benign thermal conditions, but were particularly hyperallometric relative to heat stress. Nevertheless, larval crowding did not have significant effect on MB allometry.

Thermal Pretreatment

The third study demonstrates that the administration of a mild thermal pretreatment before the acute thermal stress moderately mitigated the heat

induced volume reduction in MBs. Furthermore, DNA microarray analysis showed that heat stress significantly changed the expression level of 579 transcripts in adult fly heads, while the addition of heat pretreatment reduced that number into 127. The altered gene expression in HS and HPHS flies might be a reflection and also an adjustment of the heat stress injury. These heat stress long-term affected genes encode proteins involved in diverse biological processes. One of the heat stress downregulated genes, *CG32444,* was annotated to have the aldose 1-epimerase activity and may be involved in the hexose metabolic process (FlyBase report). The *CG32444*^{f00963} mutant showed reduced MB calyx volume, though the functional relationship between them is yet not known. Further studies for the heat stress upregulated genes by using over-expression or knocking-down might find more candidates that are important for mediating neuroanatomical and behavioral plasticity.

Heat pretreatment induces the expression of heat shock proteins, which is a key factor to guard the gene expression and protect the MBs from heat stress. Interestingly, I have found that a chronic equivalent heat stress had no inimical impact on MB anatomy (Figure 5-1). In the chronic heat stress treatment, an incubator was substituted for the water bath that produced the acute thermal stress treatment. The temperature increased much slower in the chronic thermal treatment (heat transmits slower in the air than in the water), indicating a deliberate continuous accumulating of heat shock proteins, which might be more beneficial for their function as molecular chaperones. Thus, this chronic heat stress treatment can be interpreted as a combination of a series of heat

pretreatments (continuously preparing abundant heat shock proteins) and heat stress. In the wild, *Drosophila* larvae inhabit in necrotic fruits that routinely attain temperatures greater than 35℃ and as high as 52℃ und er direct sunlight (Feder, 1997). It would be fascinating to learn how MB development is influenced by natural thermal stress.

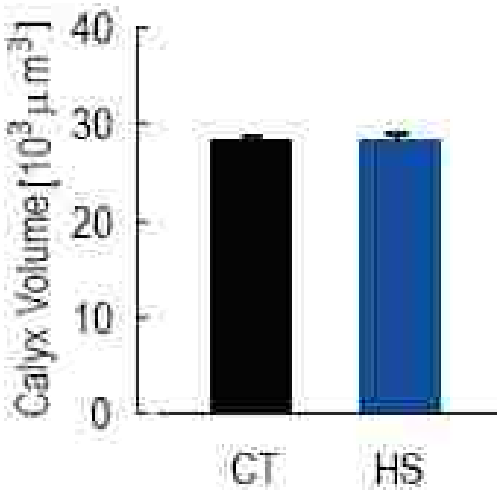

Figure 5-1. Chronic thermal stress does not affect MB development.
MB calyx volume was not significantly influenced by chronic heat stress (t-test, *P* = 0.95). Bars are mean ± SE, n_{CT} = 29, n_{HS} = 30. *Drosophila* culture vials were kept in a programmed incubator. The chronic Heat stress treatment was administered by automatically increasing the incubating temperature from 23℃ to 39.5℃ for 35 min every day throughout larval an d pupal development.

Summary

In this dissertation research, empirical linkages between thermal stress, specific targets in the brain, and consequent behavior were established in *Drosophila*. On the one hand, I discovered that MBs and associative odor learning are especially sensitive to excessive hyperthermia stress. On the other, I

showed that the brain bears resilient adaptability to retain its developing and functioning authenticity in response to certain environmental variations. Therefore, brain plasticity should be defined not only by its ability to change, but also its robust maintenance of developmental and behavioral fidelity. These findings have broad implications for societal and scientific views on genetic and environmental determinism.

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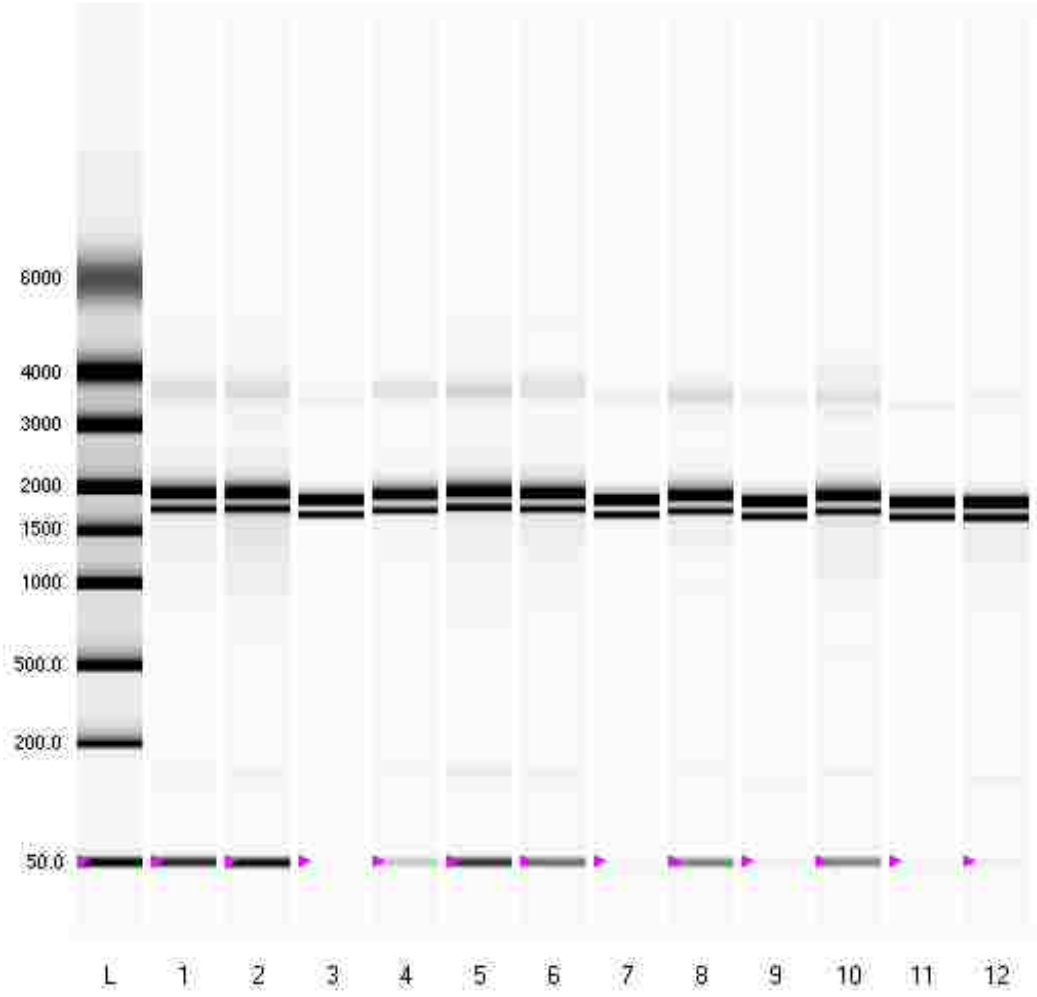
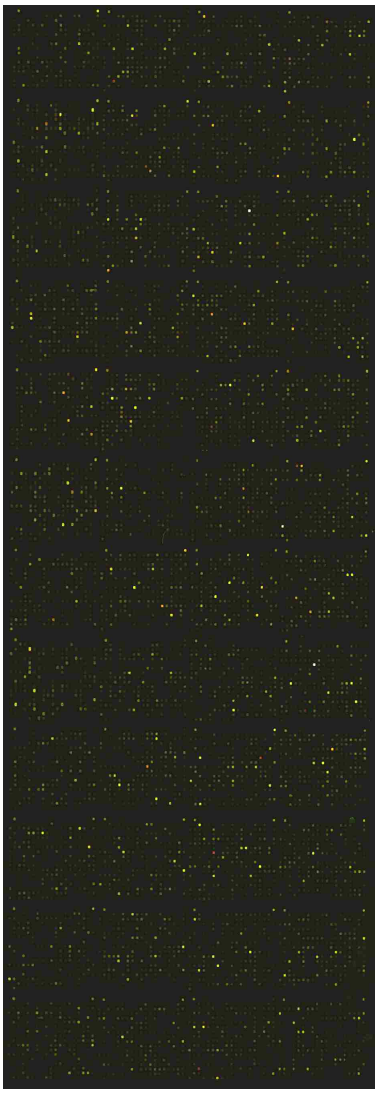
APPENDIX

SUPPLEMENTAL DATA



Supplement Figure 4-1. Electrophoresis gel image of *Drosophila* RNA.
rRNA is visible as two sharp bands half way down the gel (*Drosophila* 28S rRNA is processed into 2 fragments that migrate in a similar manner to the 18S rRNA {Ambion, #355}), whereas mRMA is the smear in the background. Lane L, RNA marker; lane 1-4, RNA of CT flies; lane 5-8, RNA of HS flies; lane 9-12, RNA of HPHS flies.



Supplement Figure 4-2. DNA microarray image.
Chip R1_Chip1_13600633_2007-10-19_6_R1. CT fly cDNA was labeled with Cy3 (green), HS fly cDNA was labeled with Cy5 (red).

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