

TRANSCRIPTIONAL RESPONSE TO SPATIAL LEARNING AND FORAGING IN HONEY
BEES: MOLECULAR BASES FOR ADULT NEUROPLASTICITY

BY

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DISSERTATION

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ABSTRACT

An important goal of neuroscience research is to discover molecular processes that enable persistent neuroplasticity in the adult and aging brain. In many species, some types of neuroplasticity typically decrease with age. Honey bees and other social hymenopterans, however, exhibit a reinstatement of structural plasticity in association with flight and foraging behavior. Adult plasticity in these species often includes growth and remodeling of processes in the mushroom bodies, a paired region of the insect brain involved in sensory integration, learning and memory. Honey bees (*Apis mellifera*) learn to navigate in the environment surrounding the hive by performing orientation flights for several days before they begin to forage; both orientation flights and foraging are cognitively challenging behaviors. Honey bee mushroom bodies show increases in neuropil volume coincident with the increased cognitive demands of orientation flights and foraging behavior, and treatment with pilocarpine, a muscarinic cholinergic receptor agonist, produces growth of the mushroom body neuropil comparable to that produced by maturation and foraging. I have examined molecular processes associated with flight and foraging experience in the mushroom bodies of honey bees to discover mechanisms that may support neuropil growth. Foraging experience regulates multiple molecular processes in the mushroom bodies, including some that may contribute directly to neuropil growth, and others that could potentially protect the brain from the effects of aging and physiological stress. My investigation of a specific behavior, the orientation flight, found that this experience triggers a rapid and transient upregulation of *egr* in the mushroom bodies. *Egr* is a homolog of *egr-1*, an immediate-early gene well known to respond to novel experience that also exhibits higher basal levels in brain regions undergoing development or remodeling in vertebrates. I found that *egr* expression was upregulated in bees prepared to perform orientation flights and in foragers relative to hive bees engaged in brood care, and established its plausibility as a link between muscarinic signaling and neuropil growth.

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CHAPTER 1

INTRODUCTION

Abstract

The discovery of molecular mechanisms that promote neuroplasticity, particularly those that could contribute to the reinstatement of neuroplasticity in aging individuals, is an important goal of neuroscience research. Social hymenopterans, including honey bees, exhibit changes in behavior driven by maturation. Naturally occurring increased structural plasticity in the mushroom bodies has been documented in several species; this structural plasticity occurs late in adult life, in association with cognitive demands related to behavioral maturation. Here I describe how studies of the molecular basis of this phenomenon in honey bees have already contributed to understanding of mechanisms that promote and maintain neuroplasticity in adulthood. The pathways that have been identified in association with mushroom body neuropil expansion are a potential focal point for future studies in bees, as well as important comparative work in other social hymenoptera.

1. Introduction

Behavioral plasticity, particularly learning, allows animal species to respond in an adaptive manner to a changing or unpredictable environment. The physiological basis for this responsiveness is neuroplasticity—the ability to change neuronal structure, activity or both in response to experience. Molecular mechanisms underlying structural and functional plasticity are highly conserved between vertebrates and insects (North and Greenspan, 2007). These commonalities, along with the huge diversity of life histories and behaviors among insect species, provide a wealth of potential study organisms to explore mechanisms underlying neuroplasticity.

Both structural and functional plasticity in the brain are crucial for learning and memory (Butz et al., 2009). Structural plasticity can be quantified on many scales, including that of total neuropil volume of a specific region; increases in neuropil volume are driven by remodeling of neuronal processes, such as lengthening or branching of dendrites, and are often accompanied by addition of synapses (Anderson, 2011). Computational theories of information storage in the brain suggest that increasing total dendritic branch length or numbers of synapses results in

increased memory capacity and computing power (Chklovskii et al., 2004). Formation of new synapses and growth of existing synapses to strengthen a particular neural circuit are both important to memory consolidation, while excess formation followed by pruning (elimination) of less active synapses also allows experience to shape the resulting neural circuitry (Holtmaat and Svoboda, 2009).

One important question is: how can structural plasticity, along with the learning ability it promotes, be maintained in adulthood? In many species including humans, many forms of neuroplasticity peak during infancy, childhood, or adolescent development, while later adulthood and senescence are accompanied by a decrease in some forms of neuroplasticity and learning abilities (Erickson and Barnes, 2003). Understanding how neuroplasticity might be upregulated in adulthood could lead to clinical applications that allow us to maintain neuroplasticity later in life (Hensch, 2004; Bavelier et al., 2010). Species in which behavioral flexibility increases instead of decreasing with age, with a corresponding increase in neuroplasticity, provide excellent opportunities to pursue answers to this question.

The adult workers of several eusocial insect species, notably honey bees, are a prime example of such organisms (Holldobler and Wilson, 2009). This introduction will first review examples of behavioral plasticity and associated structural neuroplasticity in honey bees and other social insects. I will then describe how research in honey bees, including my thesis work, has begun to elucidate the molecular basis of adult neuroplasticity in honey bees.

2. Social insects as models of adult structural plasticity in the brain

Several species of social Hymenoptera, including many species of wasps, ants, and bees, display temporal polyethism; that is, workers specialize in the performance of different sets of tasks at different ages and phases of maturation. In honey bees, younger workers (age 1-3 weeks) perform tasks such as brood care inside the hive, while older workers forage for nectar, pollen and other resources outside the hive (Winston, 1987). In almost all species studied, the behavioral progression is similar (Holldobler and Wilson, 1990), and the later phases of behavioral maturation are likely to represent greater cognitive challenges to the individual performing them (Fahrbach and Robinson, 1995). Since all hymenopterans are central place foragers (Farris and Schulmeister, 2011), accurate navigation and the accompanying requirement for spatial learning are important components of foraging behavior (Farris, 2008a). For many of

these species, foraging also represents the first significant exposure to visual stimuli and environmental odors, because the first behavioral phases are spent almost exclusively inside a dark nest. Animals whose life history involves encountering novel cognitive challenges as they age are expected to show increased neuroplasticity. This might be either in anticipation of or in response to those challenges, or both. These changes have been termed “experience-expectant” and “experience-dependent”, respectively (Greenough et al., 1987). Both types of plasticity have been reported in vertebrates, and in the adult workers of various social insects.

2.1. The mushroom bodies: a locus of adult structural plasticity in the insect brain

In social insects, effects of foraging experience on the brain have been most intensively studied in the mushroom bodies. The mushroom bodies comprise clusters of intrinsic neurons called Kenyon cells; in honey bees, there are approximately 170,000 Kenyon cells per hemisphere, meaning that together the mushroom bodies comprise 35% of the honey bee brain’s total neurons. This proportion is similar to that found in other hymenopterans, but contrast with insects such as *Drosophila*, where the percentage of Kenyon cells among total neurons is approximately 5% (Farris, 2005). Kenyon cell dendrites in each hemisphere project to form a neuropil called the calyx, while their axons bundle together to form a stalk called the peduncle, then bifurcate to form two neuropils called the vertical and medial lobes. In hymenopteran species, including honey bees, the calyx is a doubled cup-shaped structure that receives both visual and olfactory inputs. These inputs are directed to specific, concentrically arranged subregions of the calyx called the lip, the collar, and the basal ring. The lip receives olfactory input exclusively, the collar receives visual input exclusively, and the basal ring receives input from both sensory modalities (Heisenberg, 1998; Fahrbach, 2006). Because of this convergence of sensory modalities on the Kenyon cells, the mushroom bodies are thought to play a role in multimodal integration in honey bees (Strausfeld et al., 1998).

The mushroom bodies are also considered to be a primary brain region for learning and memory in the insect brain, potentially analogous to the hippocampus or the cortex of vertebrates (Capaldi et al., 1999; Farris, 2008b). Studies in *Drosophila* and other insect species have demonstrated the role of the mushroom bodies in a variety of learning and memory tasks, particularly olfactory learning (Heisenberg, 1998). For example, honey bees with unilateral mushroom body ablations induced during development show olfactory learning deficits when

training is specific to the antenna on the ablated side (Komischke et al., 2005). There is also limited evidence that suggests the involvement of the mushroom bodies in spatial learning tasks in some insect species (Farris, 2008b; Brandon and Coss, 1982; Mizunami et al., 1998).

2.2. Foraging-related structural plasticity in the mushroom bodies

Over the past two decades, a wide variety of studies have examined structural plasticity associated with foraging in the mushroom bodies of honey bees and other social insects. This literature is reviewed here, with an emphasis on studies performed in honey bees. A related body of work has shown that in some species of social wasps, mushroom body plasticity is related not only to foraging but to dominance hierarchies among nestmates (O'Donnell et al., 2007; Molina and O'Donnell, 2007; Molina and O'Donnell, 2008; Molina et al., 2009); other work has shown foraging-related mushroom body plasticity in solitary or facultatively eusocial bee species (Withers et al., 2008; Smith et al., 2010). I have chosen to focus on species in which there is evidence of a relationship between temporal polyethism (age-related shifts in task performance) and structural plasticity in the mushroom bodies of the mature adult.

Adult neuroplasticity associated with temporal polyethism was first studied in honey bees. In bees, mushroom body neurogenesis and major neuropil organization are complete before eclosion (Fahrbach et al., 1995; Farris et al., 1999). However, the mushroom bodies of newly emerged adult bees exhibit some active neuropil outgrowth and dendritic branching (Farris et al., 2001). Reinstatement of structural plasticity in the mushroom body neuropil was first discovered by Withers et al. (1993). Their study found that the neuropil of foragers is larger than that of hive bees engaged in brood care; through manipulations of colony demography that cause some bees to begin foraging far earlier than the typical age, they also showed that onset of foraging performance, not age, determined the size of the neuropil. Other work confirmed the occurrence of an expansion in anticipation of foraging onset (Withers et al., 1995; Durst et al., 1994). This growth corresponds to the time during which workers are performing orientation flights (short learning flights performed by pre-foraging bees), but is not dependent on flight experience: increased neuropil volume is observed even in similarly aged bees that have been caged and deprived of light (Fahrbach et al., 1998). This phase of growth is therefore considered to be experience-expectant, a preparation for the increased cognitive demands of flight and foraging behavior.

A second adult phase of growth in the honey bee mushroom bodies is experience-dependent, occurring in response to accumulated foraging experience. Bees that have two weeks of foraging experience have larger mushroom body neuropil volumes than do bees that have foraged for a single week, or bees that were caged and light-deprived for the second week of their foraging career (Farris et al., 2001; Ismail et al., 2006). Neuropil volume in foragers confined to the hive during the second week are intermediate between caged and active bees, suggesting that stress caused by caging cannot explain the lack of growth in less experienced foragers. Foraging-related, experience-dependent expansion is accompanied by increased dendritic branching and branch length in the visual input region of the calyx (Farris et al., 2001), and changes in microglomerular structure and density (Krofczik et al., 2008). Similarly, a single orientation flight induces experience-dependent remodeling of dendritic spines in the mushroom body calyces of workers (Brandon and Coss, 1982).

Studies in other social hymenoptera exhibiting temporal polyethism mirror the pattern of experience-expectant and -dependent growth in the mushroom body neuropil observed in honey bees. In some cases, association between neuroplasticity and the transition to foraging has been shown, but the effects of age, behavior, and experience have not yet been experimentally distinguished. Mushroom body plasticity has been found in *Pheidole*, a genus of ants in which the minor workers add foraging to their behavioral repertoire as they age while also retaining previously performed behaviors such as brood care. Foraging workers of several species (*P. dentata*, *P. morrisi*, and *P. pilifera*) have larger mushroom body neuropil volumes than do younger workers (Muscedere and Traniello, 2012); in one species (*P. dentata*) evidence of synaptic pruning and strengthening of remaining synapses was also found (Seid et al., 2005). These studies did not distinguish between the effects of experience and age. In a eusocial wasp, *Polybia aequaorialis*, older workers performing foraging tasks and those performed on the outside surface of the nest enclosure were found to have larger mushroom body calyx volumes than younger workers performing tasks inside the nest; again, this may indicate experience-expectant growth associated with age or behavioral state, or experience-dependent growth stimulated by exposure to light and environmental odors (O'Donnell et al., 2004).

In studies of other species of ants, manipulation of colony demography has facilitated the ability to distinguish experience-expectant and -dependent effects on mushroom body neuropil. An early study of carpenter ants, (*Camponotus floridanus*), found growth of the mushroom body

neuropil in foraging minor workers (Gronenberg et al., 1996). Older, “idle” workers in nests with a manipulated demography that prevented onset of foraging behavior had enlarged neuropil compared with younger workers, but not as large as same-age foragers, suggesting independent effects of both age and foraging experience. Studies in the desert ant *Cataglyphis bicolor* have yielded similar results. The timing of task progression in *Cataglyphis* is closer to that seen in bees, as are the structural changes observed in the mushroom body neuropil. Ants foraging outside the nest have larger mushroom body neuropil volumes, including larger lip (olfactory) and collar (visual) regions, than ants working inside the nest (Kuhn-Buhlmann and Wehner, 2006). Both absolute age and foraging performance contribute to this growth. Increases in the collar region are particularly large; *Cataglyphis* rely heavily on vision for navigation (Wehner and Raebler, 1979), making growth of a region for higher-order visual processing particularly important.

Research in *Cataglyphis* has also explored, more fully than in species discussed above, the possible “priming” effect of tasks involving light exposure on induction of neuroplasticity. Investigation of changes in the mushroom body neuropil on the synaptic level in two related species (*C. albicans* and *C. fortis*) suggested that neuropil growth was accompanied by pruning of excess axonal inputs to the lip and collar regions and strengthening of the remaining inputs in experienced foragers (Seid and Wehner, 2009; Stieb et al., 2010). Ants deprived of experience showed no pruning with age; instead, synaptic complexes slowly and continually proliferate in the calyces of these individuals (Stieb et al., 2010). Pruning appeared to begin in ants that had not yet transitioned to foraging, but were engaged in digging tasks likely to lead to light exposure, and old enough to perform “exploration runs” similar to orientation flights in bees. Artificially induced light exposure was also sufficient to induce pruning in interior workers (Stieb et al., 2012). Together, these results suggest age-related, experience-expectant neuropil growth, with light exposure serving as a cue to begin consolidation of visual information, and continued foraging activity stimulating further, experience-dependent neuropil growth.

The work reviewed here demonstrates the richness of social insect species as models for the study of adult neuroplasticity, and as potential models for understanding its molecular basis. In the following section and in the subsequent chapters, I have chosen to focus on the honey bee. The honey bee was the first eusocial insect with a published genome sequence (Honeybee Genome Sequencing Consortium, 2006), making it an obvious choice for molecular research. In

addition, honey bees offer the advantages of a long history of ethological study, and vastly longer history of management by humans (Winston, 1987). Although temporal polyethism dictates a predictable age-related progression of behaviors, easing their study, effects of experience can also be easily decoupled from absolute age, as described above. Flight and foraging behavior can be broken down into a variety of cognitive challenges (Giurfa, 2007), a few of which are described below, which are naturally performed and are easily studied in an ecologically relevant environment. Work with honey bees therefore combines the opportunity to explore molecular mechanisms in a naturalistic environment with extensive knowledge of life history and behavior, as well as a wide array of strategies for behavioral manipulation. These advantages have promoted the honey bee as a model organism for neurobiological study, including the investigation of neuroplasticity.

3. Plasticity-related molecular signaling in honey bee mushroom bodies

Structural neuroplasticity relies on transcription: activity-regulated genes transduce environmental and endogenous stimuli to intracellular signaling systems that direct growth, and downstream genes provide the physical substrate for structural and functional changes (Clayton, 2000; Leslie and Nedivi, 2011). Transcriptional changes associated with mushroom body neuropil growth are a subset of the wide array of transcriptional changes that accompany behavioral maturation in the honey bee brain (Whitfield et al., 2003; Whitfield et al., 2006; Chandrasekaran et al., 2011; Ament et al., 2012). These broad surveys of genomic involvement in maturation are valuable to the mechanistic understanding of behavioral plasticity, but permit only limited interpretation of connections to neuroplasticity in the mushroom bodies. Studies that focus on the transcriptional response to specific behavioral aspects of maturation (Chapters 2 and 3), as well as on specific genes (Chapters 3 and 4), are beginning to reveal pathways likely to mediate experience-expectant and -dependent phases of mushroom body structural plasticity.

3.1 Transcriptional changes associated with foraging experience

Prior to my research, the effects of two specific aspects of foraging experience on brain gene expression had been examined: the acquisition of spatial and temporal memories of floral resources. Bees' perception of distance can be manipulated by training foragers to fly through a tunnel, then altering the visual patterning inside the tunnel to simulate different amounts of optic

flow (Srinivasan et al., 2000). Sen Sarma et al. (2010) used this strategy to examine the transcriptional response in the mushroom bodies and the optic lobes (areas of primary visual input) to the perception of altered distance to a familiar sugar feeder. While a total of only 36 transcripts responsive to distance perception were detected, hindering functional analyses, coexpression network analysis revealed a more extensive set of genes correlated with response to distance perception. Genes annotated as being involved in synaptic plasticity were enriched in this set. Distance perception, an important component of spatial learning, may be acting through these and related gene products to contribute to experience-dependent neuropil growth.

Naeger et al (2011) examined transcriptional response to temporal aspects of foraging. In natural environments, foragers learn the time of day when different flowers are in bloom. This allows them to target resources while they are available, resulting in greater foraging efficiency. Bees can also be trained to visit artificial feeders at certain times of day (Moore, 2001). A microarray study that investigated whole brain gene expression in response to this type of time training identified a set of 352 genes differentially regulated in bees with specific spatiotemporal memories after excluding other factors such as time of collection. Functional analyses revealed an enrichment in this gene set of genes related to synaptogenesis. This study, like Sen Sarma et al., provides entry points to understanding the relationship between foraging-related associative learning tasks, differential expression of identified genes, and structural plasticity.

Despite the value of these insights into specific aspects of foraging experience, they do not provide a complete picture of the dynamics of gene expression throughout the foraging career, or how those dynamics may promote mushroom body neuropil growth. In Chapter 2, I addressed this question by performing a time-course microarray study to examine gene expression changes in the mushroom bodies as a function of days foraged. Clustering analysis of the differentially expressed genes suggested that the timing of a substantial proportion of foraging-related gene expression corresponds to the timing of the previously observed neuropil expansion. I used several types of bioinformatic analyses to discover functional themes that characterize the foraging-dependent genes in the mushroom bodies. Categories of genes positively correlated with foraging duration included several potentially related to dendritic remodeling and growth. Both clustering and functional annotation analyses suggest that foraging-related gene expression in the mushroom bodies could be responsible for directing and

providing materials needed for neuropil growth. This work has been published in *Developmental Neurobiology* (Lutz et al., 2012).

This study also identified a set of protein folding genes that are disparately regulated in young bees, but consistently coexpressed in foragers. Protein-folding genes may act together as part of a neuroprotective mechanism unique to forager mushroom bodies. Amdam et al. have suggested that foragers with extensive experience (>15 days of foraging) can be regarded as senescent, and serve as a model for aging research (Behrends et al., 2007; Amdam, 2011). Another study by this group found that abundance of several proteins related to synaptic plasticity is decreased in foragers with extensive experience (Wolschin et al., 2009). Although this subset of the forager population does present an interesting model for the study of cognitive senescence, most individuals die before they reach this duration of foraging (Visscher and Dukas, 1997). Results presented in Chapter 2 suggest that as experienced foragers reach this age, identified putative neuroprotective processes begin to decline. By focusing instead on foragers with 1-2 weeks of experience, a population in which active neuropil growth occurs, we have identified mechanisms by which such plasticity may be maintained in the aging brain.

3.2 Egr expression in the mushroom bodies: experience-dependent and -expectant upregulation

The work described in Chapter 2 provides a broad overview of the types of transcriptional changes that may promote and maintain neuropil growth, in response to a variety of experiences encountered during foraging. It is likely that some molecular processes that contribute to experience-dependent plasticity in the mushroom bodies are not dynamically regulated throughout foraging, but instead are upregulated and present constitutively throughout foraging, or transiently increased at the onset of flight or foraging behavior. For example, onset of foraging transiently induces alterations in Rho GTPase signaling known to promote dendritic remodeling (Dobrin and Fahrbach, 2012).

To explore the possibility of transient changes in transcription that promote subsequent neuropil growth, I focused on the bee's first experience with the environment outside the hive, the orientation flight. As discussed above, this first exposure to outside stimuli promotes structural changes in the neuropil of bees and ants (Brandon and Coss, 1982; Stieb et al., 2010; Stieb et al., 2012), and is important for subsequent foraging behavior. I also narrowed my investigation of transcriptional response to a specific gene, *egr*. *Egr* is a homolog in honey bees

of *egr-1* (also known as *zenk*), a canonical immediate-early gene. *Egr-1* is an activity-dependent transcription factor whose expression has been associated with learning and novelty detection in many vertebrate systems; its transient expression and hypothesized regulation of downstream genes related to synaptic growth made it a good candidate as an initiator of neuroplasticity in response to orientation flight (Tischmeyer and Grimm, 1999; Knapska and Kaczmarek, 2004; Moorman et al., 2011). The study described in Chapter 3 examined the transcriptional activation of *egr* in response to orientation flights, a naturally occurring behavioral paradigm for spatial learning. I used *in situ* analysis and qRT-PCR to investigate the transcriptional response of *egr* to orientation flight. I found that *egr* is rapidly, strongly, and transiently upregulated in the brain of the honey bee by orientation flight. In addition, I have shown that this experience-dependent transcriptional activity is limited to the mushroom bodies. Subsequent behavioral dissection of component experiences of the orientation flight suggests that *egr* is responsive to visual environmental novelty encountered during the flight. *Egr* may play a role in memory consolidation similar to that proposed in vertebrates, acting as a mushroom body-specific functional link between orientation flights and resulting dendritic spine remodeling (Brandon and Coss, 1982).

In addition to its well-known role as an immediate-early gene responsive to novel experience, *egr-1* exhibits higher basal levels in brain regions undergoing development or remodeling in some vertebrates (Worley et al., 1991; McCormack et al., 1992; Kaplan et al., 1995; Jin and Clayton, 1997). In the zebra finch, young birds about to enter the sensitive period for song learning exhibit a constitutive level of *egr-1* expression in the auditory forebrain; later, during the sensitive period, *egr-1* is expressed at a lower basal level, but upregulated in response to hearing a novel song (Jin and Clayton, 1997; Stripling et al., 2001). The same switch from constitutive to song-responsive expression has been observed for hundreds of other genes in the auditory forebrain (London et al., 2009), reinforcing the idea that genes promoting plasticity in response to experience may also act to induce experience-expectant plasticity at appropriate life stages. In Chapter 4, I have found evidence of a similar pattern of regulation for *egr* in the maturing adult honey bee. *Egr* expression was upregulated in the mushroom bodies of hive bees preparing to perform an orientation flight, compared with hive bees that were instead actively engaged in brood care. *Egr* was also expressed at a higher constitutive level in the mushroom bodies of foragers when compared with hive bees. These findings are consistent with a role for

egr in experience-expectant and -dependent phases of mushroom body neuropil expansion. Results in Chapter 4 support the possibility that *egr* serves as a link between muscarinic signaling, known to promote mushroom body neuropil growth in foragers (Ismail et al., 2006), and dendritic arborization in the calyx of foragers and young bees (Dobrin et al., 2011). Injection of pilocarpine induced increased expression of *egr* in the mushroom bodies of one-day old bees. Cholinergic signaling is increased in foragers compared with hive bees, particularly in the mushroom bodies (Shapira et al., 2001). It is possible that increased cholinergic signaling in bees may act through muscarinic receptors to increase basal levels of *egr*, which in turn may alter the transcription of a cascade of downstream genes involved in dendritic remodeling and growth.

4. Conclusions

Honey bees are particularly well-suited for the study of the molecular basis of adult neuroplasticity, and the findings presented here create a foundation for further investigation. One promising future direction for the work presented here is an exploration of the downstream transcriptional targets of *egr* in honey bees. By discovering the cascade of transcriptional regulation resulting from increased expression of *egr*, we may obtain a more complete picture of how environmental stimuli act through molecular signaling to promote neuroanatomical change, as well as a better understanding of how molecular state may influence the capacity of the brain to exhibit such changes. Several recent studies have used the wealth of brain gene expression data in honey bees to model the regulatory control of networks of genes by a few transcription factors, and how this regulatory control contributes to behavioral plasticity (Chandrasekaran et al., 2011; Ament et al., 2012). As genomic resources become increasingly available for a range of social insects (Werren et al., 2010; Wurm et al., 2011; Suen et al., 2011; Smith et al., 2011a; Smith et al., 2011b; Nygaard et al., 2011; Munoz-Torres et al., 2011), these types of studies can be extended to a variety of species with their own experimental advantages. Studies in taxa where foragers are longer-lived, such as *Camponotus*, or more generalist in their range of behaviors, such as *Pheidole*, may ultimately serve as better models for understanding how to promote adult neuroplasticity in humans. Identifying commonalities and differences in the molecular mechanisms supporting plasticity in different behavioral and neural characteristics would also be instructive. For example, if *egr* is part of a conserved molecular toolkit that

mediates neuroplasticity, what are the implications of potential lack of involvement in mechanisms of *Drosophila* learning and memory? Is it active in brain regions outside the mushroom bodies in species such as *Camponotus*, in which the antennal lobes also exhibit substantial adult neuropil growth (Kuhn-Buhlmann and Wehner, 2006)? How do shared or differing downstream transcriptional cascades produce different types of growth, over different time scales? The work in honey bees described here represents an important starting point to direct these and other future studies.

References

Amdam GV (2011) Social context, stress, and plasticity of aging. *Aging Cell* (England) 10:18-27.

Ament SA, Blatti CA, Alaux C, Wheeler MM, Toth AL, Le Conte Y, Hunt GJ, Guzman-Novoa E, Degrandi-Hoffman G, Uribe-Rubio JL, Amdam GV, Page RE, Jr, Rodriguez-Zas SL, Robinson GE, Sinha S (2012) New meta-analysis tools reveal common transcriptional regulatory basis for multiple determinants of behavior. *Proc Natl Acad Sci U S A* 109:1801-10.

Anderson BJ (2011) Plasticity of gray matter volume: The cellular and synaptic plasticity that underlies volumetric change. *Dev Psychobiol* 53:456-465.

Bavelier D, Levi DM, Li RW, Dan Y, Hensch TK (2010) Removing brakes on adult brain plasticity: From molecular to behavioral interventions. *J Neurosci* 30:14964-14971.

Behrends A, Scheiner R, Baker N, Amdam GV (2007) Cognitive aging is linked to social role in honey bees (*Apis mellifera*). *Exp Gerontol* 42:1146-1153.

Brandon JG, Coss RG (1982) Rapid dendritic spine stem shortening during one-trial learning: The honeybee's first orientation flight. *Brain Res* 252:51-61.

Butz M, Worgotter F, van Ooyen A (2009) Activity-dependent structural plasticity. *Brain Res Rev* 60:287-305.

Capaldi EA, Robinson GE, Fahrback SE (1999) Neuroethology of spatial learning: The birds and the bees. *Annu Rev Psychol* 50:651-682.

Chandrasekaran S, Ament SA, Eddy JA, Rodriguez-Zas SL, Schatz BR, Price ND, Robinson GE (2011) Behavior-specific changes in transcriptional modules lead to distinct and predictable neurogenomic states. *Proc Natl Acad Sci U S A* 108:18020-18025.

Chklovskii DB, Mel BW, Svoboda K Cortical rewiring and information storage. *Nature* (England) 431:782-788.

- Clayton DF (2000) The genomic action potential. *Neurobiology of Learning and Memory* 74:185-216.
- Dobrin SE, Fahrbach SE (2012) Rho GTPase activity in the honey bee mushroom bodies is correlated with age and foraging experience. *J Insect Physiol* 58:228-234.
- Dobrin SE, Herlihy JD, Robinson GE, Fahrbach SE (2011) Muscarinic regulation of Kenyon cell dendritic arborizations in adult worker honey bees. *Arthropod Struct Dev* 40:409-419.
- Durst C, Eichmüller S, Menzel R (1994) Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. *Behavioral and Neural Biology* 62:259-263.
- Erickson CA, Barnes CA (2003) The neurobiology of memory changes in normal aging. *Exp Gerontol* 38:61-69.
- Fahrbach SE (2006) Structure of the mushroom bodies of the insect brain. *Annu Rev Entomol* 51:209-232.
- Fahrbach SE, Robinson GE (1995) Behavioral development in the honey bee: Toward the study of learning under natural conditions. *Learn Mem* 2:199-224.
- Fahrbach SE, Strande JL, Robinson GE (1995) Neurogenesis is absent in the brains of adult honey bees and does not explain behavioral neuroplasticity. *Neurosci Lett* 197:145-148.
- Fahrbach SE, Moore D, Capaldi EA, Farris SM, Robinson GE (1998) Experience-expectant plasticity in the mushroom bodies of the honeybee. *Learn Mem* 5:115-123.
- Farris SM (2008a) Structural, functional and developmental convergence of the insect mushroom bodies with higher brain centers of vertebrates. *Brain Behav Evol* 72:1-15.
- Farris SM (2008b) Evolutionary convergence of higher brain centers spanning the protostome-deuterostome boundary. *Brain Behav Evol* 72:106-122.
- Farris SM, Schulmeister S (2011) Parasitoidism, not sociality, is associated with the evolution of elaborate mushroom bodies in the brains of hymenopteran insects. *Proc Biol Sci* 278:940-951.
- Farris SM, Robinson GE, Fahrbach SE (2001) Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. *J Neurosci* 21:6395-6404.
- Farris SM, Robinson GE, Davis RL, Fahrbach SE (1999) Larval and pupal development of the mushroom bodies in the honey bee, *Apis mellifera*. *J Comp Neurol* 414:97-113.
- Farris SM (2005) Evolution of insect mushroom bodies: Old clues, new insights. *Arthropod Structure & Development* 34:211-234.

Giurfa M (2007) Behavioral and neural analysis of associative learning in the honeybee: A taste from the magic well. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 193:801-824.

Greenough WT, Black JE, Wallace CS (1987) Experience and brain development. *Child Dev* 58:539-559.

Gronenberg W, Heeren S, Holldobler B (1996) Age-dependent and task-related morphological changes in the brain and the mushroom bodies of the ant *Camponotus floridanus*. *J Exp Biol* 199:2011-2019.

Heisenberg M (1998) What do the mushroom bodies do for the insect brain? an introduction. *Learn Mem* 5:1-10.

Hensch TK (2004) Critical period regulation. *Annu Rev Neurosci* 27:549-579.

Holldobler B, Wilson EO (2009) The superorganism: The beauty, elegance and strangeness of insect societies. New York, NY: Norton and Company.

Holldobler B, Wilson EO (1990) The ants. Boston, MA: Harvard University Press.

Holtmaat A, Svoboda K (2009) Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat Rev Neurosci* 10:647-658.

Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443:931-949.

Ismail N, Robinson GE, Fahrbach SE (2006) Stimulation of muscarinic receptors mimics experience-dependent plasticity in the honey bee brain. *Proc Natl Acad Sci U S A* 103:207-211.

Jin H, Clayton DF (1997) Localized changes in immediate-early gene regulation during sensory and motor learning in zebra finches. *Neuron* 19:1049-1059.

Kaplan IV, Guo Y, Mower GD (1995) Developmental expression of the immediate early gene *EGR-1* mirrors the critical period in cat visual cortex. *Brain Res Dev Brain Res* 90:174-179.

Knapska E, Kaczmarek L (2004) A gene for neuronal plasticity in the mammalian brain: *Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK*? *Prog Neurobiol* 74:183-211.

Komischke B, Sandoz JC, Malun D, Giurfa M (2005) Partial unilateral lesions of the mushroom bodies affect olfactory learning in honeybees *Apis mellifera* L. *Eur J Neurosci* 21:477-485.

Krofczik S, Khojasteh U, de Ibarra NH, Menzel R (2008) Adaptation of microglomerular complexes in the honeybee mushroom body lip to manipulations of behavioral maturation and sensory experience. *Dev Neurobiol* 68:1007-1017.

- Kuhn-Buhlmann S, Wehner R (2006) Age-dependent and task-related volume changes in the mushroom bodies of visually guided desert ants, *cataglyphis bicolor*. *J Neurobiol* 66:511-521.
- Leslie JH, Nedivi E (2011) Activity-regulated genes as mediators of neural circuit plasticity. *Prog Neurobiol* 94:223-237.
- London SE, Dong S, Replogle K, Clayton DF (2009) Developmental shifts in gene expression in the auditory forebrain during the sensitive period for song learning. *Dev Neurobiol* 69:437-450.
- Lutz CC, Rodriguez-Zas SL, Fahrbach SE, Robinson GE (2012) Transcriptional response to foraging experience in the honey bee mushroom bodies. *Dev Neurobiol* 72:153-166.
- McCormack MA, Rosen KM, Villa-Komaroff L, Mower GD (1992) Changes in immediate early gene expression during postnatal development of cat cortex and cerebellum. *Brain Res Mol Brain Res* 12:215-223.
- Mizunami M, Weibrecht JM, Strausfeld NJ (1998) Mushroom bodies of the cockroach: Their participation in place memory. *J Comp Neurol* 402:520-537.
- Molina Y, O'Donnell S (2008) Age, sex, and dominance-related mushroom body plasticity in the paperwasp *Mischocyttarus mastigophorus*. *Dev Neurobiol* 68:950-959.
- Molina Y, O'Donnell S (2007) Mushroom body volume is related to social aggression and ovary development in the paperwasp *Polistes instabilis*. *Brain Behav Evol* 70:137-144.
- Molina Y, Harris RM, O'Donnell S (2009) Brain organization mirrors caste differences, colony founding and nest architecture in paper wasps (hymenoptera: Vespidae). *Proc Biol Sci* 276:3345-3351.
- Moore D (2001) Honey bee circadian clocks: Behavioral control from individual workers to whole-colony rhythms. *J Insect Physiol* 47:843-857.
- Moorman S, Mello CV, Bolhuis JJ (2011) From songs to synapses: Molecular mechanisms of birdsong memory. molecular mechanisms of auditory learning in songbirds involve immediate early genes, including *zenk* and *arc*, the ERK/MAPK pathway and synapsins. *Bioessays* 33:377-385.
- Munoz-Torres MC, Reese JT, Childers CP, Bennett AK, Sundaram JP, Childs KL, Anzola JM, Milshina N, Elisk CG (2011) Hymenoptera genome database: Integrated community resources for insect species of the order hymenoptera. *Nucleic Acids Res* 39:658-62.
- Muscedere ML, Traniello JF (2012) Division of labor in the hyperdiverse ant genus *Pheidole* is associated with distinct subcaste- and age-related patterns of worker brain organization. *PLoS One* 7:e31618.

Naeger NL, Van Nest BN, Johnson JN, Boyd SD, Southey BR, Rodriguez-Zas SL, Moore D, Robinson GE (2011) Neurogenomic signatures of spatiotemporal memories in time-trained forager honey bees. *J Exp Biol* 214:979-987.

North G, Greenspan RJ (2007) *Invertebrate neurobiology*. (Cold Spring Harbor, NY) 665.

Nygaard S, Zhang G, Schiott M, Li C, Wurm Y, Hu H, Zhou J, Ji L, Qiu F, Rasmussen M, Pan H, Hauser F, Krogh A, Grimmelikhuijzen CJ, Wang J, Boomsma JJ (2011) The genome of the leaf-cutting ant *Acromyrmex echinator* suggests key adaptations to advanced social life and fungus farming. *Genome Res* 21:1339-1348.

O'Donnell S, Donlan N, Jones T (2007) Developmental and dominance-associated differences in mushroom body structure in the paper wasp *Mischocyttarus mastigophorus*. *Dev Neurobiol* 67:39-46.

O'Donnell S, Donlan NA, Jones TA (2004) Mushroom body structural change is associated with division of labor in eusocial wasp workers (*Polybia aequatorialis*, hymenoptera: Vespidae). *Neurosci Lett* 356:159-162.

Seid MA, Wehner R (2009) Delayed axonal pruning in the ant brain: A study of developmental trajectories. *Dev Neurobiol* 69:350-364.

Seid MA, Harris KM, Traniello JF (2005) Age-related changes in the number and structure of synapses in the lip region of the mushroom bodies in the ant *Pheidole dentata*. *J Comp Neurol* 488:269-277.

Sen Sarma M, Rodriguez-Zas SL, Gernat T, Nguyen T, Newman T, Robinson GE (2010) Distance-responsive genes found in dancing honey bees. *Genes Brain Behav* 9:825-830.

Shapira M, Thompson CK, Soreq H, Robinson GE (2001) Changes in neuronal acetylcholinesterase gene expression and division of labor in honey bee colonies. *J Mol Neurosci (United States)* 17:1-12.

Smith AR, Seid MA, Jimenez LC, Wcislo WT (2010) Socially induced brain development in a facultatively eusocial sweat bee *Megalopta genalis* (halictidae). *Proc Biol Sci* 277:2157-2163.

Smith CD et al (2011a) Draft genome of the globally widespread and invasive argentine ant (*Linepithema humile*). *Proc Natl Acad Sci U S A* 108:5673-5678.

Smith CR et al (2011b) Draft genome of the red harvester ant *Pogonomyrmex barbatus*. *Proc Natl Acad Sci U S A* 108:5667-5672.

Srinivasan MV, Zhang S, Altwein M, Tautz J (2000) Honeybee navigation: Nature and calibration of the "odometer". *Science* 287:851-853.

Stieb SM, Hellwig A, Wehner R, Rossler W (2012) Visual experience affects both behavioral and neuronal aspects in the individual life history of the desert ant *Cataglyphis fortis*. Dev Neurobiol 72:729-742.

Stieb SM, Muenz TS, Wehner R, Rossler W (2010) Visual experience and age affect synaptic organization in the mushroom bodies of the desert ant *Cataglyphis fortis*. Dev Neurobiol 70:408-423.

Strausfeld NJ, Hansen L, Li Y, Gomez RS, Ito K (1998) Evolution, discovery, and interpretations of arthropod mushroom bodies. Learn Mem 5:11-37.

Stripling R, Kruse AA, Clayton DF (2001) Development of song responses in the zebra finch caudomedial neostriatum: Role of genomic and electrophysiological activities. J Neurobiol 48:163-180.

Suen G et al (2011) The genome sequence of the leaf-cutter ant *Atta cephalotes* reveals insights into its obligate symbiotic lifestyle. PLoS Genet 7:e1002007.

Tischmeyer W, Grimm R (1999) Activation of immediate early genes and memory formation. Cell Mol Life Sci 55:564-574.

Visscher PK, Dukas R (1997) Survivorship of foraging honey bees. Insectes Sociaux 44:1-5.

Wehner R, Raeber F (1979) Visual spatial memory in desert ants, *Cataglyphis bicolor* (hymenoptera: Formicidae). Experientia 35:1569-1571.

Werren JH et al (2010) Functional and evolutionary insights from the genomes of three parasitoid nasonia species. Science 327:343-348.

Whitfield CW, Cziko AM, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. Science 302:296-299.

Whitfield CW, Ben-Shahar Y, Brillet C, Leoncini I, Crauser D, Leconte Y, Rodriguez-Zas S, Robinson GE (2006) Genomic dissection of behavioral maturation in the honey bee. Proc Natl Acad Sci U S A 103:16068-16075.

Winston ML (1987) The biology of the honey bee. Cambridge, MA: Harvard University Press.

Withers GS, Fahrbach SE, Robinson GE (1995) Effects of experience and juvenile hormone on the organization of the mushroom bodies of honey bees. J Neurobiol 26:130-144.

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238-240.

Withers GS, Day NF, Talbot EF, Dobson HE, Wallace CS (2008) Experience-dependent plasticity in the mushroom bodies of the solitary bee *Osmia lignaria* (megachilidae). *Dev Neurobiol* 68:73-82.

Wolschin F, Munch D, Amdam GV (2009) Structural and proteomic analyses reveal regional brain differences during honeybee aging. *J Exp Biol* 212:4027-4032.

Worley PF, Christy BA, Nakabeppu Y, Bhat RV, Cole AJ, Baraban JM (1991) Constitutive expression of *zif268* in neocortex is regulated by synaptic activity. *Proc Natl Acad Sci U S A* 88:5106-5110.

Wurm Y et al (2011) The genome of the fire ant *Solenopsis invicta*. *Proc Natl Acad Sci U S A* 108:5679-5684.

CHAPTER 2

TRANSCRIPTIONAL RESPONSE TO FORAGING EXPERIENCE IN THE HONEY BEE MUSHROOM BODIES¹

Abstract

Enriched environmental conditions induce neuroanatomical plasticity in a variety of vertebrate and invertebrate species. We explored the molecular processes associated with experience-induced plasticity, using naturally occurring foraging behavior in adult worker honey bees (*Apis mellifera*). In honey bees, the mushroom bodies exhibit neuroanatomical plasticity that is dependent on accumulated foraging experience. To investigate molecular processes associated with foraging experience, we performed a time-course microarray study to examine gene expression changes in the mushroom bodies as a function of days foraged. We found almost 500 genes that were regulated by duration of foraging experience. Bioinformatic analyses of these genes suggest that foraging experience is associated with multiple molecular processes in the mushroom bodies, including some that may contribute directly to neuropil growth, and others that could potentially protect the brain from the effects of aging and physiological stress.

1. Introduction

Neuroplasticity, the brain's ability to respond to experience and modulate behavior, is an important trait in many species. A question of interest is what molecular processes support this capacity. Studies examining transcriptional responses to enriched environments in the laboratory (Rampon et al., 2000; Ronnback et al., 2005; Li et al., 2007; Thiriet et al., 2008) have been valuable in the discovery of molecular processes associated with neuroanatomical plasticity. However, few studies have examined the brain's response to the extended performance of a specific, naturally occurring, ecologically relevant behavior. This approach allows for the possibility that mechanisms of neuroanatomical plasticity can be understood in the context of changes in neural functioning and behavior that occur throughout the lifespan, much the way increases and decreases in different cognitive abilities occur as a function of aging (McArdle et al., 2002). We used the honey bee to begin to understand how naturally occurring cognitive

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challenges and sensory stimulation affect molecular processes associated with neuroanatomical plasticity.

Neural development in adult worker honey bees (*Apis mellifera*) provides an opportunity to study experience-induced neuroanatomical plasticity in a naturalistic setting. The first 1-3 weeks of honey bee worker adult life are spent performing tasks inside the hive, after which bees typically forage for resources (Winston, 1987). This dramatic behavioral shift means that honey bees can be used to examine the response of a mature brain to a sudden and sustained increase in cognitive demand that is highly relevant to their natural lifestyle. Foraging exposes bees to novel sensory stimuli and involves memory-intensive tasks, such as spatial navigation and recognition of floral sources (Menzel, 1990).

The mushroom bodies, a region of the insect protocerebrum that performs multimodal integration of input from primary sensory neuropils and is involved in learning and memory (Fahrbach, 2006), is likely to participate in these cognitive tasks. Adult honey bee mushroom bodies exhibit neuropil growth in response to accumulated foraging experience; expansion is detectable between the first and second week of foraging (Farris et al., 2001; Ismail et al., 2006). This expansion is not caused by adult neurogenesis in the mushroom bodies (Fahrbach et al., 1995). Instead, experience-dependent expansion is accompanied by increased dendritic branching and branch length in the visual input region of the calyx (Farris et al., 2001), and changes in microglomerular structure and density (Krofczik et al., 2008). Behavioral studies show that foraging efficiency also improves with experience (Dukas and Visscher, 1994) and that learning may contribute to this improvement (Dukas, 2008), suggesting a link between anatomical and behavioral change.

Foraging is a physiologically demanding activity, and the average time to death once a bee has commenced foraging is one week (Visscher and Dukas, 1997); foraging-induced neuropil growth therefore represents an extreme example of plasticity in aging individuals. Neuroanatomical plasticity in other species typically decreases with age, but this decrease and the corresponding loss of cognitive ability can sometimes be prevented by experience (van Praag et al., 2000; Kolb, 1998). It is of interest to discover molecular processes that enable persistent neuroanatomical plasticity in the aging brain.

The mushroom body neuropil of adult honey bees also undergoes developmentally related growth prior to the onset of foraging behavior. The mushroom bodies of 1-day-old bees

exhibit active neuropil outgrowth and dendritic branching (Farris et al, 2001), and the neuropil shows further expansion in anticipation of foraging onset (Withers et al 1993, 1995; Durst et al, 1994). These phases of growth are apparently distinct from the plasticity observed in older foragers and independent of experience; neuropil expansion in young bees is robust even to extreme sensory deprivation (Fahrbach et al., 1998), whereas neuropil expansion in foragers requires foraging activity (Ismail et al., 2006). Are the same genes responsible for directing initial growth in development and for re-initiating neuroanatomical plasticity in response to experience?

Because knowledge of molecular processes directing adult neuropil growth is so limited, we chose to conduct a genome-wide survey for changes in gene expression that might be related to this type of growth in honey bees. We performed a detailed investigation of the long-term dynamics of gene expression associated with foraging experience, with the idea that some of these genes are potentially related to neuroanatomical plasticity. We used whole-genome microarrays to measure gene expression in the mushroom bodies of honey bees throughout their foraging lifespan.

2. Methods

2.1. Animals

Bees were observed and collected from apiaries maintained by the University of Illinois Bee Research Facility, Urbana, IL in June and July of 2008. Additional bees were collected for qRT-PCR analysis in August 2009. Experimental honey bee colonies were designed and maintained as described in Ismail et al (2006). Colonies began as one-story Langstroth hives with several empty combs and combs containing larval brood. Frames of honeycomb containing pupae were collected from 25-30 other colonies and stored in a dark, humid incubator at 34°. Adults were removed from the comb within 24 h of emergence, marked on the thorax with paint (Testor's) and introduced to each experimental colony. In total, 10,000-13,000 marked bees were added over a two-day period to each host colony. Each colony also contained a naturally-mated queen and at least 30,000 unmarked bees.

The majority of honey bee workers die before reaching 12-16 days of active foraging behavior (Visscher and Dukas, 1997). In order to collect adequate numbers of older foragers for analysis, colony demography was manipulated to ensure a large starting population of

simultaneously foraging focal bees. This was done by removing non-focal foraging bees from the colony (Huang and Robinson, 1996). Collections for microarray analysis were made from two colonies (two trials). Collections for qRT-PCR came from the same trials as those used for microarray analysis, but from groups of bees that initiated foraging at a later (for trial one) or earlier (for trial two) period. Samples from a third colony, collected a year later, were also used to confirm microarray results.

2.2. Behavioral observations

Behavioral observations were performed as in Ismail et al (2006). Briefly, beginning when focal bees were 6-7 days old, the colony entrance was observed daily for 8 h; this allowed focal bees to be identified on the first day they foraged. Focal bees observed returning with a nectar or pollen load were given a second paint mark on the abdomen to indicate their status as foragers. Based on this second paint mark, the number of days spent foraging could be determined for any double-marked bee captured. After a sufficient number of first-day foragers had been marked, brief observations of the colony every 2-3 days ensured that overall foraging activity remained high. Twelve days after this initial marking period, as many as possible of the individuals that had subsequently commenced foraging were also given an abdominal mark. This allowed the identification of age-matched, less-experienced foragers at the end of the experiment.

2.3. Collections and dissection

Individuals for microarray and qRT-PCR analyses were flash-frozen in liquid nitrogen. 1-day-old bees were collected after removal from the comb containing brood. Foragers with 4, 8, 12, and 16 days of flight experience were collected upon their return to the hive entrance with a pollen or nectar load. Samples were stored at -80° C between all subsequent processing steps.

Mushroom bodies were isolated as previously described (Sen Sarma et al., 2009). Frozen brains were treated overnight with RNAlater-ice (Ambion) and then dissected over ice. Only the calyces of the mushroom bodies were retained because of the difficulty of separating the peduncles and lobes of the mushroom bodies from the central body. The calyces contain the somata of all intrinsic mushroom body neurons (Kenyon cells) and should contain the majority of mushroom body transcripts.

2.4. Microarrays

Microarray sample preparation and hybridization procedures were similar to those described in Alaux et al (2009a). RNA was extracted from tissue using an RNeasy kit (Qiagen) and amplified (from 200 ng) with an Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion). Amplified RNA (2 µg) was labelled with a ULS aRNA labelling kit (Kreatech), and 60 ng labelled material from each sample was hybridized to honey bee whole genome oligonucleotide arrays using a Maui Hybridization System (Kreatech).

Mushroom body gene expression in five groups (1-day old bees, and bees collected 4, 8, 12, or 16 days after foraging initiation) was compared using a loop design. For each of two trials, 12 bees per group were included, and analyzed on an individual basis. Slides were scanned using an Axon 4000B scanner, and images analyzed with GENEPIX software (Agilent Technologies). Spots were removed from analysis if flagged by the GENEPIX software or if the fluorescence intensity was less than the median intensity of the negative control spots. A Loess transformation was performed using Beehive (<http://stagbeetle.animal.uiuc.edu/Beehive>) to normalize expression intensities.

2.5. Real-time RT-PCR

RNA extraction was performed as described for microarray samples. An on-column genomic DNA digest was performed with DNase 1 (Qiagen). cDNA synthesis was performed using 150 ng RNA. Quantification was performed (on samples independent from those used on microarrays) with an ABI Prism 7900 sequence detector, SYBR green detection method (Applied Biosystems), and relative quantification to a genomic DNA standard curve. Expression was normalized to the geometric mean of two constitutively expressed genes used in past studies, *eIF3-s8* (Alaux et al., 2009a) and *rp49* (Ament et al., 2008).

2.6. Statistics

A linear model including both fixed and random effects implemented using restricted maximum likelihood was used to analyze the normalized log₂-transformed fluorescence intensities for each gene, accounting for the effects of dye, bee, colony and microarray. An FDR (false discovery rate) correction was used to correct for multiple testing. Both microarray and

qRT-PCR analyses used PROC MIXED in SAS. Genes highly expressed in the hypopharyngeal glands, a nearby and potentially contaminating tissue, were filtered from the microarray results, as in previous studies (Alaux et al., 2009a). Clustering analyses were performed using MultiExperiment Viewer, part of the TM4 Microarray Software Suite (Saeed et al., 2006). The R package CMA (Classification for Microarrays), a program designed for class prediction in microarray data sets (Slawski et al., 2008), was used for this purpose; data were pre-filtered for genes with missing values. For Gene Ontology and other informatic functional analyses, known *Drosophila melanogaster* orthologs of bee genes were used as previously described (Alaux et al., 2009b), and enrichment was identified using the functional annotation clustering tool in the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Dennis et al., 2003; Huang et al., 2009). Correlation network analysis was performed using the R package Weighted Gene Co-expression Network Analysis (WGCNA). The forager bee network was constructed with values from all forager time points; the young bee network was constructed using values from day-old individuals. STEM (Short Time-series Expression Miner), a program designed for time-series microarray studies with relatively few time points, was used to identify gene expression profiles (patterns of up- or down-regulation between adjacent time points) that arose more frequently than expected by chance (Ernst and Bar-Joseph, 2006). Clustering and network analyses used log₂-transformed estimated values for individual samples, as described previously (Whitfield et al., 2006).

3. Results

3.1. Foraging induces a restricted and temporally focused transcriptional response

We compared gene expression in the mushroom bodies of five groups: 1-day old bees, and foragers with 4, 8, 12, or 16 days of flight experience. A total of 6,219 genes of the 11,761 included in the analysis (53%) were significant in the overall ANOVA (FDR<0.05). Of these, 5,839 genes (50%) were differentially regulated in the mushroom bodies of 1-day-olds and foragers. This large number of regulated genes is consistent with previous comparisons of whole-brain gene expression in foragers and hive bees (Alaux et al., 2009a; Whitfield et al., 2003) or foragers and day-olds (Whitfield et al., 2006; Sen Sarma et al., 2007).

By contrast, the number of genes in the mushroom bodies differentially regulated by increasing amount of foraging experience was much smaller: only 498 genes (4%) were

differentially regulated between any two foraging time points, and 239 genes were differentially regulated between adjacent time points (pair-wise comparisons). A total of 200 of these 239 showed significant regulation between 8 and 12 days of foraging. A disproportionate amount of experience-related expression differences thus occurred within the previously identified time frame for experience-dependent neuropil expansion (Ismail et al., 2006).

Analysis of microarray gene expression data through ANOVA relies on expression differences in individual genes, identified by a somewhat arbitrary significance threshold. A relatively new alternative method for understanding expression data, weighted gene co-expression network analysis (WGCNA), can supplement other methods and provide a more complete view of molecular processes associated with a factor of interest. Networks of genes are identified by their coexpression (positive or negative correlation) in multiple individuals across different experimental conditions. Within-group variation, otherwise detrimental to detection of expression differences for a single gene across groups of individuals, can in WGCNA be used to reveal potential interactions between groups of genes; correlation of gene expression is likely to reflect coregulation (Zhang and Horvath, 2005).

We applied WGCNA to the expression of 9754 genes in individual foragers: the resulting network contained 17 groups, or modules, of genes that exhibit high expression overlap (Figure 2.1A). The stereotypical expression of genes in a module can be described by the eigengene, which represents the first principal component of variation in expression for those genes. Modules with expression influenced by amount of foraging experience were identified by calculating the correlation between eigengene expression and number of days spent foraging. Expression in five modules showed a positive correlation with amount of foraging experience, while expression in two modules showed a negative correlation (Figure 2.1A, B). Genes in these modules displayed too much individual variability to be detected by ANOVA, but their membership in foraging-related modules provides support for their regulation by foraging experience.

We used gene expression data from the mushroom bodies of 1-day-old individuals to construct a separate co-expression network that describes transcriptional regulation at this earlier (“young bee”) developmental stage. Comparison with the forager network revealed that all but one forager module had significant overlap with at least one of the modules in the young bee network. Among foraging-related modules, only one, module K, showed no significant overlap

with any of the young bee modules. This result suggests that the genes in module K are involved in disparate functions in 1-day-olds, but act together to perform some activity- or plasticity-related function in forager mushroom bodies. All other foraging-related modules showed significant, >10% overlap with at least one of the young bee modules (Fisher's exact test, $p < 0.0005$). Modules C, F and R, overlapped with the same large module in 1-day-olds. This widespread overlap indicates that coregulation of expression is similar in the two distinct phases of mushroom body development exhibited by 1-day-old and forager bees.

3.2. Individual experience level is reflected in distinct gene expression profiles

Given that the complexity and vagaries of natural foraging behavior lead to considerable individual variability in experience, can foraging-related genes serve as reliable measures of foraging experience? The coincident timing of expression differences in this study and previously observed neuropil expansion suggests a natural division of foragers into phenotypically distinguishable groups: “less experienced” (4- and 8-day foragers) and “more experienced” foragers (12- and 16-day foragers.) Two statistical methods were used to evaluate the strength of this distinction.

An unsupervised clustering method, principal component analysis, was performed on estimated values of foraging-regulated genes for individual foragers. A 2D plot of the two largest components of variation identified by this analysis revealed approximate separation of less and more experienced foragers into two clusters (Figure 2.2). This separation can be attributed to the largest principal component of variation in foraging-related gene expression (PC1), which describes 21% of total variation, indicating that duration of foraging experience is a moderate but important factor contributing to individual differences in gene expression. Other potential sources of variation in mushroom body gene expression likely include genetic background and uncontrolled aspects of foraging experience.

Additional results supporting the division of foragers into these two categories were obtained by class prediction analysis (Leung and Cavalieri, 2003). We used class prediction analysis to select a list of 100 genes best able to classify individual foragers as less or more experienced. An algorithm trained with expression values from our study performed leave-one-out cross-validation with 78.1% accuracy, and the number of incorrect assignments was roughly equal for the two groups. This rate is significantly better than what would be expected by chance

(Chi-square test, $p < 0.0001$) and demonstrates the ability of gene expression to accurately predict foraging experience, despite substantial within-group variation. All but four of these 100 most predictive genes were upregulated in more experienced foragers (Figure 2.3).

3.3. Validation of microarray results by qPCR

We selected 10 genes to measure with qRT-PCR to evaluate the microarray results (Table 2.1). Independently collected samples were used, both from the same colonies as the microarray study and from an additional colony. We also used an additional control group of bees that were the same age as 16-day foragers, but had only 4 days of foraging experience. This group was used to help distinguish between effects of experience and age on gene expression. Four out of 10 genes showed significant differences in the direction predicted by the microarray results (*inos*, *jip*, *GB13145*, and *GB30322*, Figure 2.4.) As in the microarray study, expression was increased in bees with greater foraging experience, and greatest in 16-day foragers. Expression in the mushroom bodies of bees of the same age as 16-day foragers, but with minimal flight experience, was comparable to 4-day foragers for each of these four genes. This demonstrates that the increase in expression was likely related to experience itself, not increasing age. Expression of a fifth gene examined, *GB12552*, showed a weak but significant positive correlation with number of days spent foraging (Spearman's $\rho = 0.173$, $p = 0.039$). Additionally, for two heat shock protein genes, *hsp90* and *GB14435*, expression was increased in 16-day foragers in two out of three trials. In the third trial, an approximately 4-fold upregulation of these genes in 12-day foragers, a much larger change than any observed in previous trials, suggested that these genes were responding to some acute stimulus in these individuals, rather than reflecting the accumulated effect of foraging experience. In summary, 7 out of 10 genes showed expression changes in some way consistent with the microarray results, with *inos*, *jip*, *GB13145*, and *GB30322* being the most consistent. Both genes with *Drosophila* annotations have potential functional connections to neural plasticity. *jip* encodes JNK interacting protein, a scaffolding protein that modulates MAPK signaling pathways (Dickens et al, 1997). Inositol-3-phosphate is synthesized *de novo* by the enzyme encoded by *inos* (Park et al, 2000); inositol signaling is important to neuronal development and plasticity, and alterations in inositol phosphate synthesis affect neural development in mice (Alebous et al, 2009). Inositol signaling

has been previously implicated in honey bee foraging (Kucharski and Maleszka, 2002; Whitfield et al, 2003).

3.4. Functional processes regulated by foraging experience

To discover functional processes regulated by foraging experience, we first grouped foraging-regulated genes according to their temporal expression patterns; such groups can be valuable for identifying functionally related genes (Wang et al., 2008). We used STEM (Short Time-series Expression Miner) to classify foraging-related genes by their pattern of expression over the 16-day period of foraging experience. Of the 498 foraging-related genes, 382 were clustered into 8 significantly over-represented expression profiles (FDR<0.01, Figure 2.5); the remaining genes exhibited less common patterns of expression. STEM additionally grouped profiles according to pattern similarity. Significantly overrepresented categories could be described as linearly increasing (profiles 31 and 43), increased in a step-wise fashion in experienced foragers (profiles 23, 17 and 32), linearly decreasing (profiles 12 and 20), or increasing in experienced foragers (profile 16). Expression in the largest and most significant profile, profile 23, closely resembles the pattern of expression seen in the highly predictive genes identified with class prediction analysis (Figure 2.3).

Gene Ontology (GO) enrichment analysis of genes from expression profiles identified by STEM is shown in Table 2.2. Genes upregulated in experienced foragers (profile 23) were enriched for genes related to protein kinase activity and G-protein coupled receptor signaling. Another group of genes upregulated in experienced foragers, but showing a more cubic trend (profile 17), was enriched for genes involved in protein folding.

It was not possible to identify significantly enriched categories in profiles 31 and 43, because each contained a relatively small number of genes. When the two clusters were pooled, however, enriched terms included DNA-dependent regulation of transcription and RNA processing. Several genes in this category (*achaete*, *diminutive* and *extradenticle*) are involved in cell fate determination and patterning in *Drosophila melanogaster* larval development (Tweedie et al., 2009). Because neurogenesis does not occur in the adult honey bee mushroom bodies and is therefore not the basis for neuropil expansion (Fahrbach et al., 1995), it is not expected that these genes are performing a similar function here. Rather, if functional, they may play an unknown organizational role in adult dendritic morphogenesis.

Another way to strengthen functional interpretation of genes showing a pattern of linear increase is to include those identified through co-expression network analysis. Eigengenes of 4 of the 5 modules positively correlated with foraging experience were clustered into profile 31 by STEM, confirming the association of this broader set of genes with a linearly increasing expression pattern. In order to understand the biological processes represented by these modules, we again used GO enrichment analysis to discover overrepresented functional categories. Representative examples of the most enriched categories are shown in Table 2.3. Categories in modules positively correlated with foraging included small GTPase mediated signal transduction, nuclear export, protein kinase activity, regulation of RNA splicing, plasma membrane part, actin cytoskeleton organization, and cell morphogenesis. Genes involved in small GTPase mediated signal transduction regulate membrane trafficking, and several, including *Rab11* and *Cdc42*, are involved in neurite outgrowth (Ng and Tang, 2008; Rodal et al., 2008). Another enriched category was plasma membrane part, which consists of genes encoding proteins present in the cellular membrane. Genes in this category included several related to synaptic signaling, including *dopamine receptor 2*, *serotonin receptor 1a*, and *shaker*. Genes in the category nuclear export are involved in transporting mRNA from the nucleus, reinforcing the suggestion of increased transcription associated with foraging experience. Together, these results suggest that foraging-related gene expression in the mushroom bodies is responsible for both directing and providing materials needed for neuropil growth.

To discover functional processes downregulated by foraging experience, genes in profiles 12 and 20 were also analyzed together. The only overrepresented category was oxidation reduction. This is in agreement with previous findings that metabolic processes are downregulated in forager brains compared with hive bees (Ament et al., 2008; Alaux et al., 2009c). Our result indicates that the decrease in metabolic activity in the mushroom bodies is not only seen during the transition to foraging activity, but is ongoing throughout foraging experience.

Eigengenes of negatively correlated modules N and O were associated with profiles 12 and 20, respectively. These modules were enriched for genes related to mitotic spindle elongation and sensory perception of chemical stimuli. Several genes in the latter category are orthologs of odorant-binding proteins; it is surprising to see expression of these genes in the mushroom bodies. However, our previous studies have also detected expression of these genes

in whole brain as well as mushroom bodies (Sen Sarma et al., 2010; Alaux et al., 2009b; Alaux et al., 2009c). In addition, a study examining regional expression of odorant-binding proteins found several expressed in whole bee brain, as well as other parts of the body, suggesting currently unknown functions for these genes (Foret and Maleszka, 2006). The number of genes in enriched categories for both negatively correlated modules was relatively small, hindering biological interpretation of their importance.

4. Discussion

We investigated the transcriptional response to naturally occurring foraging experience in the mushroom bodies of honey bees. Because neuropil outgrowth is taking place in the mushroom bodies during this time, it is likely that part of this transcriptional response represents molecular processes involved in neuroanatomical plasticity, and our functional analyses of genes identified here are consistent with that interpretation. Future studies could extend and further support these findings. For example, it might be possible to identify a pharmacological inhibitor of one or more of the genes discussed here that could be administered in the field; the ability of such a drug to prevent neuropil expansion in foraging bees would provide evidence for a causal role of the target in MB neuropil outgrowth. Another type of experiment that would provide more associational data, but would be more practical within the constraints of a field experiment, could examine the specificity of potential growth-related genes by examining their expression in brain regions in which no large-scale neuropil growth is occurring. However, neuroanatomical plasticity is present in other regions of the bee brain (Winnington et al., 1996; Sigg et al., 1997; Brown et al., 2002), and it might be difficult to find such regions.

Investigation of multiple time points allowed us to discover a linear increase in gene expression with increasing experience, and stepwise upregulation between 8 and 12 days of foraging. These distinct temporal expression patterns may reflect several distinct molecular processes associated with neuropil outgrowth. Comparison of gene expression networks in the mushroom bodies of 1-day old bees and foragers also suggested that similar molecular processes support outgrowth at different stages of development. Parallels to findings from analyses of enriched environments in the laboratory demonstrate the robustness of the brain's molecular responses to experience.

Our study identified genes whose expression continually increased in response to foraging experience. The temporal expression pattern of these genes suggests that they may be necessary for dendritic growth, with continued upregulation reflecting accelerated growth in more experienced foragers. Investigation of enriched environment-induced gene expression in mouse cortex identified a set of genes similarly regulated at 2 and 14 days after the onset of experience (Rampon et al., 2000), and genes with functions related to functional neuroplasticity are upregulated throughout the critical period in the visual cortex of mice (Lyckman et al., 2008).

The most common pattern of gene expression we observed in forager mushroom bodies was higher expression in more experienced foragers. Genes with this profile were part of the gene expression signature that could distinguish foragers on the basis of their experience. Functional analysis suggests that these genes could be involved in processes supporting neuropil growth. Included in this group were several genes involved in signal transduction or protein kinase activity, both of which are functional groups related to plasticity in other organisms. These functional categories were also implicated in a laboratory study of genes differentially regulated by enriched environment in the striatum of adolescent mice (Thiriet et al., 2008). Protein kinase activity is well known to be involved in molecular studies of memory and synaptic plasticity, and post-translational modification has been proposed as a mechanism underlying long-term memory consolidation (Routtenberg and Rekart, 2005).

Another possible role for genes exhibiting higher expression in more experienced foragers is the stabilization of newly formed dendrites. Long-term maintenance of dendritic arbors is thought to require constitutive molecular signaling distinct from that responsible for outgrowth (Benard and Hobert, 2009). A few specific genes involved in stabilization have been identified so far, including several protein kinases (Moresco et al., 2005; Emoto et al., 2006). The coincidence of upregulation and neuropil expansion and the overrepresentation of protein kinases among the genes with this expression profile make them candidates for involvement in a mechanism of stabilization in forager mushroom bodies that have experienced recent dendritic growth.

We also found that many foraging-related modules overlapped significantly with modules in the gene expression network for 1-day-old bees, with shared enrichment of neuroanatomical plasticity-related functional categories. These results suggest that similar sets of genes could mediate some aspects of two phases of mushroom body neuropil growth in honey bees that have

been documented, the early developmental phase in 1-day-old bees and the later experience-dependent phase (Farris et al., 2001). If this is the case, it might be expected that the same pathways are also involved in the experience-expectant phase of mushroom body neuropil growth that occurs before the onset of foraging in hive bees.

One foraging-related gene module was notable because it did not overlap with any module in the young bee network. Module K in foragers was enriched for protein folding genes, several of which are heat shock protein genes. Expression in this module was significantly correlated with amount of foraging experience. Experienced foragers are rapidly approaching the end of their lifespan (Visscher and Dukas, 1997) and the act of foraging is associated with a measure of accumulation of oxidative damage in the brain (Seehuus et al., 2006). Heat shock proteins upregulated in the mushroom bodies may be necessary to prevent or delay neurological aging. Moderate downregulation of protein-folding genes in 16-day foragers could represent the onset of neurodegenerative processes. The specificity of this module in the forager network, in comparison with the young bee network, reinforces the idea that protein-folding genes are acting together to perform some function unique to forager mushroom bodies.

Given the ability of muscarinic agonists to induce neuropil growth (Ismail et al., 2006) and dendritic arborization (Dobrin et al., 2011) in foragers, we expected that genes directly related to cholinergic signaling would also be among those regulated by foraging experience. We found no significant expression differences among foragers in genes directly related to cholinergic signaling, including *AChE*, *ChaT*, *VACAT*, and *mAChR*. However, among the larger set of foraging-regulated genes identified by network analysis were several involved in the regulation of the MAPKKK cascade, the downstream target of which is the MAPK signaling pathway. Upregulation of these genes represents a possible convergence of mechanisms with muscarinic receptor stimulation. In vertebrates, muscarinic signaling modulates synaptic plasticity by increasing intracellular calcium, thereby activating MAPK and other protein kinases (Hamilton and Nathanson, 2001; Gu, 2003). Our results suggest that transcriptional responses to experience may enhance the effects of muscarinic signaling on neuropil growth by enhancing these downstream processes.

The relatively small number of foraging-related genes identified in the mushroom bodies in this study also provides a marked contrast to the much broader transcriptional changes in the honey bee brain associated with changes in behavioral state seen in comparisons of 1-day old

and forager gene expression (this study and Alaux et al., 2009b; Whitfield et al., 2003). Several factors could contribute to the relatively small number of significantly regulated foraging-related genes. The study of neuroanatomical plasticity in a naturalistic setting necessitates considerable loss of control over variation in individual experience; this variation may be caused by effects of genetic background, individual differences in foraging activity, as well as the complexity of foraging behavior, which might result in qualitatively different types of experience among individuals visiting different floral sources. It is likely that some real effects of foraging on gene expression may be hidden by within-group variation. However, comparison of our results to those from laboratory studies of other species reveal a general trend for relatively small gene lists associated with experience-dependent neuroanatomical plasticity (Rampon et al., 2000; Thiriet et al., 2008).

We found that foraging experience drives transcriptional changes in genes in the mushroom bodies. Because foraging is a natural behavior, it is expected that the brain's responses to foraging experience should occur on a time scale consistent with the life history of the bee. This perspective, together with a consideration of the functions of the foraging-related genes we identified in this study, suggests that experience-dependent neuroanatomical changes in the mushroom bodies represent a balance between plasticity in response to novel sensory input, and stability and protection in the face of aging and physiological stress. The balance appears to shift toward the latter in experienced foragers, which is consistent with findings that suggest a qualitative difference in cognitive ability in more experienced foragers (Behrends et al., 2007). Manipulation of genes associated with these processes could lead to a more complete understanding of how neuroplasticity is maintained in the adult and aging brain.

References

- Alaux C, Le Conte Y, Adams HA, Rodriguez-Zas S, Grozinger CM, Sinha S, Robinson GE (2009a) Regulation of brain gene expression in honey bees by brood pheromone. *Genes Brain Behav* 8:309-319.
- Alaux C, Duong N, Schneider SS, Southey BR, Rodriguez-Zas S, Robinson GE (2009b) Modulatory communication signal performance is associated with a distinct neurogenomic state in honey bees. *PLoS One* 4:e6694.

Alaux C, Sinha S, Hasadsri L, Hunt GJ, Guzman-Novoa E, DeGrandi-Hoffman G, Uribe-Rubio JL, Southey BR, Rodriguez-Zas S, Robinson GE (2009c) Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proc Natl Acad Sci U S A* 106:15400-15405.

Ament SA, Corona M, Pollock HS, Robinson GE (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc Natl Acad Sci U S A* 105:4226-4231.

Behrends A, Scheiner R, Baker N, Amdam GV (2007) Cognitive aging is linked to social role in honey bees (*Apis mellifera*). *Exp Gerontol* 42:1146-1153.

Benard C, Hobert O (2009) Looking beyond development: Maintaining nervous system architecture. *Curr Top Dev Biol* 87:175-194.

Brown SM, Napper RM, Thompson CM, Mercer AR (2002) Stereological analysis reveals striking differences in the structural plasticity of two readily identifiable glomeruli in the antennal lobes of the adult worker honeybee. *J Neurosci* 22:8514-8522.

Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA (2003) DAVID: Database for annotation, visualization, and integrated discovery. *Genome Biol* 4:R60.

Dobrin SE, Herlihy JD, Robinson GE, Fahrbach SE (2011) Muscarinic regulation of kenyon cell dendritic arborizations in adult worker honey bees. *Arthropod Struct Dev* 40:409-419.

Dukas R (2008) Life history of learning: Performance curves of honeybees in the wild. *Ethology* 114:1195-1200.

Dukas R, Visscher PK (1994) Lifetime learning by foraging honey bees. *Anim Behav* 48:1007-1012.

Durst C, Eichmueller S, Menzel R (1994) Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. *Behav Neural Biol* 62: 259-263.

Emoto K, Parrish JZ, Jan LY, Jan YN (2006) The tumour suppressor hippo acts with the NDR kinases in dendritic tiling and maintenance. *Nature* 443:210-213.

Ernst J, Bar-Joseph Z (2006) STEM: A tool for the analysis of short time series gene expression data. *BMC Bioinformatics* 7:191.

Fahrbach SE (2006) Structure of the mushroom bodies of the insect brain. *Annu Rev Entomol* 51:209-232.

Fahrbach SE, Moore D, Capaldi EA, Farris SM, Robinson GE (1998) Experience-expectant plasticity in the mushroom bodies of the honeybee. *Learn Mem* 5:115-123.

- Fahrbach SE, Strande JL, Robinson GE (1995) Neurogenesis is absent in the brains of adult honey bees and does not explain behavioral neuroplasticity. *Neurosci Lett* 197:145-148.
- Farris SM, Robinson GE, Fahrbach SE (2001) Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. *J Neurosci* 21:6395-6404.
- Foret S, Maleszka R (2006) Function and evolution of a gene family encoding odorant binding-like proteins in a social insect, the honey bee (*Apis mellifera*). *Genome Res* 16:1404-1413.
- Gu Q (2003) Contribution of acetylcholine to visual cortex plasticity. *Neurobiol Learn Mem* 80:291-301.
- Hamilton SE, Nathanson NM (2001) The M1 receptor is required for muscarinic activation of mitogen-activated protein (MAP) kinase in murine cerebral cortical neurons. *J Biol Chem* 276:15850-15853.
- Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc (England)* 4:44-57.
- Huang Z, Robinson GE (1996) Regulation of honey bee division of labor by colony age demography. *Behavioral Ecology and Sociobiology* 39:147-158.
- Ismail N, Robinson GE, Fahrbach SE (2006) Stimulation of muscarinic receptors mimics experience-dependent plasticity in the honey bee brain. *Proc Natl Acad Sci U S A* 103:207-211.
- Kolb (1998) Brain plasticity and behavior. *Annu Rev Psychol* 49:43-64.
- Krofczik S, Khojasteh U, de Ibarra NH, Menzel R (2008) Adaptation of microglomerular complexes in the honeybee mushroom body lip to manipulations of behavioral maturation and sensory experience. *Dev Neurobiol* 68:1007-1017.
- Kucharski R, Maleszka R (2002) Molecular profiling of behavioural development: differential expression of mRNAs for inositol 1,4,5-trisphosphate 3-kinase isoforms in naive and experienced honeybees (*Apis mellifera*). *Brain Res Mol Brain Res* 99: 92-101.
- Leung YF, Cavalieri D (2003) Fundamentals of cDNA microarray data analysis. *Trends Genet* 19:649-659.
- Li C, Niu W, Jiang CH, Hu Y (2007) Effects of enriched environment on gene expression and signal pathways in cortex of hippocampal CA1 specific NMDAR1 knockout mice. *Brain Res Bull* 71:568-577.
- Lyckman AW, Horng S, Leamey CA, Tropea D, Watakabe A, Van Wart A, McCurry C, Yamamori T, Sur M (2008) Gene expression patterns in visual cortex during the critical period: Synaptic stabilization and reversal by visual deprivation. *Proc Natl Acad Sci U S A* 105:9409-9414.

McArdle JJ, Ferrer-Caja E, Hamagami F, Woodcock RW (2002) Comparative longitudinal structural analyses of the growth and decline of multiple intellectual abilities over the life span. *Dev Psychol (United States)* 38:115-142.

Menzel R (1990) Learning, memory, and "cognition" in honey bees. In: *Neurobiology of comparative cognition* (Kesner RP, Olton DS, eds), pp 237-292. Hillsdale, NJ: Erlbaum Associates, Inc.

Moresco EM, Donaldson S, Williamson A, Koleske AJ (2005) Integrin-mediated dendrite branch maintenance requires abelson (abl) family kinases. *J Neurosci* 25:6105-6118.

Ng EL, Tang BL (2008) Rab GTPases and their roles in brain neurons and glia. *Brain Res Rev* 58:236-246.

Rampon C, Jiang CH, Dong H, Tang YP, Lockhart DJ, Schultz PG, Tsien JZ, Hu Y (2000) Effects of environmental enrichment on gene expression in the brain. *Proc Natl Acad Sci U S A* 97:12880-12884.

Rodal AA, Motola-Barnes RN, Littleton JT (2008) Nervous wreck and Cdc42 cooperate to regulate endocytic actin assembly during synaptic growth. *J Neurosci* 28:8316-8325.

Ronnback A, Dahlqvist P, Svensson PA, Jernas M, Carlsson B, Carlsson LM, Olsson T (2005) Gene expression profiling of the rat hippocampus one month after focal cerebral ischemia followed by enriched environment. *Neurosci Lett* 385:173-178.

Rosenzweig MR (2007) Modification of brain circuits through experience. In: *Neural plasticity and memory: From genes to brain imaging* (Bermudez-Rattoni F, ed), Boca Raton (FL): Taylor & Francis Group, LLC.

Routtenberg A, Rekart JL (2005) Post-translational protein modification as the substrate for long-lasting memory. *Trends Neurosci* 28:12-19.

Saeed AI, Bhagabati NK, Braisted JC, Liang W, Sharov V, Howe EA, Li J, Thiagarajan M, White JA, Quackenbush J (2006) TM4 microarray software suite. *Methods Enzymol* 411:134-193.

Seehuus SC, Krekling T, Amdam GV (2006) Cellular senescence in honey bee brain is largely independent of chronological age. *Exp Gerontol* 41:1117-1125.

Sen Sarma M, Whitfield CW, Robinson GE (2007) Species differences in brain gene expression profiles associated with adult behavioral maturation in honey bees. *BMC Genomics* 8:202.

Sen Sarma M, Rodriguez-Zas SL, Hong F, Zhong S, Robinson GE (2009) Transcriptomic profiling of central nervous system regions in three species of honey bee during dance communication behavior. *PLoS One* 4:e6408.

- Sen Sarma M, Rodriguez-Zas SL, Gernat T, Nguyen T, Newman T, Robinson GE (2010) Distance-responsive genes found in dancing honey bees. *Genes Brain Behav* 9:825-830.
- Sigg (1997) Activity-dependent changes to the brain and behavior of the honey bee, *apis mellifera* (L.). *The Journal of Neuroscience* 17:7148.
- Slawski M, Daumer M, Boulesteix AL (2008) CMA: A comprehensive bioconductor package for supervised classification with high dimensional data. *BMC Bioinformatics* 9:439.
- Thiriet N, Amar L, Toussay X, Lardeux V, Ladenheim B, Becker KG, Cadet JL, Solinas M, Jaber M (2008) Environmental enrichment during adolescence regulates gene expression in the striatum of mice. *Brain Res* 1222:31-41.
- Tweedie S, Ashburner M, Falls K, Leyland P, McQuilton P, Marygold S, Millburn G, Osumi-Sutherland D, Schroeder A, Seal R, Zhang H, FlyBase Consortium (2009) FlyBase: Enhancing *Drosophila* gene ontology annotations. *Nucleic Acids Res* 37:D555-9.
- van Praag H, Kempermann G, Gage FH (2000) Neural consequences of environmental enrichment. *Nat Rev Neurosci* 1:191-198.
- Visscher PK, Dukas R (1997) Survivorship of foraging honey bees. *Insectes Soc* 44:1-5.
- Wang X, Wu M, Li Z, Chan C (2008) Short time-series microarray analysis: Methods and challenges. *BMC Syst Biol* 2:58.
- Whitfield CW, Cziko AM, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. *Science* 302:296-299.
- Whitfield CW, Ben-Shahar Y, Brillet C, Leoncini I, Crauser D, Leconte Y, Rodriguez-Zas S, Robinson GE (2006) Genomic dissection of behavioral maturation in the honey bee. *Proc Natl Acad Sci U S A* 103:16068-16075.
- Winnington (1996) Structural plasticity of identified glomeruli in the antennal lobes of the adult worker honey bee. *Journal of Comparative Neurology* 365:479.
- Winston ML (1987) *The biology of the honey bee*. Cambridge, MA: Harvard University Press.
- Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. *Nature* 364: 238-240.
- Withers GS, Fahrbach SE, Robinson GE (1995) Effects of experience and juvenile hormone on the organization of the mushroom bodies of honeybees. *J Neurobiol* 26: 130-144.
- Zhang B, Horvath S (2005) A general framework for weighted gene co-expression network analysis. *Stat Appl Genet Mol Biol* 4:Article17.

Figures and Tables

Figure 2.1

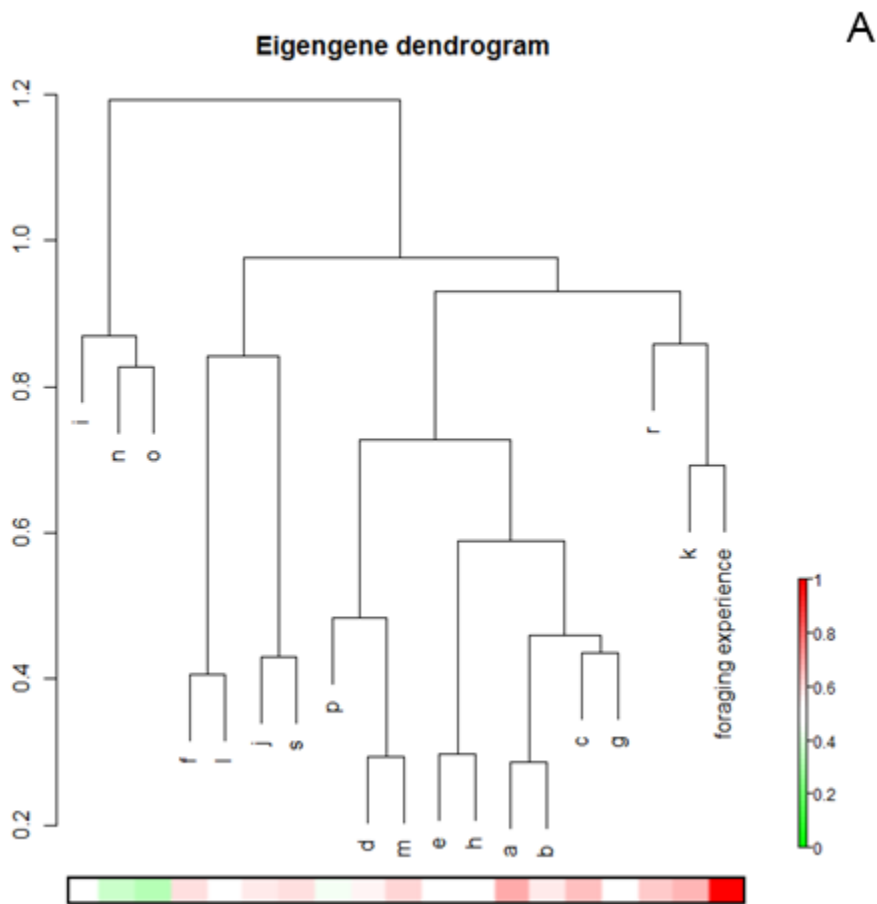


Figure 2.1 (continued)

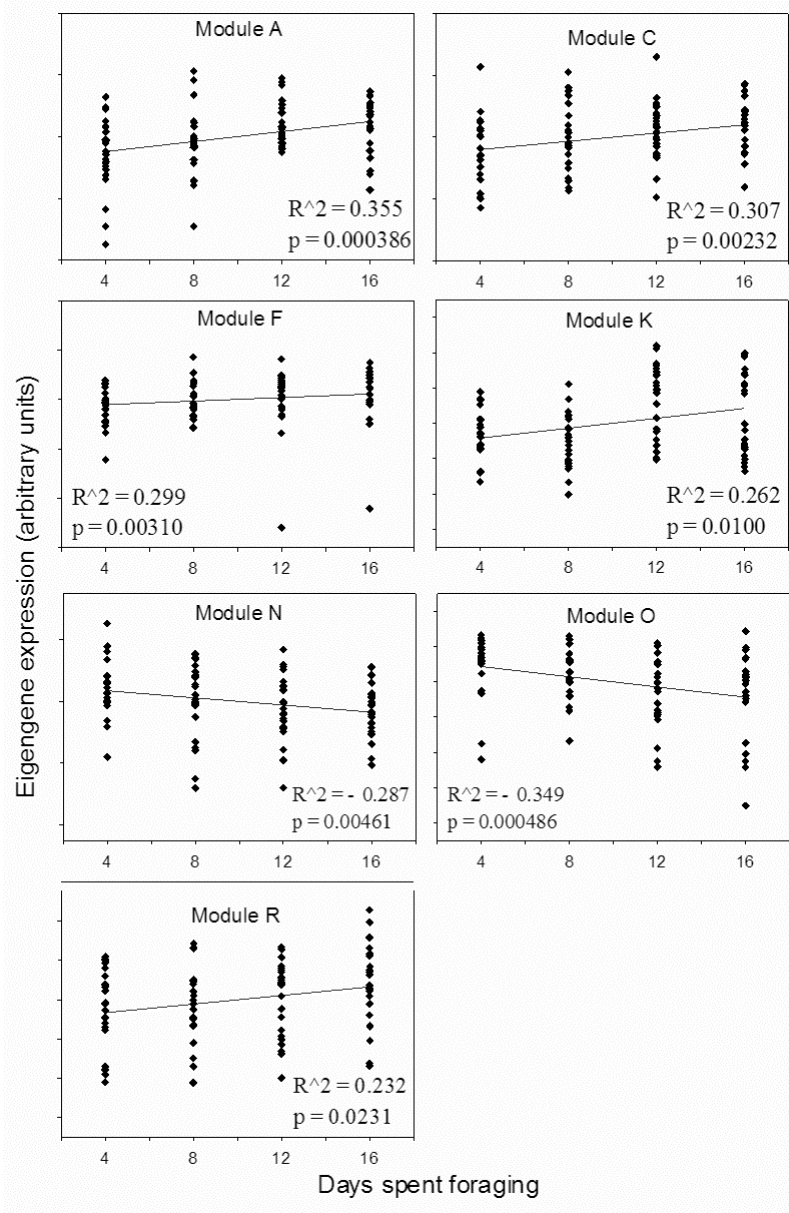


Figure 2.1. A. Hierarchical clustering of coexpression modules in the forager network. The number of days each individual had foraged was included in the analysis and is shown here as the arm labeled “foraging experience.” The heatmap below the dendrogram shows the R^2 value for the correlation between expression in each module with days of foraging experience, according to the legend on the right. B. Days spent foraging vs. module eigengene expression for each module showing a significant correlation with foraging experience (Spearman’s $\rho < 0.05$). Each point represents an individual forager.

Figure 2.2

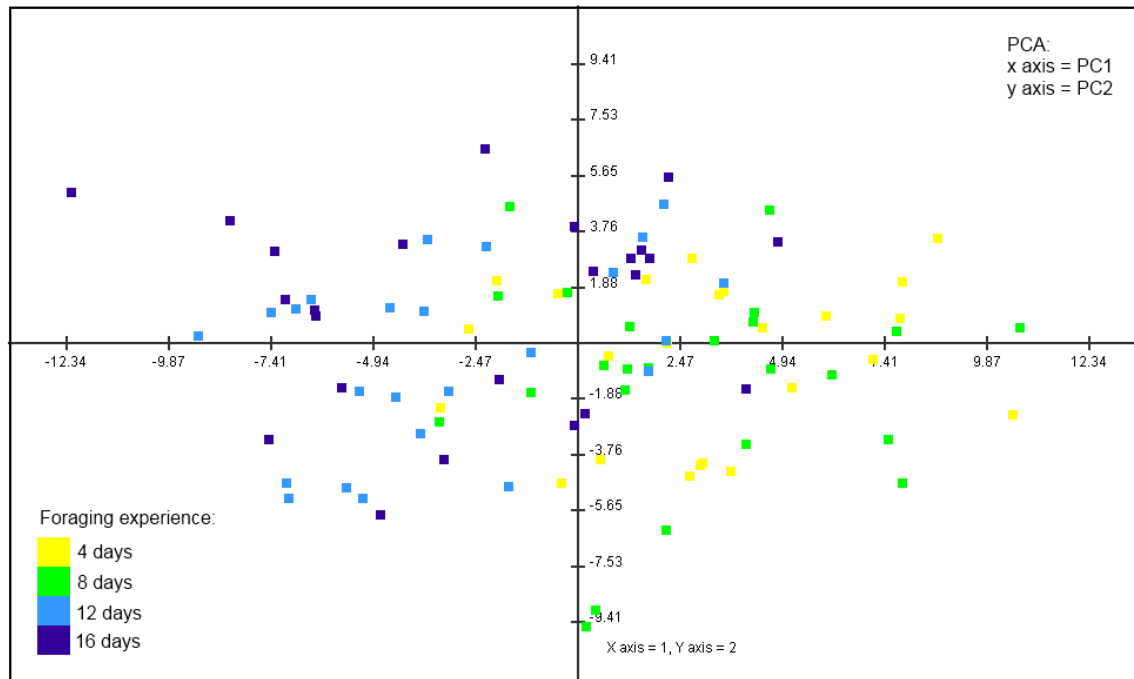


Figure 2.2. Principal component analysis of foraging individuals shows a division between less (4- and 8-day) and more experienced (12- and 16-day) foragers.

Figure 2.3

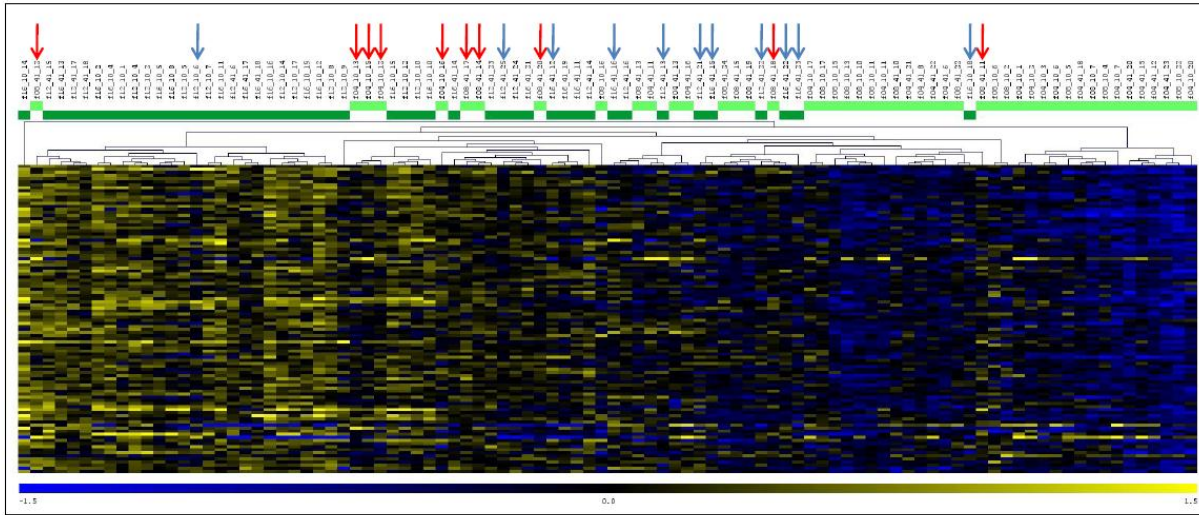


Figure 2.3. Heat map depicting expression differences for 100 most predictive genes identified by class prediction analysis. The heat map displays expression profiles of all foraging individuals. At the top, light green indicates novice foragers and dark green indicates experienced foragers. Misclassified individuals are marked with red or blue arrows, respectively. Yellow represents upregulation, blue represents downregulation.

Figure 2.4

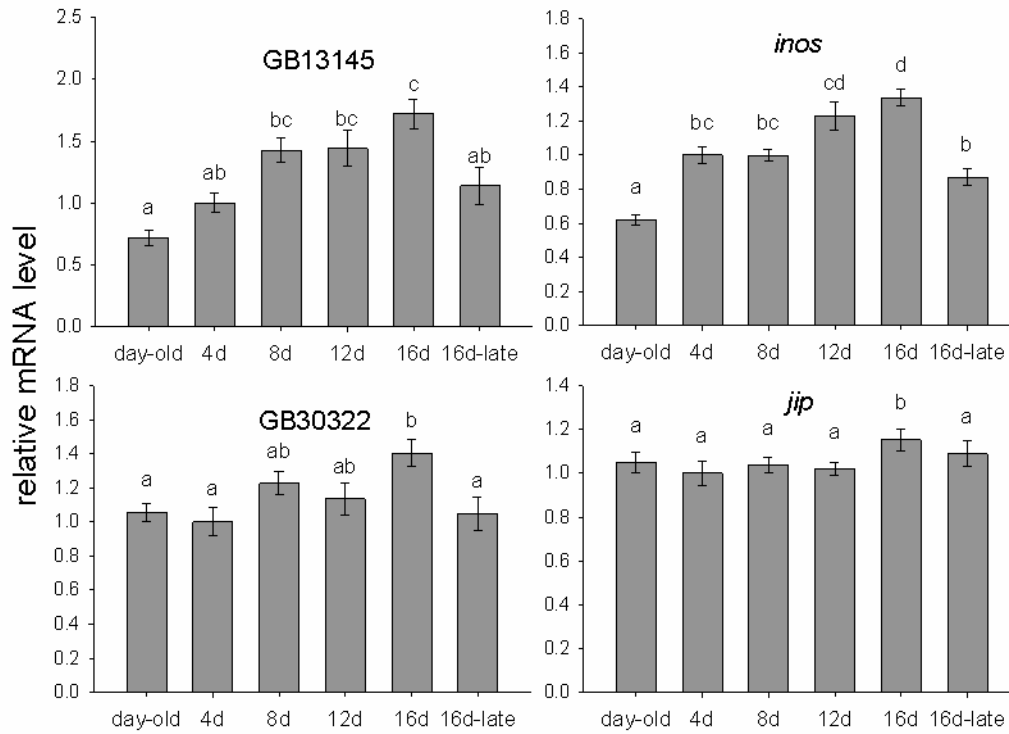


Figure 2.4. Expression of four genes measured with qRT-PCR. Letters indicate significant differences ($p < 0.05$, mixed model ANOVA). Expression is shown relative to average expression in 4-day foragers, which was normalized to 1. The group name 16d-late indicates bees of the same age as 16-day foragers, but with only 4 days of foraging experience.

Figure 2.5

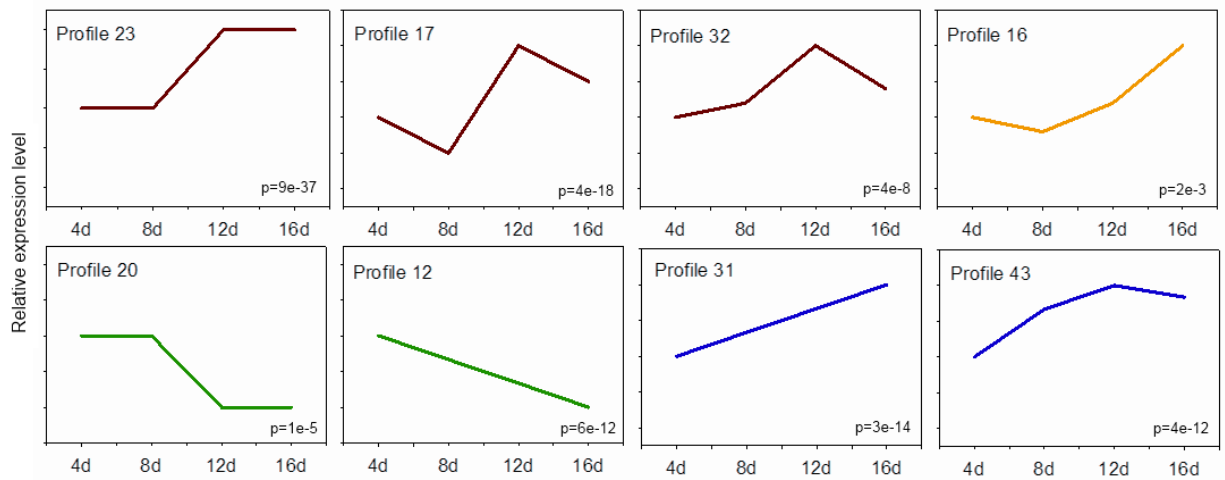


Figure 2.5. Overrepresented gene expression profiles identified by STEM. Lines represent pattern of expression across the four foraging time points. Profiles with similar expression patterns were assigned the same color. Numbers in the bottom left are p-values representing significance of overrepresentation.

Table 2.1

Gene	Name	Putative Function	<i>Drosophila</i> best hit
GB12552		unknown	FBgn0052512
GB30322		similar to insulin-like receptor	FBgn0013984
GB14758	<i>hsp90</i>	response to heat	FBgn0001233
GB13145		unknown	
GB14435		response to heat	FBgn0011296
GB11572	<i>inos</i>	inositol synthesis	FBgn0025885
GB19694	<i>samdc</i>	S-adenosylmethionine decarboxylase	FBgn0019932
GB14151	<i>jip</i>	JNK-interacting protein	FBgn0040281
GB10565	<i>trbl</i>	kinase-like protein	FBgn0028978
GB18093		transcription factor	FBgn003 9169

Table 2.1. Genes selected for validation and further analysis using qRT-PCR. Gene names and putative function based on orthology to *Drosophila melanogaster*.

Table 2.2

Profile	Functional Category	Number of genes
23	protein kinase activity	7
	regulation of JNK cascade	3
	G-protein coupled receptor protein signaling pathway	6
	nuclear export	4
17	protein folding	7
31 and 43	nuclear lumen	6
	regulation of transcription, DNA-dependent	6
	RNA processing	4
12 and 20	oxidation reduction	7

Table 2.2. Gene Ontology functional processes enriched in temporal expression profiles identified by STEM. Clusters of enriched categories (EASE<0.05) were identified in DAVID, with a representative category from each cluster shown here.

Table 2.3

Module	Functional Category	Number of genes
A	small GTPase mediated signal transduction	11
	nuclear export	6
	translation initiation factor activity, nucleic acid	
	binding	10
	ncRNA metabolic process	15
	nucleotide binding	64
	establishment of protein localization	21
C	protein kinase activity	35
	regulation of RNA splicing	13
	ATP-dependent helicase activity	14
	chromatin modification	17
	guanylate kinase activity	5
	synaptic vesicle transport	15
	regulation of MAPKKK cascade	10
	learning	10
F	chromatin organization	5
R	plasma membrane part	93
	multicellular organism reproduction	118
	cellular component morphogenesis	108
	regulation of nervous system development	32
	cell motion	65
	cell adhesion	38
	learning or memory	24
	sensory organ development	69
	tissue morphogenesis	53
	neuron projection	17

Table 2.3 (continued)

	growth	28
	locomotor behavior	34
	negative regulation of gene expression	41
	asymmetric protein localization	14
	actin filament-based process	33
	molecular adaptor activity	9
	cation channel activity	25
	regulation of Ras protein signal transduction	26
	metal ion binding	200
	courtship behavior	12
	gliogenesis	16
	Notch signaling pathway	12
K	protein folding	7
	ATP binding	10
	epigenetic regulation of gene expression	5
N	structural constituent of cuticle	4
	sensory perception of chemical stimulus	5
	microsome	3
	extracellular space	3
O	mitotic spindle elongation	6
	ATP biosynthetic process	4
	hexose catabolic process	3

Table 2.3. Gene Ontology functional processes enriched in modules showing significant correlations with amount of foraging experience. Module F was not significantly enriched for any functional category. Clusters of enriched categories (EASE<0.05) were identified in DAVID, with a representative category from each cluster shown here.

CHAPTER 3

ACTIVITY-DEPENDENT GENE EXPRESSION IN HONEY BEE MUSHROOM BODIES IN RESPONSE TO A SPATIAL LEARNING EVENT

Abstract

The natural history of adult worker honey bees (*Apis mellifera*) provides an opportunity to study the molecular basis of spatial learning in an ecological context. Foragers must learn to navigate between the hive and floral locations that may be up to miles away. Young pre-foragers prepare for this task by performing orientation flights near the hive, during which they begin to learn navigational cues such as the appearance of the hive, position of landmarks, and the movement of the sun. Despite well-described spatial learning and navigation behavior, there is currently limited information on the neural basis of insect spatial learning. We examined immediate early gene expression (IEG) in the brains of orienting bees to begin to elucidate both the molecular and neural bases of spatial learning in honey bees. We found that *egr*, an insect homolog of *egr-1*, is rapidly and transiently upregulated in the mushroom bodies in response to orientation; this is the first example of an *egr-1* homolog acting as a learning-related IEG in an insect. This transcriptional response occurred both in naïve bees and foragers induced to re-orient. Further experiments suggest that visual environmental novelty, rather than exercise or memorization of specific visual cues, acts as the stimulus for *egr* upregulation. Our results implicate the mushroom bodies in spatial learning and emphasize the deep conservation of *egr*-related pathways in experience-dependent plasticity.

1. Introduction

The need for navigation to survive in a natural environment is common to many species. Brain regions involved in vertebrate, particularly mammalian, spatial learning have been identified and extensively studied (Mizumori et al., 2004; Moser et al., 2008). Less is known about the neural basis for spatial learning in insects, but there is evidence to suggest that the mushroom bodies, a region of the insect brain involved in sensory integration and memory, support spatial learning in some species (Mizunami et al., 1998; Farris 2008). However, investigations of spatial learning in *Drosophila* have consistently shown a lack of mushroom body involvement (Zars, 2011).

Honey bees provide an advantageous system for studies of the neural basis of ecologically relevant spatial learning. Honey bees are central place foragers that depend on ephemeral and scattered floral resources, and form robust long-term memories of floral and hive locations (Robinson and Dyer, 1993; Menzel et al., 2006). Bees prepare for the navigational challenges of foraging by first performing short learning flights called orientation flights, during which bees acquire information about landmarks and learn to associate position of the sun, time of day, and directionality (Winston, 1987; Capaldi and Dyer, 1999). This information is necessary for later foraging behavior (Becker, 1958), during which bees must accurately navigate between floral sources and the hive. Orientation flights thus offer an exceptional opportunity for analysis because they are discrete, natural learning events driven by innate behavior.

Egr-1 is a canonical immediate-early gene (IEG), a transcription factor whose expression is activity-dependent and associated with learning and novelty detection in many vertebrate systems (Knapska and Kaczmarek, 2004). *Egr-1* expression is induced in the hippocampus of rodents engaged in spatial learning tasks (Bozon et al., 2002). Recent investigation of IEG activity in insects has yielded growing evidence for activity- and learning-related expression similar to that in vertebrates (Alaux and Robinson, 2007; Ghosal et al., 2010; Kiya and Kubo, 2011; Kiya et al., 2008; Sen Sarma et al., 2010). However, the insect homolog of *egr-1*, here named *egr*, has mainly been studied for its role in muscle development (Volk, 1999), though it has been shown to be upregulated in the brains of flies after seizure, suggesting that it may also be induced by neuronal activity (Guan et al., 2005).

We explored whether we could use *egr* expression in the honey bee brain as an indicator of neuronal activity, as in vertebrates. We used this IEG to identify the mushroom bodies as a brain region that is involved in cognitive processes during orientation flights. Taking advantage of the ability to manipulate natural bee behavior in both the laboratory and the field, we also tested four hypotheses to identify what kind of stimuli associated with orientation flight evoke the *egr* mushroom body response (Table 3.1).

2. Materials and Methods

2.1. Phylogenetic analyses

We obtained the nucleotide and amino acid sequences for *egr-1*, *stripe* (original *Drosophila* name for *egr* ortholog), *GB50091* and *AGAP005288* in *Mus musculus*, *Drosophila*

melanogaster, *Apis mellifera*, and *Anopheles gambiae*, respectively, from GenBank. Sequence orthology was examined using CLUSTALW2 alignments (Goujon et al., 2010) and displayed with Boxshade.

2.2. Animals

Single-cohort colonies were created as in Robinson et al. (1989). Briefly, we placed a laying queen and ~3000 one-day-old adult workers bees into a small Styrofoam hive box with several frames with honeycomb containing nectar and pollen. To ensure that bees were naïve to flight, the entrance to each colony was blocked, and colonies were kept inside at 21-23 °C and protected from light for 5-6 days after assembly. In colonies constructed exclusively from young bees, a subset will undergo precocious maturation to produce a foraging workforce (Huang and Robinson, 1996). A previous study found that when flight is restricted for several days, these bees become “primed” to make an orientation flight, exiting the colony to do so as soon as the entrance is opened (Capaldi and Dyer, 1999). This process therefore creates a convenient population of naïve bees ready to perform orientation flights. On the day of experimentation, colonies were moved outside and allowed to acclimate for at least 30 minutes.

2.3. Collections and gene expression analyses

Orienting bees were captured on their return to the hive, placed in individual cages, and returned to the hive for 30 minutes (in one experiment, 60 minutes). Expression of *egr-1* typically peaks 30 minutes after the initiating stimulus in vertebrate studies (Zangenehpour and Chaudhuri, 2002). Bees were then flash-frozen in liquid nitrogen (for qRT-PCR analysis) or chilled on ice until brain dissection (for *in situ* analysis).

In situ hybridization was performed as in Velarde et al (2006). Whole brains were dissected in cold physiological saline (Fahrbach et al., 1995), frozen in OCT, and cut in 12 µm frontal sections. Sections were mounted on slides, fixed in 4% paraformaldehyde and hybridized overnight with a DIG-labeled RNA probe. After incubation with anti-DIG AP, slides were developed in NBT/BCIP until a clear signal was visible. Expression was quantified in a single trial (n=5) by counting positively-labeled cells in one hemisphere of a randomly selected section from a defined plane in the center of the brain (Groh et al., 2004).

qRT-PCR on mushroom body tissue was performed as described in Lutz et al (2012). Mushroom bodies were isolated as described in that study after treatment of flash-frozen heads with RNAlater-ice. RNA was extracted from isolated mushroom bodies or other brain regions using Picopure extraction kits (Arcturus). Abundance of transcript was analyzed with a SYBRgreen probe, quantified relative to a genomic DNA standard curve, and normalized to *eif-s8*, a constitutively expressed endogenous control gene used in previous studies (Alaux et al., 2009). Results shown are an average of two trials (for the initial investigation of orientation flight, three trials); n=6-8 for all groups. Two-factor ANOVAs were performed for multiple trials using PROC MIXED in SAS.

Sequences of primers used to generate the probe for *in situ* hybridization, as well as for qRT-PCR, are listed in Table 3.2.

2.4. *Is egr upregulated by orientation flight?*

To observe and collect bees performing orientation flights, groups of 6-10 bees were allowed to exit a colony, and an observer confirmed that the flight pattern and duration were typical of orienting bees (Winston, 1987). Control bees were captured while exiting the hive, held for 6-7 minutes in individual cages next to the hive, and then treated as described above for orienting bees. The 6-7 minute holding period allowed control bees to experience sunlight, outdoor odors and other environmental stimuli for an amount of time roughly equivalent to an orientation flight. Because control bees were collected while exiting the hive, it is presumed that these individuals would have performed an orientation flight had they not been captured. The conditions used in this experiment were adapted in order to test alternative hypotheses for the upregulation of *egr* (Table 3.1), as described below.

2.5. *Is egr upregulated by exercise in the absence of flight? (Hypothesis I)*

We tested this hypothesis by studying exercise in a familiar environment. Fanning bees (bees exhibiting vigorous, flight-like wing movement while remaining stationary) were collected by setting up a colony as described for naïve orientation flight collections, but in a small glass-walled hive housed indoors; only 1000 worker bees were added. After 7 days, the colony was heated to 40 °C for 45 minutes. This induced some bees to beat their wings vigorously at the hive entrance to provide ventilation (Southwick and Moritz, 1987). Bees that engaged in this

fanning behavior for at least 2 minutes were collected, along with several inactive bees to serve as controls.

2.6. Is egr upregulated by motor learning associated with flight? (Hypothesis II)

We tested this hypothesis by inducing orientation flights by foragers. Foragers, bees with extensive flight experience, perform additional re-orientation flights after a hive is moved, or after moving to a new nest site as part of a swarm (Winston, 1987). These re-orientation flights allow bees to learn their new environment. To induce re-orientation, small colonies with natural age demographics were closed after sunset and transported to a new location at a distance outside the maximum foraging range (Capaldi and Dyer, 1999; Capaldi et al., 2000). This treatment induces bees with foraging experience to perform additional orientation flights in their new environment. The morning after the colony move, orienting foragers and controls were collected as described above, including observations of flight structure and duration. Returning bees were examined to ensure that they had not gathered a pollen or nectar load, indicating that they were performing an orientation flight and not foraging.

2.7. Is egr upregulated by exposure to specific visual cues? (Hypothesis III)

We tested this hypothesis by studying orientation flights in deprived conditions. Bees will perform orientation flights even in indoor arenas of limited size, devoid of distant landmarks (Brandon and Coss, 1982); such environments are in extreme contrast to the sensorially enriched outdoors. The experimental conditions described above for testing response to a typical first orientation flight were repeated for tethered bees in a small indoor flight enclosure relatively devoid of visual patterning. Temperature in the enclosure was 29.5 °C during the day and 24 °C at night, and lights were on a 12/12 hour light/dark cycle.

Bees used in tethered flight experiments came from colonies prepared as for the naïve orientation flight collections. Bees were captured as they exited the colony entrance in preparation for an orientation flight. Bees were immediately placed on ice just until anesthetized. One end of a thread was then fixed to the thorax using Superglue; the other end was fixed to the end of a rod. Tethered bees were placed on the floor of a white box and allowed to recover until able to walk in a coordinated manner. Flight was induced by lifting the bee's feet from the ground (Feller and Nachtigall, 1989); this action was repeated as often as necessary

to sustain flight for 2 minutes, usually 0-2 additional times. Controls were treated in the same manner as foragers, but restrained in an individual plastic cage rather than being induced to fly.

2.8. Is *egr* upregulated by exposure to environmental novelty? (Hypothesis IV)

We tested this hypothesis by studying flights performed in the absence of visual novelty; that is, flights performed by bees completely deprived of visual input. The tethered flight experiment described above was repeated using red lighting, which is not visible to bees (Peitsch et al., 1992).

3. Results

3.1. Honey bee *egr* is a homolog of the vertebrate *egr* gene family

We investigated GB50091, a 2448 bp transcript encoding an 815-amino acid protein. This gene is an ortholog of the *Drosophila* gene *stripe*; the genes are reciprocal best blast hits and share 53.8% sequence identity (e-value: 1e-95). Like *stripe*, the honey bee gene is a homolog of genes in the *egr* gene family in vertebrates, including *egr-1*, with which it shares 44.5% sequence identity (e-value: 2e-46). The DNA binding domain, which consists of three zinc-finger domains (Pavletich and Pabo, 1991), is highly conserved between bee, fly, mouse, and mosquito (Figure 3.1). The amino acid sequence identity between the honey bee and *Drosophila* zinc-finger domains is 94%; between the honey bee and mouse zinc-finger domains is 89%. Because of the apparent functional similarity (see below) to the vertebrate gene *egr-1*, we have used the name *egr* to refer to the honey bee gene.

3.2. *Egr* expression is rapidly and transiently increased in the mushroom bodies of orienting bees

We first assessed the spatial distribution of *egr* mRNA in the honey bee brain, using *in situ* hybridization and qPCR. *In situ* analysis revealed visible *egr* mRNA expression only in scattered Kenyon cells in the mushroom bodies in both orienting bees and controls (Figure 3.2A). Similarly, qRT-PCR analysis showed that *egr* expression was enriched in the mushroom bodies compared with the rest of the brain (Figure 3.3).

We next assessed activity-dependent changes in *egr* expression in the mushroom bodies. In many vertebrate species, activity-dependent *egr-1* mRNA expression peaks ~30 minutes after

the relevant stimulus and returns to baseline after an hour (Zangenehpour and Chaudhuri, 2002). We investigated the temporal dynamics of *egr* expression in orienting bees by analyzing transcript abundance in the mushroom bodies of bees collected 30 m and 1 h after flight. Orienting bees showed an almost 2-fold upregulation of *egr* in comparison to controls ($p < 0.005$; Figure 3.3) 30 minutes after flight; analysis of *in situ* images suggested that this difference was due to greater numbers of neurons expressing *egr* in orienting bees (Figure 3.2B, C). Because control bees were collected as they attempted to perform an orientation flight, and were exposed to sunlight and environmental odors, we hypothesized that increased *egr* expression in the mushroom bodies was caused by an aspect of the orientation flight other than preparedness or exposure to stimuli.

Upregulation of *egr* was limited to the mushroom bodies: the low level of detectable *egr* expression in the rest of the brain was not differentially regulated by the experience of an orientation flight. The consistent upregulation of *egr* in the mushroom bodies after orientation provides circumstantial evidence for the involvement of the mushroom bodies in honey bee spatial learning and suggests that this activity-dependent mechanism is specific to the mushroom bodies. Presumably an orientation flight is also associated with neuronal activity in other brain regions such as the optic lobes, but any such changes appear to be *egr*-independent.

The orientation flight, although most significant as an example of spatial learning, can be broken down into several component experiences: exercise, motor learning, exposure to visual cues, and exposure to novelty. Any of these components could conceivably induce an IEG response. We performed a series of experiments to test predictions stemming from specific hypotheses on the relationship between the experience of an orientation flight and upregulation of *egr* (Table 3.1).

3.3. *Egr is not upregulated by exercise in the absence of flight.*

In rodents, *c-fos*, *arc* and *egr-1* expression in the hippocampus increase in response to exercise (Rhodes et al., 2003; Clark et al., 2011). Flight in honey bees represents an abrupt increase in metabolic demand that could mediate an exercise-dependent increase in *egr* expression. We devised a behavioral manipulation that decoupled the exercise associated with rapid wing movement from flight outside the hive. The exercise examined was wing fanning inside the hive entrance to ventilate the hive after heat stress. Bees collected while engaged in

fanning behavior experienced exercise roughly comparable with flight, but in a familiar environment (Yang et al., 2010). Fanning bees showed no *egr* upregulation in response to this form of exercise compared with controls ($p = 0.859$, Figure 3.4), although bees from the same colony did show the expected *egr* upregulation in response to an orientation flight ($p < 0.001$). This experiment suggests that exercise alone cannot induce *egr* upregulation in the mushroom bodies.

3.4. Egr is not upregulated by motor learning associated with flight.

Experienced foragers will perform additional orientation flights after participating in a swarm, or after a colony is moved by humans (Winston, 1987). We took advantage of this to examine *egr* expression in the mushroom bodies of foragers that performed a re-orientation flight after a colony move. Although experienced in flight, these bees exhibited upregulation of mushroom body *egr* expression in comparison to controls ($p < 0.0005$; Figure 3.5A). This difference indicated that *egr* upregulation cannot be attributed to motor learning.

3.5. Egr is not upregulated by exposure to specific visual cues.

To examine the relationship between feature memorization and *egr* upregulation, we investigated the transcriptional response to orientation flight in visually deprived environments. We collected bees after they performed an orientation flight in a small indoor flight arena with white floors and bare screen walls and ceiling. Bees that performed an orientation flight in this environment showed upregulation of mushroom body *egr* expression compared with controls ($p < 0.0001$, Figure 3.5B).

To examine the effect of even more extreme visual deprivation during flight, naïve bees preparing for an orientation flight were tethered to a restraint that allowed freedom of movement and induced to fly in a blank, white arena. Bees that experienced two minutes of tethered flight in a white box devoid of visual cues also showed increased *egr* expression compared with tethered and untreated controls ($p < 0.0001$, Figure 3.5C). Although tethered bees were exposed to a novel environment during flight, exposure to specific visual cues was not necessary to induce *egr* upregulation.

3.6. Egr is upregulated by exposure to environmental novelty.

IEG expression in many vertebrate species is upregulated in response to the novelty of a stimulus (Tischmeyer and Grimm, 1999; Clayton, 2000); orientation flights expose bees to novel stimuli. To test the necessity of visual novelty to induce *egr* upregulation, we designed an experiment to give bees the experience of flight without visual novelty. Bees experienced tethered flight as described above, but in a room with red lighting at a wavelength too long for them to detect (Peitsch et al., 1992). These tethered bees showed no upregulation of *egr* in comparison with controls ($p = 0.303$, Figure 3.6), unlike orienting bees from the same colony ($p < 0.005$). This experiment suggests that visual environmental novelty during the orientation flight is necessary to induce *egr* upregulation in the mushroom bodies.

4. Discussion

We investigated the transcriptional response of *egr*, an insect homolog of *egr-1*, in the brains of honey bees after the performance of orientation flights and related behaviors. We found that a single orientation flight upregulates *egr* expression exclusively in the mushroom bodies. This increased expression was seen in comparison with bees exiting the hive for a flight, and therefore associated with the experience rather than the anticipation of a spatial learning event.

We also found that flight in several contexts and environments was sufficient to cause this upregulation, as long as the environment was novel. Exercise was insufficient to cause *egr* upregulation without the visual perception of environmental novelty. Our results suggest a previously undescribed learning-related role for *egr* in an insect species, and support the hypothesis that the mushroom bodies may be involved in spatial learning in honey bees.

Our results provide evidence for the involvement of the mushroom bodies in spatial learning in honey bees, which contrasts with previous research on *Drosophila* (Neuser et al., 2008; Wolf et al., 1998; Putz and Heisenberg, 2002; Sitaraman et al., 2008; Zars et al., 2000; Ofstad et al., 2011). However, prior work in other species besides *Drosophila* has suggested a possible link between the mushroom bodies and spatial learning. Insect species whose feeding ecologies depend more heavily on navigation have larger, more elaborated mushroom bodies (Farris, 2008; Mizunami et al., 1998). Cockroaches with lesioned mushroom bodies perform poorly on a spatial learning task similar to the Morris water maze (Mizunami et al., 1998). In honey bees, mushroom body neuropil undergoes age-related expansion roughly coincident with

orientation flights in both workers and reproductives (Fahrbach et al., 1995; Brandon and Coss, 1982; Withers et al., 1993; Fahrbach et al., 1997), and one study found rapid remodeling of dendritic spines in the mushroom bodies after an orientation flight (Brandon and Coss, 1982). In addition, one previous study (Kiya et al., 2007) found evidence of neuronal activation in the mushroom bodies of re-orienting foragers. *Drosophila* have minimal mushroom bodies that receive olfactory input almost exclusively (Heisenberg, 2003), while those of honey bees comprise a substantial portion of total brain volume and receive significant visual input. It has been recently suggested that *Drosophila* spatial learning may not involve the mushroom bodies because this species, which does not exhibit central place foraging or other fidelity to particular locations, has no need for long-term spatial memories (Zeil, 2012). Hymenopterans and other insects that must navigate a fairly constant environment for days, weeks, or months would be predicted to exhibit mushroom body involvement in spatial learning. Given the similarity in mushroom body structure in hymenopterans and the shared ecological pressures that may have shaped them (Farris, 2008), a role in spatial learning is more likely to be shared in bees, ants and wasps, and perhaps other species with elaborate mushroom bodies as well.

Egr-1 is hypothesized to play a role in memory consolidation by promoting structural neuroplasticity in the vertebrate brain following exposure to novel or salient stimuli (Knapska and Kaczmarek, 2004; Tischmeyer and Grimm, 1999; Moorman et al., 2011). Many regulatory targets of *egr-1* have been identified, and several are involved in synaptic formation and remodeling. Given this new demonstration of the deep evolutionary conservation of *egr* as a possible mediator of experience-dependent plasticity, it is plausible that *egr* may function similarly in the bee brain. Although the present study did not examine potential downstream targets, *egr* is a good candidate for the functional link between orientation flights and resulting dendritic spine remodeling (Brandon and Coss, 1982). In addition, chronic administration of pilocarpine, a muscarinic cholinergic agonist, has been shown to stimulate dendritic growth in honey bee mushroom bodies (Ismail et al., 2008; Dobrin et al., 2011), and cholinergic signaling in the brain increases with behavioral maturation in honey bees (Shapira et al., 2001). Muscarinic stimulation was found to produce neurite outgrowth through *egr* expression in neuroblastoma cell culture (Salani et al., 2009), again suggesting the possibility of a link between *egr* expression and structural neuroplasticity in the bee brain.

To our knowledge, we are the first to report that *egr* displays activity-dependent expression in an insect, providing evidence for a conserved role as an immediate early gene. Despite extensive literature linking the *egr* gene family to neuronal activation and learning in vertebrates, there is little work on the role of this gene in the *Drosophila* nervous system, and no study linking this gene to novelty or learning. Our own finding that honey bee *egr* was, as in vertebrates, rapidly transcribed and degraded in response to environmental novelty strongly supports a role for *egr* as an IEG in insects as well. These results invite investigation of *egr* expression in other behavioral contexts, in honey bees and other insect species. Future studies should involve determining the behavioral and downstream molecular consequences of manipulations of *egr* signaling through pharmacological manipulations or RNAi knockdown. Such work could provide a more causal link between *egr*, downstream signaling, and the resulting consolidation of learning that is believed to occur in vertebrates (Bozon et al., 2003; Davis et al., 2003; Perez-Cadahia et al., 2011).

Our results suggest that the interaction between flight activity and visual novelty is sufficient to stimulate *egr* upregulation, as well as spatial learning itself. To clarify whether flight activity, in combination with visual novelty, is necessary for *egr* upregulation, future experiments could examine gene expression or homing behavior after bees are transported mechanically in a manner that imitates flight.

Because *egr-1* is widely responsive in vertebrates (Knapska and Kaczmarek, 2004), it is often difficult to distinguish between transcription resulting from the behavior or stimulus of interest and that resulting from exercise, stress, or other confounding factors. Honey bee orientation flights provide an excellent opportunity to further understanding of the role of *egr* and related genes in learning. Orientation flights are self-motivated and have a defined and quantifiable goal (successful navigation), so their study avoids the confounds of stress, lack of motivation, and attention to irrelevant stimuli. Future work in honey bees could also help to clarify the molecular role of *egr* in learning. Despite years of study, the exact relationship between *egr-1* expression, structural neuroplasticity and learning remains uncertain. The short duration and well-defined timing of orientation flight performance may facilitate the study of transcriptional targets of *egr* in learning-related contexts.

The possible involvement of the mushroom bodies in spatial learning in some, if not many, insect species has remained an open question for years. Similarly, the dearth of learning-

related findings for insect *egr* orthologs is mystifying. The results of the present study provide a new avenue of investigation for both of these intriguing issues. Pursuit of both is likely to provide new insight into the molecular functions and circuits underlying the ability of large and small brains to acquire the spatial information needed to navigate successfully.

References

Alaux C, Robinson GE (2007) Alarm pheromone induces immediate-early gene expression and slow behavioral response in honey bees. *J Chem Ecol* 33:1346-1350.

Alaux C, Le Conte Y, Adams HA, Rodriguez-Zas S, Grozinger CM, Sinha S, Robinson GE (2009) Regulation of brain gene expression in honey bees by brood pheromone. *Genes Brain Behav* 8:309-319.

Becker, L. (1958) Untersuchungen ueber das Heimfindevermoegen der Bienen. *Z Vergl Physiol* 41:1-25.

Bozon B, Davis S, Laroche S (2002) Regulated transcription of the immediate-early gene *Zif268*: Mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. *Hippocampus* 12:570-577.

Bozon B, Kelly A, Josselyn SA, Silva AJ, Davis S, Laroche S (2003) MAPK, CREB and *zif268* are all required for the consolidation of recognition memory. *Philos Trans R Soc Lond B Biol Sci* 358:805-814.

Brandon JG, Coss RG (1982) Rapid dendritic spine stem shortening during one-trial learning: The honeybee's first orientation flight. *Brain Res* 252:51-61.

Capaldi EA, Dyer FC (1999) The role of orientation flights on homing performance in honeybees. *J Exp Biol* 202:1655-1666.

Capaldi EA, Smith AD, Osborne JL, Fahrbach SE, Farris SM, Reynolds DR, Edwards AS, Martin A, Robinson GE, Poppy GM, Riley JR (2000) Ontogeny of orientation flight in the honeybee revealed by harmonic radar. *Nature* 403:537-540.

Clark PJ, Bhattacharya TK, Miller DS, Rhodes JS (2011) Induction of *c-fos*, *Zif268*, and *arc* from acute bouts of voluntary wheel running in new and pre-existing adult mouse hippocampal granule neurons. *Neuroscience* 184:16-27.

Clayton DF (2000) The genomic action potential. *Neurobiol Learn Mem* 74:185-216.

Davis S, Bozon B, Laroche S (2003) How necessary is the activation of the immediate early gene *zif268* in synaptic plasticity and learning? *Behav Brain Res* 142:17-30.

Dobrin SE, Herlihy JD, Robinson GE, Fahrbach SE (2011) Muscarinic regulation of Kenyon cell dendritic arborizations in adult worker honey bees. *Arthropod Struct Dev* 40:409-419.

- Fahrback SE, Giray T, Robinson GE (1995) Volume changes in the mushroom bodies of adult honey bee queens. *Neurobiol Learn Mem* 63:181-191.
- Fahrback SE, Giray T, Farris SM, Robinson GE (1997) Expansion of the neuropil of the mushroom bodies in male honey bees is coincident with initiation of flight. *Neurosci Lett* 236:135-138.
- Farris SM (2008) Evolutionary convergence of higher brain centers spanning the protostome-deuterostome boundary. *Brain Behav Evol* 72:106-122.
- Feller P, Nachtigall W (1989) Flight of the honey bee. *J Comp Physiol B* 158:719-727.
- Ghosal K, Naples SP, Rabe AR, Killian KA (2010) Agonistic behavior and electrical stimulation of the antennae induces fos-like protein expression in the male cricket brain. *Arch Insect Biochem Physiol* 74:38-51.
- Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez R (2010) A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res* 38:W695-9.
- Groh C, Tautz J, Rossler W (2004) Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. *Proc Natl Acad Sci U S A* 101:4268-4273.
- Guan Z, Saraswati S, Adolfsen B, Littleton JT (2005) Genome-wide transcriptional changes associated with enhanced activity in the drosophila nervous system. *Neuron* 48:91-107.
- Heisenberg M (2003) Mushroom body memoir: From maps to models. *Nat Rev Neurosci* 4:266-275.
- Huang Z, Robinson GE (1996) Regulation of honey bee division of labor by colony age demography. *Behav Ecol Sociobiol* 39:147-158.
- Ismail N, Christine S, Robinson GE, Fahrback SE (2008) Pilocarpine improves recognition of nestmates in young honey bees. *Neurosci Lett* 439:178-181.
- Kiya T, Kubo T (2011) Dance type and flight parameters are associated with different mushroom body neural activities in worker honeybee brains. *PLoS One* 6:e19301.
- Kiya T, Kunieda T, Kubo T (2007) Increased neural activity of a mushroom body neuron subtype in the brains of forager honeybees. *PLoS One* 2:e371.
- Kiya T, Kunieda T, Kubo T (2008) Inducible- and constitutive-type transcript variants of kakusei, a novel non-coding immediate early gene, in the honeybee brain. *Insect Mol Biol* 17:531-536.
- Knapska E, Kaczmarek L (2004) A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? *Prog Neurobiol* 74:183-211.
- Lutz CC, Rodriguez-Zas SL, Fahrback SE, Robinson GE (2012) Transcriptional response to foraging experience in the honey bee mushroom bodies. *Dev Neurobiol* 72:153-166.

- Menzel R, De Marco RJ, Greggers U (2006) Spatial memory, navigation and dance behaviour in *Apis mellifera*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 192:889-903.
- Mizumori SJ, Yeshenko O, Gill KM, Davis DM (2004) Parallel processing across neural systems: Implications for a multiple memory system hypothesis. *Neurobiol Learn Mem* 82:278-298.
- Mizunami M, Weibrecht JM, Strausfeld NJ (1998) Mushroom bodies of the cockroach: Their participation in place memory. *J Comp Neurol* 402:520-537.
- Moorman S, Mello CV, Bolhuis JJ (2011) From songs to synapses: Molecular mechanisms of birdsong memory. molecular mechanisms of auditory learning in songbirds involve immediate early genes, including *zenk* and *arc*, the ERK/MAPK pathway and synapsins. *Bioessays* 33:377-385.
- Moser EI, Kropff E, Moser MB (2008) Place cells, grid cells, and the brain's spatial representation system. *Annu Rev Neurosci* 31:69-89.
- Neuser K, Triphan T, Mronz M, Poeck B, Strauss R (2008) Analysis of a spatial orientation memory in *Drosophila*. *Nature* 453:1244-1247.
- Ofstad TA, Zuker CS, Reiser MB (2011) Visual place learning in *Drosophila melanogaster*. *Nature* 474:204-207.
- Pavletich NP, Pabo CO (1991) Zinc finger-DNA recognition: Crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* 252:809-817.
- Peitsch D, Fietz A, Hertel H, de Souza J, Ventura DF, Menzel R (1992) The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J Comp Physiol A* 170:23-40.
- Perez-Cadahia B, Drobic B, Davie JR (2011) Activation and function of immediate-early genes in the nervous system. *Biochem Cell Biol* 89:61-73.
- Putz G, Heisenberg M (2002) Memories in *Drosophila* heat-box learning. *Learn Mem* 9:349-359.
- Rhodes JS, Garland T, Jr, Gammie SC (2003) Patterns of brain activity associated with variation in voluntary wheel-running behavior. *Behav Neurosci* 117:1243-1256.
- Robinson GE, Dyer FC (1993) Plasticity of spatial memory in honey bees: Reorientation following colony fission. *Anim Behav* 46:311-320.
- Robinson GE, Page RE, Jr, Strambi C, Strambi A (1989) Hormonal and genetic control of behavioral integration in honey bee colonies. *Science* 246:109-112.
- Salani M, Anelli T, Tocco GA, Lucarini E, Mozzetta C, Poiana G, Tata AM, Biagioni S (2009) Acetylcholine-induced neuronal differentiation: Muscarinic receptor activation regulates *EGR-1* and *REST* expression in neuroblastoma cells. *J Neurochem* 108:821-834.
- Sen Sarma M, Rodriguez-Zas SL, Gernat T, Nguyen T, Newman T, Robinson GE (2010) Distance-responsive genes found in dancing honey bees. *Genes Brain Behav* 9:825-830.

- Shapira M, Thompson CK, Soreq H, Robinson GE (2001) Changes in neuronal acetylcholinesterase gene expression and division of labor in honey bee colonies. *J Mol Neurosci* 17:1-12.
- Sitaraman D, Zars M, Laferriere H, Chen YC, Sable-Smith A, Kitamoto T, Rottinghaus GE, Zars T (2008) Serotonin is necessary for place memory in drosophila. *Proc Natl Acad Sci U S A* 105:5579-5584.
- Southwick EE, Moritz RFA (1987) Social control of air ventilation in colonies of honey bees. *J Insect Physiol* 33:623-626.
- Tischmeyer W, Grimm R (1999) Activation of immediate early genes and memory formation. *Cell Mol Life Sci* 55:564-574.
- Velarde RA, Robinson GE, Fahrbach SE (2006) Nuclear receptors of the honey bee: Annotation and expression in the adult brain. *Insect Mol Biol* 15:583-595.
- Volk T (1999) Singling out *Drosophila* tendon cells: A dialogue between two distinct cell types. *Trends Genet* 15:448-453.
- Winston ML (1987) The biology of the honey bee. Cambridge, MA: Harvard University Press.
- Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. *Nature* 364:238-240.
- Wolf R, Wittig T, Liu L, Wustmann G, Eyding D, Heisenberg M (1998) *Drosophila* mushroom bodies are dispensable for visual, tactile, and motor learning. *Learn Mem* 5:166-178.
- Yang MY, Radloff S, Tan K, Hepburn R (2010) Anti-predator fan-blowing in guard bees, *Apis mellifera capensis* esch. *J Insect Behav* 23:12-18.
- Zangenehpour S, Chaudhuri A (2002) Differential induction and decay curves of c-fos and zif268 revealed through dual activity maps. *Brain Res Mol Brain Res* 109:221-225.
- Zars T (2011) Neuroscience: Flies race to a safe place. *Nature* 474:169-170.
- Zars T, Wolf R, Davis R, Heisenberg M (2000) Tissue-specific expression of a type I adenylyl cyclase rescues the rutabaga mutant memory defect: In search of the engram. *Learn Mem* 7:18-31.
- Zeil J (2012) Visual homing: An insect perspective. *Curr Opin Neurobiol* 22:285-293.

Figures and Tables

Figure 3.1

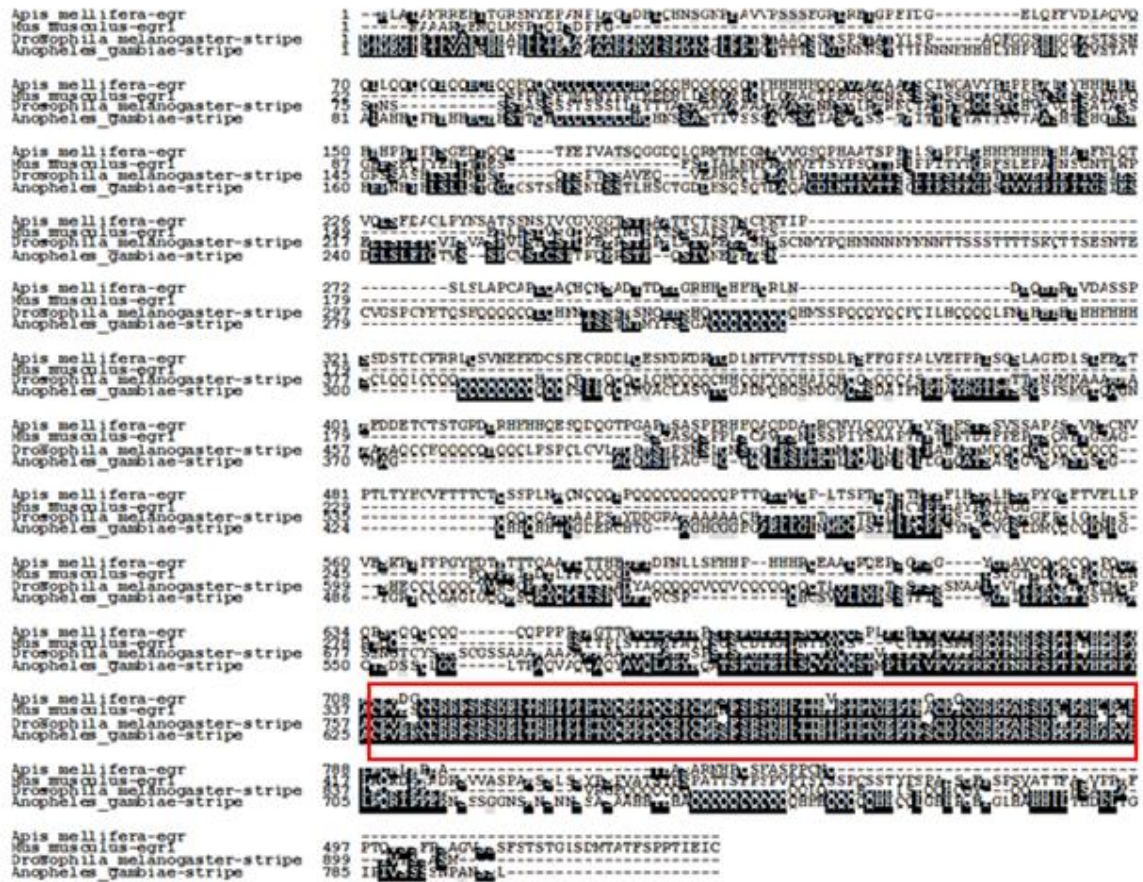


Figure 3.1. Alignment of *egr* with a vertebrate homolog in mouse (*Mus musculus*), and orthologs in fruit fly (*Drosophila melanogaster*) and mosquito (*Anopheles gambiae*). Conserved residues are highlighted in dark gray. The DNA-binding domain is outlined with a red box.

Figure 3.2

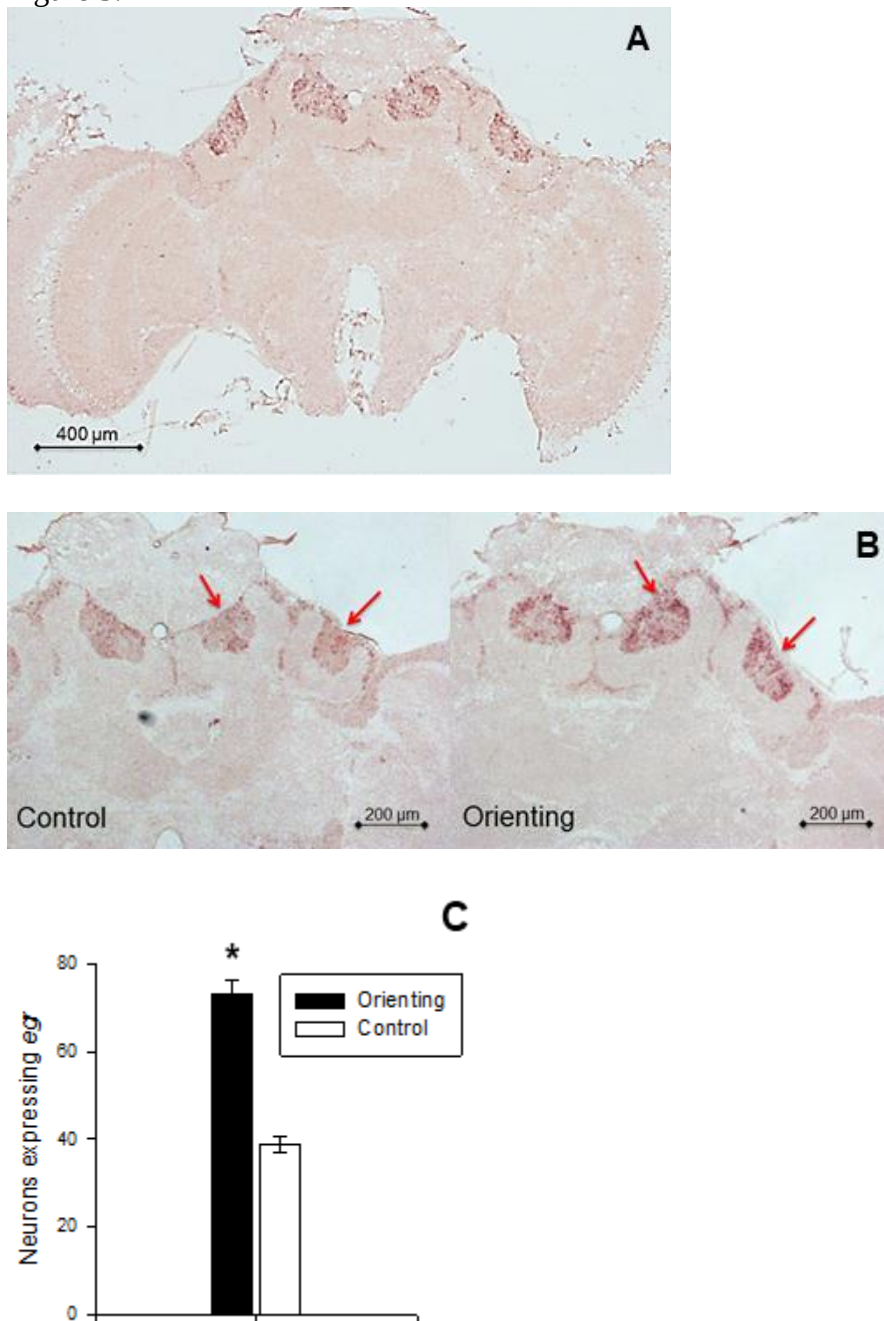


Figure 3.2.

A. Frontal sections of a brain from an orienting bee. Dark red staining indicates *egr* expression in mushroom body neuron somata. Staining was not observed in any other brain region. B. Mushroom bodies in frontal sections of brains from control and orienting bees. Dark red staining indicates *egr* expression in mushroom body neuron somata (red arrows). C. *Egr* was detectably expressed in a greater number of neurons in orienting bees than controls ($p < 0.01$).

Figure 3.3

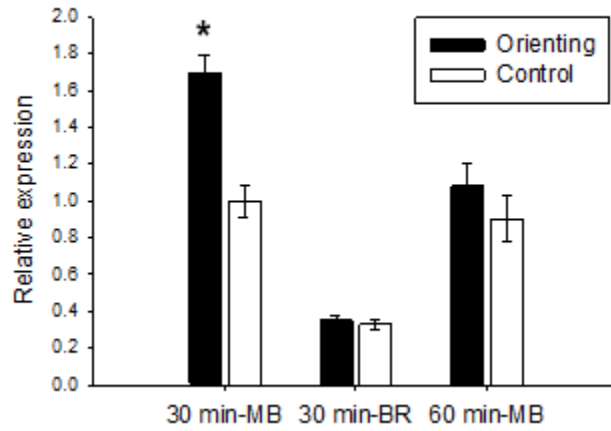


Figure 3.3. Expression of *egr* in the brains of orienting and control bees collected either 30 or 60 minutes post-collection, measured using qRT-PCR. MB denotes mushroom bodies, BR denotes all brain tissue other than mushroom bodies. *Egr* showed nearly 2-fold upregulation in mushroom bodies 30 minutes after orientation flight ($p < 0.0001$). *Egr* was not differentially expressed between orienting bees and controls in BR samples at 30 minutes, or in MB 60 minutes after orientation flight.

Figure 3.4

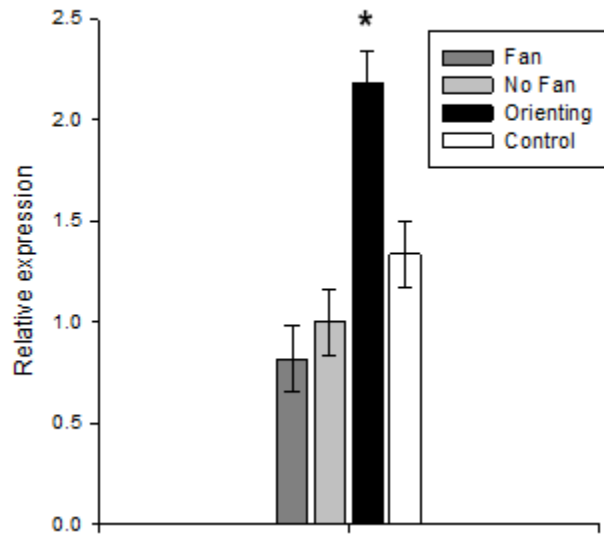


Figure 3.4. Test of Hypothesis 1: *Egr* is upregulated by exercise alone. Expression of *egr* in the mushroom bodies of bees vigorously fanning their wings for ~2 minutes to ventilate the hive, compared with orienting bees and controls. *Egr* was upregulated in response to flight ($p < 0.001$), but not fanning ($p = 0.859$).

Figure 3.5

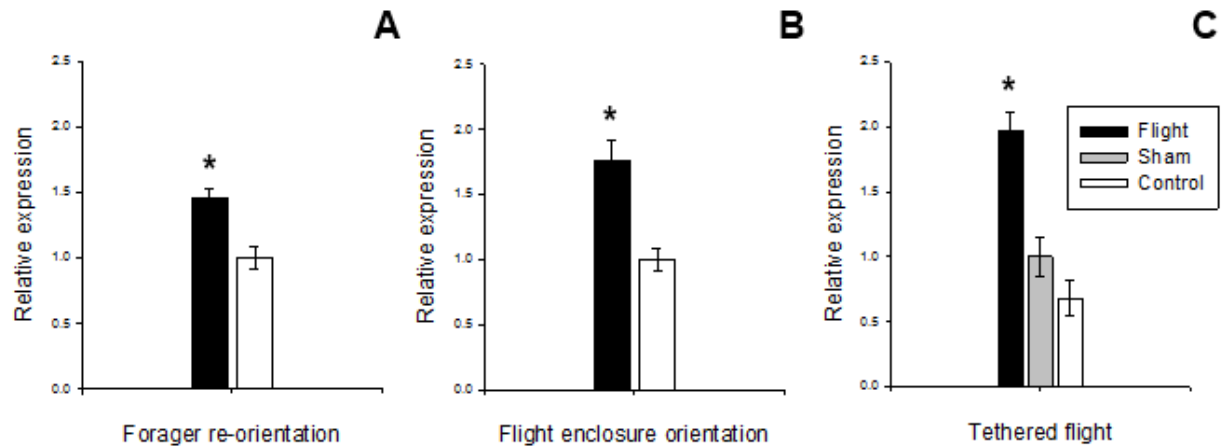


Figure 3.5.

A. Test of Hypothesis II: *Egr* is upregulated by motor learning associated with flight. Expression of *egr* in the mushroom bodies of re-orienting and control foragers. *Egr* showed upregulation in response to re-orientation by experienced foragers in a novel environment ($p < 0.0005$). B-C. Test of Hypothesis III: *Egr* is upregulated by exposure to specific visual cues. B. Expression of *egr* in the mushroom bodies of bees orienting in an indoor flight enclosure, compared with controls. *Egr* was upregulated in response to an orientation flight in this more deprived environment ($p < 0.0001$). C. Expression of *egr* in the mushroom bodies of tethered bees flying for two minutes in a white enclosure. Flight indicates bees that experienced flight while tethered. Sham bees were anesthetized and tethered, but not allowed to fly. Controls were simultaneously collected, unmanipulated bees. *Egr* was upregulated in response to flight ($p < 0.0001$).

Figure 3.6

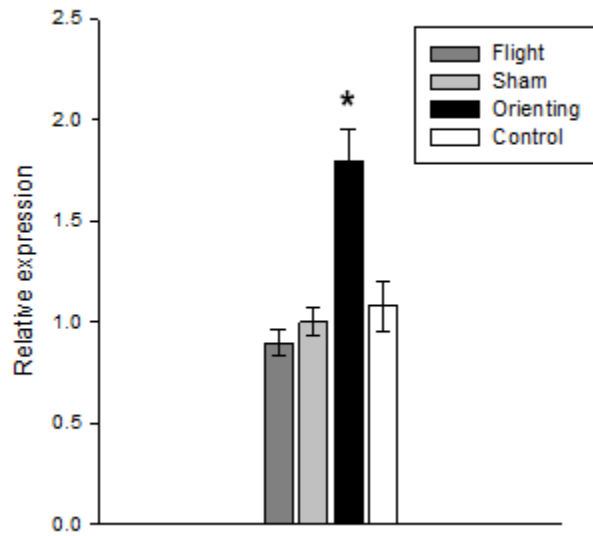


Figure 3.6. Test of Hypothesis IV: *Egr* is upregulated by exposure to environmental novelty. Expression of *egr* in the mushroom bodies of tethered bees flying for two minutes in a red light, compared with orienting bees and controls. *Egr* was upregulated in response to flight ($p < 0.005$), but not to tethered flight in red light ($p = 0.303$). Primers used to produce RNA probe for *in situ* hybridization, and for qRT-PCR.

Table 3.1

Hypothesis	Prediction	Result	Supported?
I. <i>Egr</i> is upregulated by exercise alone.	<i>Egr</i> will be upregulated by exercise in the absence of environmental novelty.	<i>Egr</i> was not upregulated in the mushroom bodies of bees after performing vigorous wing fanning inside the hive (Figure 3.4).	No
II. <i>Egr</i> is upregulated by motor learning associated with flight.	<i>Egr</i> will not be upregulated by re-orientation flight in foragers with prior flight experience.	<i>Egr</i> was upregulated in the mushroom bodies of foragers after a re-orientation flight (Figure 3.5A).	No
III. <i>Egr</i> is upregulated by exposure to visual cues.	<i>Egr</i> will not be upregulated by flight in environments devoid of visual cues.	<i>Egr</i> was upregulated in the mushroom bodies of bees after an orientation flight in deprived environments, and after flight in a white enclosure (Figures 3.5B,C).	No
IV. <i>Egr</i> is upregulated by exposure to environmental novelty.	<i>Egr</i> will not be upregulated by exercise when environmental novelty cannot be detected.	<i>Egr</i> was not upregulated in the mushroom bodies of bees after flight without visual information (Figure 3.6).	Yes

Table 3.1. Hypotheses tested to determine what aspects of the orientation flight experience are necessary for *egr* induction in the honey bee mushroom bodies

Table 3.2

Primers	Primer Name	Sequence 5'-3'
ISH probe	<i>egr</i> -ISH-F	TAA TAC GAC TCA CTA TAG GGC GCA AGT ACC CGA ATC GAC
	<i>egr</i> -ISH-R	GAT TTA GGT GAC ACT ATA GGC TTC TTC TCG TCG CTC CTC
qRT-PCR	<i>egr</i> -F	GCA AAC GGT GCA GCT CAG T
	<i>egr</i> -R	CCG CAT ACG ATC GAA TTC G
	<i>elf3-s8</i> -F	TGA GTG TCT GCT ATG GAT TGC AA
	<i>elf3-s8</i> -R	TCG CGG CTC GTG GTA AA
	<i>rp49</i> -F	GGG ACA ATA TTT GAT GCC CAA T
	<i>rp49</i> -R	CTT GAC ATT ATG TAC CAA AAC TTT TCT

Table 3.2. Primers used to produce RNA probe for *in situ* hybridization, and for qRT-PCR.

CHAPTER 4

MATURATIONAL REGULATION OF MUSHROOM BODY *EGR* EXPRESSION IN HONEY BEES

Abstract

Honey bees (*Apis mellifera*) learn to navigate in the environment surrounding the hive by performing orientation flights for several days before they begin to forage. Honey bee mushroom bodies, a paired region of the insect brain involved in learning, show both experience-expectant and experience-dependent changes in neuropil volume, coincident with the increased cognitive demands of orientation flights and foraging behavior. It also has been shown that treatment with pilocarpine, a muscarinic cholinergic receptor agonist, produces growth of the mushroom body neuropil comparable to that produced by maturation and foraging. My previous work found that orientation flights trigger in the mushroom bodies a rapid and transient upregulation of *egr*: *egr* is a homolog of *egr-1*, an immediate-early gene well known to respond to novel experience. *Egr-1* also exhibits higher basal levels in brain regions undergoing development or remodeling in vertebrates. I hypothesized that increases in basal *egr* mushroom body expression occurs in association with neuropil growth, and that these increases may be mediated by cholinergic signaling. Consistent with my hypothesis, I found that *egr* expression was upregulated in bees prepared to perform orientation flights and in foragers relative to hive bees engaged in brood care. Moreover, pilocarpine administration in young bees also upregulated *egr*, supporting the possibility of *egr* as a link between muscarinic signaling and neuropil growth.

1. Introduction

Honey bees (*Apis mellifera*) are an excellent model system for the study of neurobiology and behavior, and in particular the study of the modulation of neuroplasticity by maturation and experience. Honey bees exhibit temporal polyethism; workers age 1-3 weeks perform in-hive tasks such as brood care, while older workers forage outside the hive for resources such as nectar and pollen (Winston, 1987). Bees learn to navigate in the environment surrounding the hive by performing “orientation” flights for several days before they begin to forage. The transition from in-hive tasks to foraging involves several novel cognitive challenges. Initial orientation flights

and foraging trips represent the first significant exposure to visual stimuli and environmental odors, in contrast to the dark nest interior. In addition, orientation flights and foraging require spatial learning for successful navigation, while efficient foraging depends on learned recognition of preferred flowers (Menzel, 1990).

In honey bees the mushroom bodies, a paired region of the insect brain involved in learning, exhibit two phases of structural plasticity in parallel with these increased cognitive demands. Mushroom body neuropil volume increases in anticipation of foraging onset (Withers et al., 1993; Withers et al., 1995; Durst et al., 1994), coinciding with but not dependent on performance of orientation flights (Fahrbach et al., 1998). The mushroom bodies are also responsive to the experience of orientation flight; a single flight is associated with the remodeling of dendritic spines in the mushroom body calyces (Brandon and Coss, 1982). A second adult phase of growth in the honey bee mushroom bodies occurs in response to accumulated foraging experience. Prolonged foraging experience induces further expansion of the mushroom body neuropil (Farris et al., 2001; Ismail et al., 2006), increased dendritic branching and branch length in the visual input region of the calyx (Farris et al., 2001), and changes in microglomerular structure and density (Krofczik et al., 2008). Such neuroanatomical changes are thought to be the physical substrate for consolidation of memories (Fu and Zuo, 2011). Molecular processes underlying neuroanatomical change create a permissive state for growth, transduce endogenous and environmental signals, and provide the building blocks for new processes and synapses (Leslie and Nedivi, 2011). What molecular events are important for neural growth in aging individuals is a question of broad interest in the field of neuroscience, with implications for human health.

Previous studies have examined molecular correlates in the mushroom bodies of particular aspects of foraging experience (Sen Sarma et al., 2009; Sen Sarma et al., 2010; Kiya and Kubo, 2011), or of foraging experience itself (Wolschin et al., 2009; Lutz et al., 2012; Dobrin and Fahrbach, 2012). Other work has surveyed transcriptional changes across the whole brain that accompany the transition from in-hive to foraging behavior (Whitfield et al., 2003; Whitfield et al., 2006; Chandrasekaran et al., 2011; Ament et al., 2012). While these studies have identified classes of molecular changes that may be associated with structural plasticity, in most cases connections between specific pathways and neuropil growth have not yet been shown.

One exception to this is cholinergic signaling, which has been shown to promote structural plasticity in the mushroom bodies. Pilocarpine, a cholinergic muscarinic receptor agonist, is known to promote mushroom body neuropil growth in foragers (Ismail et al., 2006), and dendritic growth in the calyx of foragers and young bees (Dobrin et al., 2011). Cholinergic signaling is increased in foragers compared with hive bees (Shapira et al., 2001), suggesting that the neuroanatomical effects are biologically relevant. However, no connection has been established between muscarinic stimulation and downstream signaling that could contribute to neuropil growth.

One possible downstream response to muscarinic stimulation is transcription of *egr*, an insect homolog of the vertebrate immediate-early gene *egr-1*. In the previous chapter, I described my findings that expression of *egr* expression was enriched in mushroom bodies, and upregulated by exposure to environmental novelty during orientation flights (Chapter 3). The latter result suggests a functional homology between *egr* and vertebrate *egr-1*. Although I know of no previous work examining transcriptional regulation of *egr* in bees, experiments in vertebrate cell culture have repeatedly demonstrated that muscarinic receptor stimulation results in transient increases in expression of *egr-1* (Ebihara and Saffen, 1997; von der Kammer et al., 1998; Hirabayashi and Saffen, 2000; Rossler et al., 2008). Salani et al. further showed that *egr-1* expression was necessary for neurite outgrowth induced by muscarinic signaling (2009). *Egr-1* is believed to promote structural plasticity, not only through increased transcription in response to discrete events, but during periods of neural development (Knapska and Kaczmarek, 2004). *Egr-1* has been found in several species to be constitutively expressed at higher levels in areas of the brain undergoing development. For example, *egr-1* signaling in the visual cortex is higher during the critical period for establishment of ocular dominance patterns (Worley et al., 1991; McCormack et al., 1992; Kaplan et al., 1995; Herms et al., 1994), and in the forebrains of zebra finches during the sensitive period for learning their conspecific song (Jin and Clayton, 1997; Stripling et al., 2001).

I hypothesized that phases of structural plasticity in the mushroom bodies of honey bees, including growth mediated by acetylcholine (Ismail et al., 2006; Dobrin et al., 2011), are similarly accompanied by higher levels of *egr*. This hypothesis predicts that *egr* expression is upregulated in bees preparing to transition to foraging behavior, and in bees actively accumulating foraging experience, times when neuropil growth is occurring. If *egr* acts as a link

between cholinergic signaling and mushroom body neuropil growth, it is expected that muscarinic stimulation should also induce upregulation of *egr*. To test these predictions, I compared the mushroom body expression levels of same-aged bees at different stages of behavioral maturation, and in response to pilocarpine treatment.

2. Methods

2.1. Animals

Bees were obtained from apiaries maintained by the University of Illinois Bee Research Facility, Urbana, IL in April-August of 2012. Single-cohort colonies were created as in Robinson et al. (1989). Briefly, I placed a laying queen and ~3000 one-day-old workers (bees that had emerged as adults within the past 24 hours) into a small Styrofoam hive box with several honeycomb frames containing nectar and pollen. Day 1 for the experiment was therefore also day 1 of adult life for all focal bees in following experiments. On the day after assembly, colonies were moved outside. An additional frame with many uncapped cells containing young larvae was removed from a mature colony and, after any adult bees had been removed, transferred to the experimental colony. This allowed the easy identification (Robinson, 1987) of hive bees engaged in brood care (nursing) in subsequent experiments. In one experiment, one-day old bees were introduced to cages in groups of 15 and fed with a 50/50 honey and pollen mixture and water *ad libitum*. Cages were kept in a dark, humid incubator at 32 °C for up to 10 days.

2.2. Behavioral observations and collections

For all experiments, collected bees were flash-frozen in liquid nitrogen to preserve RNA, and then stored at -80 °C between all subsequent processing steps. Experiments were designed to compare *egr* expression in bees whose maturational state or treatment predicted varying levels of neuropil growth and muscarinic signaling.

2.2.1. Comparison among hive bees

Hive bees were collected on day 4. The colony was observed from 2 pm onward, to identify the onset of the peak time for orientation flights. For each of three trials performed with three different colonies, this time was between 3:30-4:00 PM. Bees that were seen leaving the

colony to perform orientation flights before this time were captured and eliminated. As the number of bees exiting the hive increased, several were collected as they crawled through the entrance and immediately collected. Thirty minutes later, while orientation flight activity was still intense, the frame of honeycomb containing cells with uncapped larvae was briefly removed from the colony, and several hive bees observed with their heads inside these cells were collected (Robinson, 1987). The age of the colony and the absence of previously observed flight or foraging activity suggested that bees collected at the entrance (“OR-ready”) were preparing to perform orientation flights. Bees collected with their heads inside cells containing larvae (“nurses”) during the peak of orientation flight activity were presumed to be less likely to perform an orientation flight that same day. In one trial, a second collection of nurses was made on day 8, at the same time of day and in the same manner as the first.

2.2.2. Age effects in caged bees

Collections of caged bees were performed every other day for 10 days, starting on day two. At approximately 2 PM on each day of collection, one cage was removed from the incubator, and the bees inside were immediately transferred to liquid nitrogen.

2.2.3. Comparison between hive bees and foragers

Collections of same-age nurses and foragers were performed for three trials (three separate colonies) at varying ages between day 8 and day 21. Colonies were observed between 3:30-4 pm on a warm day with clear weather. Bees returning to the hive with a load of pollen or an abdomen distended with nectar (confirmed by squeezing the abdomen and looking for regurgitated liquid) were identified as foragers and collected. Shortly thereafter, nurses were collected in the manner described above.

2.2.4. Comparisons among foragers

An additional collection of foragers was made from one colony on day 17. The colony was observed in the morning, beginning at 7:30 AM, when there was little flight activity. Several bees (“departing foragers”) were captured as they exited the hive, placed in individual cages, and returned to the hive for 30 minutes. Other bees (“returning foragers”) were allowed to depart, captured on their return with a pollen or nectar load as described above, placed in

individual cages, and returned to the hive for 30 minutes. Each bee was then collected and frozen at the end of its 30-minute interval. This collection procedure was repeated from 1:45-2:15 PM on the same day, when foraging activity was high, but high orientation flight activity was not apparent.

2.3. *Pilocarpine injections*

This experiment was repeated in two separate trials. Young (>24-hour) adult bees were collected after emergence and housed in groups of 100+ individuals in a large, ventilated container. Bees were kept in a dark, humid incubator at 32 °C and had access to a 50:50 honey/pollen mixture *ad libitum*. Several minutes before treatment, individual bees were anesthetized briefly on ice, and then immobilized on a mount of modeling clay. A small area of cuticle was removed from the median ocellus; 100 nL of insect saline (Fahrbach et al., 1995), or of pilocarpine HCl (Sigma) dissolved in saline, were then injected just under the cuticle using a 36-gauge needle on a Hamilton syringe. Each bee was then removed from the mount, placed in an individual cage, and placed in the incubator for 30 minutes before being flash-frozen in liquid nitrogen. Bees were mobile within 2-3 minutes of the time of injection, and locomotor behavior appeared normal at the time of collection.

2.4. *Molecular analyses*

qRT-PCR on mushroom body tissue was performed as described in Chapters 2 and 3. Mushroom bodies were isolated as described in that study after treatment of flash-frozen heads with RNAlater-ice (Ambion). RNA was extracted from isolated mushroom bodies or other brain regions using Picopure extraction kits (Arcturus). Abundance of transcript was analyzed with a SYBRgreen probe, quantified relative to a genomic DNA standard curve, and normalized to *eif-s8*, a constitutively expressed endogenous control gene used in previous studies (Alaux et al., 2009). One-factor and two-factor ANOVAs (for multiple trials) with Tukey's post-hoc tests were performed using PROC MIXED in SAS; a Pearson correlation test was performed in R.

3. Results

3.1. *Egr expression was increased in anticipation of orientation flights*

I investigated the expression of *egr* in the mushroom bodies of 5-day old nurses, compared with that in same-age hive bees exiting the hive to perform an orientation flight. *Egr* was significantly upregulated in two of three trials, as well as in a combined analysis of all trials (Figure 4.1A-D, $p < 0.0001$). This increased expression in anticipation of orientation flight was separate from the previously shown upregulation in *egr* caused by orientation, which was dependent on novel visual experience encountered during the flight (Chapter 3).

In trial 3, I examined *egr* expression in a second group of older nurses, collected four days later from the same colony. Pairwise comparison revealed no significant difference in *egr* expression between 4- and 8-day old nurses (Figure 4.1C, $p = 0.64$). This corresponds to *egr* expression levels I observed in the mushroom bodies of caged bees confined to a dark incubator for up to 10 days. In a single trial, *egr* expression trended upward over time but was not significantly different at any age examined (Figure 4.2, $p = 0.12$). These data do not exclude the possibility of a subtle effect of age, undetected in these limited experiments.

3.2. Egr expression was upregulated in response to muscarinic signaling, and constitutively in foragers

One-day old bees injected with pilocarpine, a cholinergic agonist specific to muscarinic receptors, exhibited increased *egr* expression relative to control bees (Figure 4.3A, $p < 0.0001$). The lowest concentration solution injected, 10^{-3} M pilocarpine, showed no effect compared with saline ($p = 0.99$), while 5-fold and 10-fold higher concentrations elicited expression levels significantly different from controls and from each other ($p < 0.005$). *Egr* expression was significantly correlated with concentration of injected pilocarpine (Figure 4.3B; $r_{\text{Pearson}} = 0.729$, $p < 0.0001$).

Comparison of *egr* expression in the mushroom bodies of hive bees engaged in nursing tasks and same-age foragers found significantly higher levels in foragers (Figure 4.4; $p < 0.0001$). This was true in every trial performed, regardless of the age of nurses and foragers examined (8-21 days), again emphasizing the influence of maturational state rather than age. *egr* expression in foragers was almost 3-fold higher than in nurses, a much greater increase than that seen in anticipation of orientation flights.

In a previous study, I observed *egr* upregulation to re-orientation flights performed by experienced foragers after relocation to a new area (Chapter 4.3). This suggested that in

foragers, as well as in bees naïve to flight, *egr* expression is responsive to environmental novelty. To test the possibility that a component of the increased expression observed in foragers in the present study was related to a specific experience encountered in the act of foraging, I compared *egr* expression in several groups of foragers with different recent experiences.

In bees, an increase in *egr* expression is observed 30 minutes after a stimulus, but disappears 60 minutes after the stimulus (Chapter 3); the same temporal dynamics are well-established for *egr-1* in several vertebrate species (Zangenehpour and Chaudhuri, 2002). *Egr* expression in response to specific stimuli should therefore reflect experiences encountered ~30 minutes prior. Bees captured while returning from the first foraging trip of the morning and collected 30 minutes later exhibited no increase in *egr* expression compared with bees captured while exiting the hive after a night of inactivity (Figure 4.5; $p=0.15$). The same was true of foragers captured in the afternoon (Figure 4.5; $p=0.15$), either while returning to the hive, or while exiting the hive after a probable pause in flight activity for behaviors such as unloading resources or resting (Winston, 1987). There was also no significant difference related to time of day ($p=0.20$). These results support the interpretation that increased *egr* levels observed in foragers are constitutive.

4. Discussion

I examined expression of *egr*, an insect immediate-early gene, in bees at stages of behavioral maturation known to predict varying levels of structural plasticity in the mushroom bodies. Mushroom body *egr* was higher in hive bees prepared for orientation flights and in foragers, compared with hive bees engaged in nursing tasks; *egr* was also upregulated in response to cholinergic signaling, which is enhanced in the mushroom bodies of foragers compared with hive bees (Shapira et al., 2001). These findings are consistent with the possibility that *egr* supports mushroom body neuropil growth associated with the transition to foraging (Withers et al., 1993; Withers et al., 1995; Durst et al., 1994), and with continued foraging experience (Farris et al., 2001; Ismail et al., 2006). A similar connection has been found in vertebrates between periods of rapid development in a brain region, and constitutively increased expression of *egr-1* in that region (Worley et al., 1991; McCormack et al., 1992; Kaplan et al., 1995; Herms et al., 1994; Jin and Clayton, 1997; Stripling et al., 2001). My findings support that idea that *egr* in honey bees, like *egr-1* in vertebrates, may initiate a cascade of transcription that

promotes the growth and remodeling of neuronal processes (Knapska and Kaczmarek, 2004; Clayton, 2000; Moorman et al., 2011).

Egr expression was moderately higher in hive bees in anticipation of orientation flight performance. Expression levels in nurses and caged bees over a range of ages and comparisons between nurses and same-age foragers suggested that maturational stage had a larger impact on regulation of *egr* than did age, although the results presented here do not exclude the possibility of an age effect. Brain expression levels of some genes differentially regulated in hive bees and foragers reflect maturational state, independent of age (Whitfield et al., 2003; Whitfield et al., 2006), while others respond to age, independent of behavior (Whitfield et al., 2006). Effects of maturation independent from age have been shown for mushroom body neuropil growth (Withers et al., 1993). Further investigation of *egr* expression in caged bees and bees confined to the hive could clarify the possible effect of age.

Increased levels of *egr* transcript in hive bees preparing for orientation flight might be related to exposure to light at the hive entrance. First exposure to light upregulates *egr-1* in the visual cortex of dark-reared young and adult vertebrates (Herms et al., 1994; Rosen et al., 1992; Kaplan et al., 1996; Mower and Kaplan, 2002), while continued exposure maintains but does not further increase expression levels. Orientation flights in hive bees, like flight activity in foragers, may be related to positive phototaxis (Ben-Shahar et al., 2003). Hive bees preparing for flight may crowd at the entrance long enough for the limited exposure to light to induce upregulation of *egr*. Further exposure encountered outside the hive might then be insufficient to further increase expression in the absence of sufficiently relevant stimuli, or visual experience paired with flight (Chapter 3). Future studies could continue to explore the relationship between *egr* expression in pre-flight hive bees and light exposure, perhaps by administering a light stimulus to hive bees in afternoon hours when orientation flights are likely and examining *egr* expression in bees exhibiting positive and negative phototaxis. By comparing expression in bees before and after a time interval that allows for transcription to take place, one might separate endogenous and light-induced effects on regulation of *egr*.

In this experiment, it was impossible to definitively determine the future behavior of sampled bees because RNA abundance measurements in the brain require destructive sampling. I therefore assumed that because I sampled bees engaged in nursing tasks during a time when many other bees were performing orientation flights, a majority of the sampled bees would have

been much less likely to also perform an orientation flight later that same day than those bees captured at the entrance. For some individuals, however, this assumption may have been incorrect. If the average expression level for nurses included some orientation-ready individuals with higher values, it is possible that such errors decreased the magnitude of the detected difference.

I observed higher levels of *egr* expression in foragers compared with nurses. This increased expression, unlike that observed in bees after an orientation flight (Chapter 3), appeared unrelated to specific experiences encountered on a single foraging trip. This is consistent with patterns of *egr* expression observed in previous microarray studies. *Egr* expression was previously found to be higher in the mushroom bodies of foragers than in one-day old bees, but not differentially regulated by duration of foraging experience (Lutz et al., 2012) or perception of distance while visiting a food source (Sen Sarma et al., 2010). *Egr* expression in foragers may reflect constitutively higher neuronal activity in the forager mushroom bodies. However, in both cat and zebra finch, higher *egr-1* expression during critical periods of development does not necessarily correspond to higher electrophysiological activity (Stripling et al., 2001; Kaplan et al., 1996). Instead, it may be related to molecular changes that produce a more permissive state for transcription.

My finding that pilocarpine, a muscarinic agonist, upregulates *egr* in the mushroom bodies of young bees suggests one possible pathway through which level of transcription might be modulated. Levels of acetylcholinesterase, the enzyme responsible for terminating cholinergic signaling by breaking down acetylcholine after its release, are lower in the mushroom bodies of foragers than in hive bees (Shapira et al., 2001). Thus, similar levels of acetylcholine release in hive bees and foragers could result in somewhat prolonged muscarinic signaling in foragers only. In vertebrate cell culture, muscarinic signaling acts at least in part through the IP₃ signaling pathway to upregulate *egr-1* (Ebihara and Saffen, 1997; von der Kammer et al., 1998; Rossler et al., 2008). In honey bees, several components of this pathway are enriched in the mushroom bodies (Kamikouchi et al., 1998; Kamikouchi et al., 2000). In addition, two genes that contribute to the metabolism of inositol, *inos* and *IP3K*, are upregulated in the brains of foragers compared with hive bees (Lutz et al., 2012; Whitfield et al., 2003; Kucharski and Maleszka, 2002). My data, interpreted in the context of these findings, do not exclude the likely possibility that other signaling systems also contribute, as they do in

vertebrates (Beckmann and Wilce, 1997), to the regulation of *egr*. They do, however, present a coherent picture of how muscarinic signaling, acting through IP₃ signaling, could upregulate *egr* and promote structural plasticity specifically in the forager mushroom bodies.

Past studies examining the relationship between *egr-1* and critical periods of development in vertebrates have focused on young and juvenile animals. An investigation of *egr-1* in the hippocampus of aging rats revealed a decline in expression levels, accompanied by morphological signs of senescence (Desjardins, 1997). In honey bees, I have found increased *egr* expression in adulthood, in association with maturational states that predict renewed structural plasticity. Further investigation of the mechanisms that drive this relationship in honey bees may hold promise for the reinstatement of neuroplasticity in adulthood and aging in other species, including humans.

Works Cited

Alaux C, Le Conte Y, Adams HA, Rodriguez-Zas S, Grozinger CM, Sinha S, Robinson GE (2009) Regulation of brain gene expression in honey bees by brood pheromone. *Genes Brain Behav* 8:309-319.

Ament SA, Blatti CA, Alaux C, Wheeler MM, Toth AL, Le Conte Y, Hunt GJ, Guzman-Novoa E, Degrandi-Hoffman G, Uribe-Rubio JL, Amdam GV, Page RE, Jr, Rodriguez-Zas SL, Robinson GE, Sinha S (2012) New meta-analysis tools reveal common transcriptional regulatory basis for multiple determinants of behavior. *Proc Natl Acad Sci U S A* 109:E1801-10.

Beckmann AM, Wilce PA (1997) Egr transcription factors in the nervous system. *Neurochem Int* 31:477-510; discussion 517-6.

Ben-Shahar Y, Leung HT, Pak WL, Sokolowski MB, Robinson GE (2003) cGMP-dependent changes in phototaxis: A possible role for the foraging gene in honey bee division of labor. *J Exp Biol* 206:2507-2515.

Brandon JG, Coss RG (1982) Rapid dendritic spine stem shortening during one-trial learning: The honeybee's first orientation flight. *Brain Res* 252:51-61.

Capaldi EA, Dyer FC (1999) The role of orientation flights on homing performance in honeybees. *J Exp Biol* 202:1655-1666.

Chandrasekaran S, Ament SA, Eddy JA, Rodriguez-Zas SL, Schatz BR, Price ND, Robinson GE (2011) Behavior-specific changes in transcriptional modules lead to distinct and predictable neurogenomic states. *Proc Natl Acad Sci U S A* 108:18020-18025.

Clayton DF (2000) The genomic action potential. *Neurobiology of Learning and Memory* 74:185-216.

Desjardins S, Mayo W, Vallee M, Hancock D, Le Moal M, Simon H, Abrous DN (1997) Effect of Aging on the Basal Expression of c-Fos, c-Jun, and Egr-1 proteins in the hippocampus. *Neurobiol Aging* 18:37-44.

Dobrin SE, Fahrbach SE (2012) Rho GTPase activity in the honey bee mushroom bodies is correlated with age and foraging experience. *J Insect Physiol* 58:228-234.

Dobrin SE, Herlihy JD, Robinson GE, Fahrbach SE (2011) Muscarinic regulation of Kenyon cell dendritic arborizations in adult worker honey bees. *Arthropod Struct Dev* 40:409-419.

Durst C, Eichmüller S, Menzel R (1994) Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. *Behavioral and Neural Biology* 62:259-263.

Ebihara T, Saffen D (1997) Muscarinic acetylcholine receptor-mediated induction of *zif268* mRNA in PC12D cells requires protein kinase C and the influx of extracellular calcium. *J Neurochem* 68:1001-1010.

Fahrbach SE, Giray T, Robinson GE (1995) Volume changes in the mushroom bodies of adult honey bee queens. *Neurobiol Learn Mem* 63:181-191.

Fahrbach SE, Moore D, Capaldi EA, Farris SM, Robinson GE (1998) Experience-expectant plasticity in the mushroom bodies of the honeybee. *Learn Mem* 5:115-123.

Farris SM, Robinson GE, Fahrbach SE (2001) Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. *J Neurosci* 21:6395-6404.

Fu M, Zuo Y (2011) Experience-dependent structural plasticity in the cortex. *Trends Neurosci* 34:177-187.

Hermes J, Zurmohle U, Schlingensiepen R, Brysch W, Schlingensiepen KH (1994) Developmental expression of the transcription factor *zif268* in rat brain. *Neurosci Lett* 165:171-174.

Hirabayashi T, Saffen D (2000) M1 muscarinic acetylcholine receptors activate *zif268* gene expression via small G-protein rho-dependent and lambda-independent pathways in PC12D cells. *Eur J Biochem* 267:2525-2532.

Ismail N, Robinson GE, Fahrbach SE (2006) Stimulation of muscarinic receptors mimics experience-dependent plasticity in the honey bee brain. *Proc Natl Acad Sci U S A* 103:207-211.

Jin H, Clayton DF (1997) Localized changes in immediate-early gene regulation during sensory and motor learning in zebra finches. *Neuron* 19:1049-1059.

- Kamikouchi A, Takeuchi H, Sawata M, Natori S, Kubo T (2000) Concentrated expression of Ca²⁺/calmodulin-dependent protein kinase II and protein kinase C in the mushroom bodies of the brain of the honeybee *Apis mellifera* L. *J Comp Neurol* 417:501-510.
- Kamikouchi A, Takeuchi H, Sawata M, Ohashi K, Natori S, Kubo T (1998) Preferential expression of the gene for a putative inositol 1,4,5-trisphosphate receptor homologue in the mushroom bodies of the brain of the worker honeybee, *Apis mellifera* L. *Biochem Biophys Res Commun* 242:181-186.
- Kaplan IV, Guo Y, Mower GD (1996) Immediate early gene expression in cat visual cortex during and after the critical period: Differences between EGR-1 and fos proteins. *Brain Res Mol Brain Res* 36:12-22.
- Kaplan IV, Guo Y, Mower GD (1995) Developmental expression of the immediate early gene EGR-1 mirrors the critical period in cat visual cortex. *Brain Res Dev Brain Res* 90:174-179.
- Kiya T, Kubo T (2011) Dance type and flight parameters are associated with different mushroom body neural activities in worker honeybee brains. *PLoS One* 6:e19301.
- Knapska E, Kaczmarek L (2004) A gene for neuronal plasticity in the mammalian brain: *Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK*? *Prog Neurobiol* 74:183-211.
- Krofczik S, Khojasteh U, de Ibarra NH, Menzel R (2008) Adaptation of microglomerular complexes in the honeybee mushroom body lip to manipulations of behavioral maturation and sensory experience. *Dev Neurobiol* 68:1007-1017.
- Kucharski R, Maleszka R (2002) Molecular profiling of behavioural development: Differential expression of mRNAs for inositol 1,4,5-trisphosphate 3-kinase isoforms in naive and experienced honeybees (*Apis mellifera*). *Mol Brain Res* 99:92-101.
- Leslie JH, Nedivi E (2011) Activity-regulated genes as mediators of neural circuit plasticity. *Prog Neurobiol (England)* 94:223-237.
- Lutz CC, Rodriguez-Zas SL, Fahrbach SE, Robinson GE (2012) Transcriptional response to foraging experience in the honey bee mushroom bodies. *Dev Neurobiol (United States)* 72:153-166.
- McCormack MA, Rosen KM, Villa-Komaroff L, Mower GD (1992) Changes in immediate early gene expression during postnatal development of cat cortex and cerebellum. *Brain Res Mol Brain Res* 12:215-223.
- Menzel R (1990) Learning, memory, and "cognition" in honey bees. In: *Neurobiology of comparative cognition* (Kesner RP, Olton DS, eds), pp237-292. Lawrence Erlbaum Associates, Inc.

- Moorman S, Mello CV, Bolhuis JJ (2011) From songs to synapses: Molecular mechanisms of birdsong memory. molecular mechanisms of auditory learning in songbirds involve immediate early genes, including zenk and arc, the ERK/MAPK pathway and synapsins. *Bioessays* 33:377-385.
- Mower GD, Kaplan IV (2002) Immediate early gene expression in the visual cortex of normal and dark reared cats: Differences between fos and egr-1. *Brain Res Mol Brain Res* 105:157-160.
- Robinson GE (1987) Regulation of honey bee age polyethism by juvenile hormone. *Behav Ecol Sociobiol* 20:329-338.
- Rosen KM, McCormack MA, Villa-Komaroff L, Mower GD (1992) Brief visual experience induces immediate early gene expression in the cat visual cortex. *Proc Natl Acad Sci U S A* 89:5437-5441.
- Rossler OG, Henss I, Thiel G (2008) Transcriptional response to muscarinic acetylcholine receptor stimulation: Regulation of egr-1 biosynthesis by ERK, elk-1, MKP-1, and calcineurin in carbachol-stimulated human neuroblastoma cells. *Arch Biochem Biophys* 470:93-102.
- Salani M, Anelli T, Tocco GA, Lucarini E, Mozzetta C, Poiana G, Tata AM, Biagioni S (2009) Acetylcholine-induced neuronal differentiation: Muscarinic receptor activation regulates EGR-1 and REST expression in neuroblastoma cells. *J Neurochem* 108:821-834.
- Sen Sarma M, Rodriguez-Zas SL, Hong F, Zhong S, Robinson GE (2009) Transcriptomic profiling of central nervous system regions in three species of honey bee during dance communication behavior. *PLoS One* 4:e6408.
- Sen Sarma M, Rodriguez-Zas SL, Gernat T, Nguyen T, Newman T, Robinson GE (2010) Distance-responsive genes found in dancing honey bees. *Genes Brain Behav* 9:825-830.
- Shapira M, Thompson CK, Soreq H, Robinson GE (2001) Changes in neuronal acetylcholinesterase gene expression and division of labor in honey bee colonies. *J Mol Neurosci* 17:1-12.
- Stripling R, Kruse AA, Clayton DF (2001) Development of song responses in the zebra finch caudomedial neostriatum: Role of genomic and electrophysiological activities. *J Neurobiol* 48:163-180.
- von der Kammer H, Mayhaus M, Albrecht C, Enderich J, Wegner M, Nitsch RM (1998) Muscarinic acetylcholine receptors activate expression of the EGR gene family of transcription factors. *J Biol Chem* 273:14538-14544.
- Whitfield CW, Cziko AM, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. *Science* 302:296-299.

Whitfield CW, Ben-Shahar Y, Brillet C, Leoncini I, Crauser D, Leconte Y, Rodriguez-Zas S, Robinson GE (2006) Genomic dissection of behavioral maturation in the honey bee. *Proc Natl Acad Sci U S A* 103:16068-16075.

Winston ML (1987) *The biology of the honey bee*. Cambridge, MA: Harvard University Press.

Withers GS, Fahrbach SE, Robinson GE (1995) Effects of experience and juvenile hormone on the organization of the mushroom bodies of honey bees. *J Neurobiol* 26:130-144.

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. *Nature* 364:238-240.

Wolschin F, Munch D, Amdam GV (2009) Structural and proteomic analyses reveal regional brain differences during honeybee aging. *J Exp Biol (England)* 212:4027-4032.

Worley PF, Christy BA, Nakabeppu Y, Bhat RV, Cole AJ, Baraban JM (1991) Constitutive expression of *zif268* in neocortex is regulated by synaptic activity. *Proc Natl Acad Sci U S A* 88:5106-5110.

Zangenehpour S, Chaudhuri A (2002) Differential induction and decay curves of *c-fos* and *zif268* revealed through dual activity maps. *Brain Res Mol Brain Res* 109:221-225.

Figures

Figure 4.1. Expression of *egr* in the MBs of 4- and 8-day old hive bees engaged in brood care (nurse), or exiting the hive entrance for orientation flight (OR-ready). Data shown represent individual trials (A-C) and pooled data (D). Expression values for individual trials were normalized to average expression in 4-day nurses. A. *Egr* was upregulated in OR-ready bees compared with nurses ($p < 0.01$). N=6-8. B. *Egr* was not significantly upregulated, but was higher, in OR-ready bees than in nurses. N=8. C. *Egr* was significantly upregulated in OR-ready bees, compared with 5- or 8-day old nurses ($p < 0.005$). N=13-15. D. *Egr* was upregulated in OR-ready bees ($p < 0.0001$). Effect of trial was not significant.

Figure 4.2. Basal expression of *egr* in the MBs of caged bees at two, four, six, eight and 10 days of age. *Egr* was not differentially regulated by age in socially isolated bees ($p = 0.12$). N=6-8.

Figure 4.3. Expression of *egr* in the MBs of one-day-old bees injected with either saline or varying concentrations of pilocarpine in saline. Values are pooled data from two trials; in each trial, N=6-8. Effect of trial was not significant.

A. *Egr* was upregulated by pilocarpine ($p < 0.0001$).

B. *Egr* expression was significantly correlated with pilocarpine concentration ($r_{\text{Pearson}} = 0.729$, $p < 0.0001$).

Figure 4.4. Expression of *egr* in the MBs of hive bees engaged in brood care (nurse), or returning to hive with a pollen or nectar load (forager). *Egr* was upregulated in foragers ($p < 0.0001$). Values are pooled data from three trials; in each trial, N=7-8. Effect of trial was not significant.

Figure 4.5. Expression of *egr* in the MBs of foragers departing or returning to the hive. *Egr* was not differentially regulated by recent behavior ($p = .15$) or by time of day ($p = 0.20$).

Figure 4.1

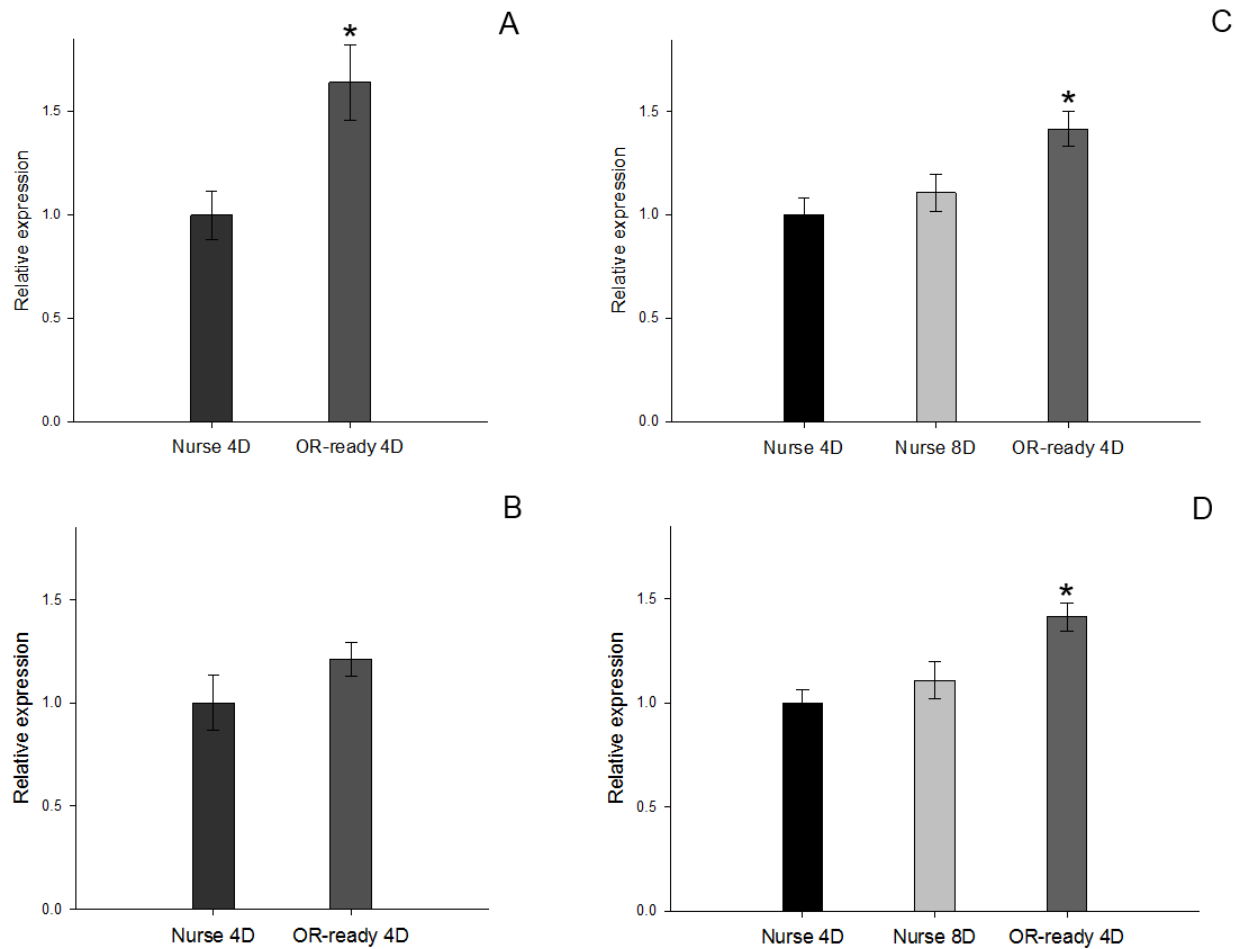


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Figure 4.2

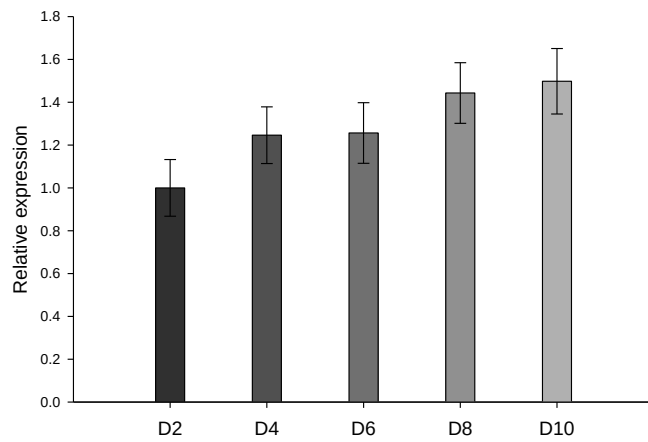


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Figure 4.3

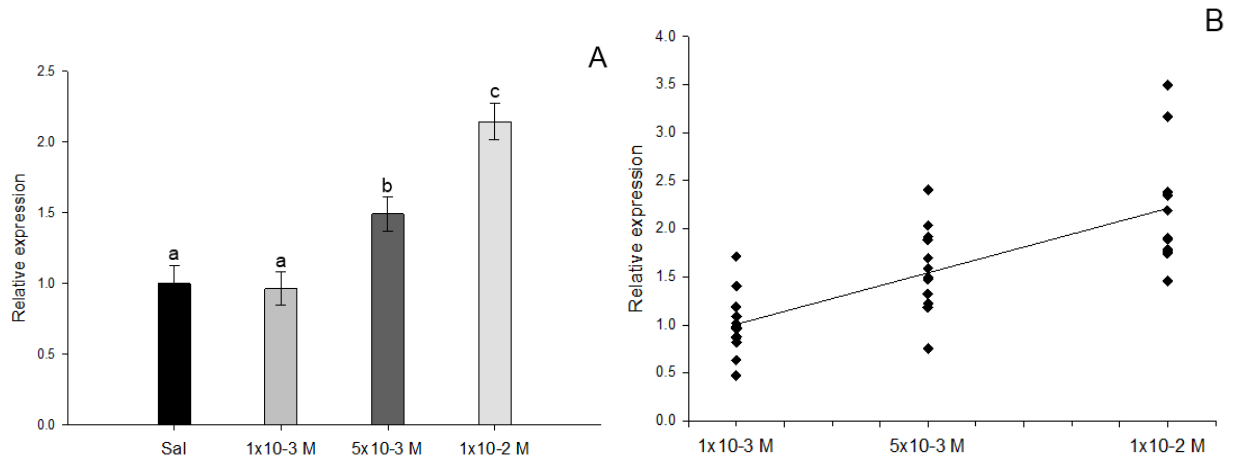


Figure 4.3. Expression of *egr* in the MBs of one-day-old bees injected with either saline or varying concentrations of pilocarpine in saline. Values are pooled data from two trials; in each trial, N=6-8. Effect of trial was not significant. A. *Egr* was upregulated by pilocarpine ($p < 0.0001$). B. *Egr* expression was significantly correlated with pilocarpine concentration ($r_{\text{Pearson}} = 0.729$, $p < 0.0001$).

Figure 4.4

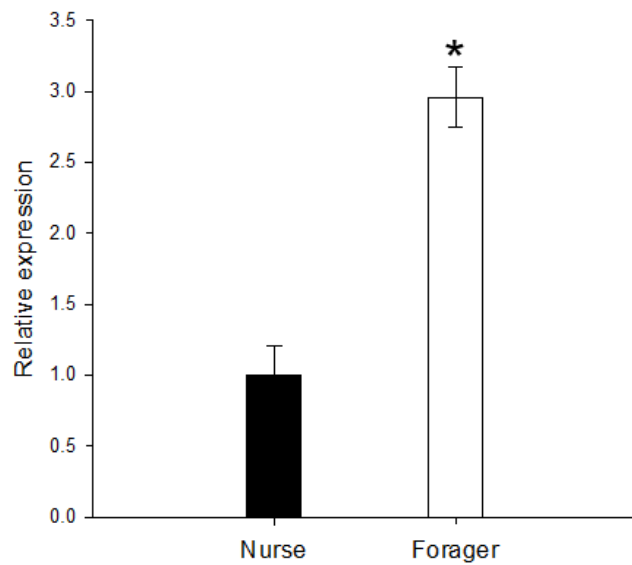


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Figure 4.5

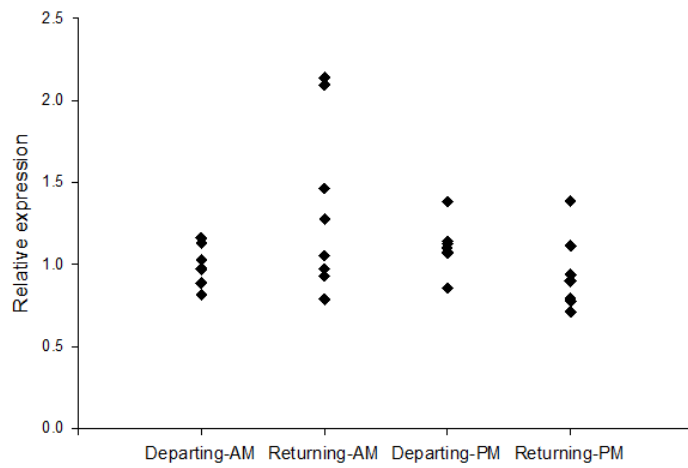


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CHAPTER 5

CONCLUSION

A detailed understanding of changes in gene expression that support neuroplasticity could eventually lead to treatments that improve recovery after brain injury or stroke. In addition, exploration of how behaviors, particularly those performed in an ecological setting, relate to gene expression and resulting neuroplasticity can suggest less invasive behavioral therapies to facilitate recovery. In Chapter 1, I discussed the usefulness of social hymenopterans, especially honey bees, to the study of adult neuroplasticity. Structural plasticity in the mushroom bodies that occurs late in adult life has been documented in several species including honey bees, coincident with cognitive demands related to behavioral maturation. A rich variety of complex behaviors, ease of manipulability, access to annotated genomic information, and a long history of neurobiological and ethological research have made honey bees an excellent model organism for the study of molecular neuroscience. In this thesis, I investigated the molecular mechanisms supporting several phases of adult structural plasticity in the honey bee brain.

Honey bee workers exhibit temporal polyethism; young workers perform tasks inside the hive, while older workers forage for resources outside the hive. Young pre-foragers prepare for this task by performing orientation flights near the hive, during which they begin to learn navigational cues such as the appearance of the hive, position of landmarks, and the movement of the sun. Foragers must learn to navigate between the hive and floral locations that may be up to miles away, as well as form associative memories for preferred floral sources. Honey bee mushroom bodies, a paired region of the insect brain involved in learning, show both experience-expectant and experience-dependent changes in neuropil volume. Treatment with a muscarinic cholinergic receptor agonist produces growth of the mushroom body neuropil comparable to that produced by maturation and foraging. I examined gene expression in the mushroom bodies of honey bees in response to two behaviors (orientation flights and foraging) associated with phases of structural plasticity, in order to identify molecular processes supporting these phases of growth. My results give new insight into the temporal dynamics of plasticity-related gene expression in the adult honey bee. In addition, they support conservation of plasticity-related

gene function between vertebrates and honey bees, and provide a basis for work to better understand the functional roles of these genes in both insects and vertebrates.

I began my thesis work with a broad survey of temporal dynamics of molecular processes associated with experience-dependent plasticity; this work was described in Chapter 2. To discover molecular processes associated with foraging experience, I performed a time-course microarray study to examine gene expression changes in the mushroom bodies as a function of days foraged. Clustering analyses suggested a natural division of samples into two groups, foragers with less or more experience; this division corresponds to the timing of the previously observed neuropil expansion. Functional analysis suggests that these genes could be involved in processes supporting neuropil growth. Included in this group were several genes involved in signal transduction or protein kinase activity, both of which are functional groups related to plasticity in other organisms. These results suggest that many foraging-related changes in mushroom body gene expression are involved in promoting or directing neuropil growth, or possibly in the stabilization of new dendritic processes.

I also used weighted gene co-expression network analysis to examine potential patterns of coregulation in both 1-day-old bees and foragers. Results of this comparison suggest that similar sets of genes could mediate some aspects of two phases of mushroom body neuropil growth in honey bees that have been documented, the early developmental phase in 1-day-old bees and the later experience-dependent phase. A notable exception was a group of protein folding genes co-expressed in foragers, but not in younger bees: expression of these genes was significantly correlated with amount of foraging experience. Upregulation of these genes in the mushroom bodies may be necessary to prevent or delay neurological aging and facilitate continued neuropil growth.

In Chapter 3, I focused in on a specific and important aspect of flight behaviors: spatial learning. This focus had particular relevance due to the currently dearth of information on the neural and molecular basis of insect spatial learning. To begin to address this lack in honey bees, I examined immediate early gene (IEG) expression in the brains of orienting bees. I found that *egr*, an insect homolog of *egr-1*, is rapidly and transiently upregulated in the mushroom bodies in response to orientation; this is the first example of an *egr-1* homolog acting as a learning-related IEG in an insect. Further experiments suggest that visual environmental novelty, rather than other aspects of the orientation flight, stimulates *egr* upregulation.

Despite many years of study, the functional relationship between *egr-1* and learning is still unknown. My demonstration of conservation of *egr*-related pathways in experience-dependent plasticity lays the groundwork for new studies of the relationship between *egr* function and learning. My results also implicate the mushroom bodies in spatial learning. When interpreted in conjunction with prior work, this points to an intriguing, though untested, hypothesis that the neural circuits involved in spatial learning may vary substantially between species that rely heavily on navigation, and those that do not.

In Chapter 4, I continued to investigate the role in honey bees of a specific gene, *egr*, while making connections back to the experience-expectant and -dependent growth that were the focus of Chapters 1 and 2. *Egr-1* exhibits higher basal levels in brain regions undergoing development or remodeling in vertebrates; I hypothesized that increases in basal *egr* mushroom body expression occur in association with neuropil growth, and that these increases may be mediated by cholinergic signaling. I found that *egr* expression was upregulated in bees prepared to perform orientation flights and in foragers relative to hive bees engaged in brood care, consistent with the hypothesis that *egr* upregulation facilitates neuropil growth. Pilocarpine administration in young bees also upregulated *egr*, supporting the possibility that signaling via *egr* serves as a link between muscarinic stimulation and neuropil growth.

As a whole, my thesis work suggests the importance of dynamic regulation of a single putative transcription factor, *egr*, as well as groups of genes working together to promote neuroplasticity. Some of these functional groups of genes are involved in several distinct phases of structural change, while others may be specific to maintenance of plasticity in the face of approaching senescence. Future experimental and bioinformatic work could uncover a connection between an altered regulatory state created by the changes in *egr* expression observed in Chapters 3 and 4, and the long-term expression dynamics found in Chapter 2. Further studies could also examine the roles of specific molecular processes in promoting neuropil growth. More complete understanding of how individual pathways contribute to structural plasticity could lead to development of targeted therapies that permit more efficient and successful learning or re-learning in adults, while avoiding the undesirable consequences of excessive plasticity observed in some developmental disorders. Such studies in the honey bee may ultimately enable us to control not only plasticity, but how plasticity and stability balance each other to facilitate cognition.