Metamorphosis and Adult Development of the Mushroom Bodies of the Red Flour Beetle, *Tribolium castaneum*

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**ABSTRACT:** The insect mushroom bodies play important roles in a number of higher processing functions such as sensory integration, higher level olfactory processing, and spatial and associative learning and memory. These functions have been established through studies in a handful of tractable model systems, of which only the fruit fly *Drosophila melanogaster* has been readily amenable to genetic manipulations. The red flour beetle *Tribolium castaneum* has a sequenced genome and has been subject to the development of molecular tools for the ready manipulation of gene expression; however, little is known about the development and organization of the mushroom bodies of this insect. The present account bridges this gap by demonstrating that the organization of the *Tribolium* mushroom bodies is strikingly like that of the fruit fly, with the significant exception that the timeline of neurogenesis is shifted so that the last population of Kenyon cells is born entirely after adult eclosion. *Tribolium* Kenyon cells are generated by two large neuroblasts per hemisphere and segregate into an early-born δ lobe subpopulation followed by clear homologs of the *Drosophila* γ, α′/β′ and α/β lobe subpopulations, with the larval-born cohorts undergoing dendritic reorganization during metamorphosis. BrdU labeling and immunohistochemical staining also reveal that a proportion of individual *Tribolium* have variable numbers of mushroom body neuroblasts. If heritable, this variation represents a unique opportunity for further studies of the genetic control of brain region size through the control of neuroblast number and cell cycle dynamics.

**Keywords:** evolution; olfaction; neuroblast; plasticity; pruning

**INTRODUCTION**

A relationship between the insect mushroom bodies and higher cognitive functions was first posited by (Dujardin, 1850), who noted that the largest mushroom bodies were to be found in the brains of the social Hymenoptera. In the intervening decades, a wealth of studies have revealed the importance of the mushroom bodies in complex behaviors such as sensory integration (Schildberger, 1984; Li and Strausfeld, 1997, 1999), place memory and motor control (Mizunami et al., 1993), higher-level olfactory processing (Laurent and Naramghi, 1994; Perez-Orive et al., 2002; Cassenae and Laurent, 2007), and associative and context-dependent learning and memory (Liu et al., 1999; for reviews see Waddell and Quinn, 2001; Heisenberg, 2003). Using the powerful tools for the manipulation of gene expression that are available in the fruit fly *Drosophila melanogaster*, it has also been possible to elucidate the molecular pathways for memory formation and their localization within subpopulations of mushroom body neurons (Zars et al., 2000; Pascual and Prétat, 2001; McGuire et al., 2003; Krashes et al., 2007; Schwaerzel et al., 2007; Thum et al., 2007).

Mushroom body intrinsic neurons, called Kenyon cells, undergo a dramatic reorganization during
metamorphosis in Drosophila, and display adult plasticity of axonal and dendritic outgrowth that is associated with behavioral experience in both Drosophila and the honey bee Apis mellifera (Technau and Heisenberg, 1982; Technau, 1984; Armstrong et al., 1998; Lee et al., 1999; Farris et al., 2001). Another form of neural plasticity, adult neurogenesis, is of great interest in the vertebrate literature because of the possibility that newborn neurons mediate higher cognitive processes such as learning, and the potential for neural stem cells to be utilized in a therapeutic context (Van Praag et al., 2002, 2005; Aimone et al., 2006; Bruel-Jungerman et al., 2006; Sohur et al., 2006; Okano and Sawamoto, 2008). In this area, however, Drosophila has not been a useful model as the available evidence suggests that the mushroom body neuroblasts do not persist beyond adult eclosion (Technau and Heisenberg, 1982; Technau, 1984). Adult neurogenesis is present in the mushroom bodies of crickets (Acheta domesticus), and neuron production dynamics have been linked to environmental enrichment, olfactory stimulation, and performance on olfactory learning tasks in both associative and operant contexts (Scotto Lomassese et al., 2000, 2002, 2003; Cayre et al., 2007). Tools for the manipulation of gene expression are not available for the cricket, however, so although neurogenesis in this insect has been shown to be under the control of both external and internal factors (Cayre et al., 1997a,b, 2001, 2005), it has not been possible to gain more detailed insight into the regulatory pathways that mediate adult neurogenesis.

The red flour beetle Tribolium castaneum is a stored products pest that has recently proven valuable as a point of comparison with Drosophila in studies of the genetic control of body plan organization and the specification of appendage identity (DeCamillis et al., 2003; Schröder, 2003; Wheeler et al., 2003; Copf et al., 2004; Tomoyasu et al., 2005; van der Zee et al., 2005; Choe et al., 2006; Choe and Brown, 2007). Like Drosophila, Tribolium is amenable to genetic manipulation through germ line transformation, transgene expression vectors and RNAi; the genome has also been sequenced and assembled into linkage maps that are available online via Beetlebase (Browne and Scholtz, 1999; Lorenzen et al., 2002, 2003, 2005; Wang et al., 2007). Unlike the fruit fly and similar to the cricket, the mushroom body neuroblasts of beetles persist into adulthood and undergo active neurogenesis as indicated by BrdU incorporation (Cayre et al., 1996). Little else is known about the mushroom bodies of Tribolium; however, mushroom body morphology has been described in detail for only one family of beetles (the Scarabaeidae; Larsson et al., 2004; Farris and Roberts, 2005) despite the fact that the beetles (Coleoptera) are the most speciose taxon on Earth.

The present account of Tribolium mushroom body structure and development is intended to put this species at the forefront of comparative studies that will lay the groundwork for studies of the genetic regulation and experimental manipulation of adult neurogenesis. Immunohistochemistry and BrdU labeling reveals a mushroom body organization much like that of Drosophila but with key differences in cellular composition and developmental events. The mushroom bodies undergo significant remodeling of dendritic structures but not of axons during metamorphosis, and continuous proliferation of mushroom body neuroblasts are observed until nearly 3-months post-eclosion. An unexpected result of this study is that unlike in Drosophila (Ito and Hotta, 1992; Ito et al., 1997), neuroblast number is variable across individuals and appears to impact mushroom body size. If the variation be heritable, the Tribolium model system will be uniquely suited to future studies of the regulation of neuroblast number and cell division dynamics, the effects of different sized populations of intrinsic neurons on afferent and efferent connections, and the relationship between mushroom body structure and behavioral output.

METHODS

Insects

Tribolium castaneum beetles were purchased from Carolina Biological Supply Company (Burlington, NC) and kept in an incubator at 28°C, with a 12:12 light:dark cycle. Prepared Tribolium medium was purchased from the same company and was composed of four parts white flour, four parts whole wheat flour, and one part brewer’s yeast.

Last instar larvae were identified and selected for immunostaining according to their body size, which was the largest among all of the larvae in the colony. Pre-pupae were selected from the media based on their large size and restricted movement and kept separately in petri dishes until pupation. Pre-pupae were monitored daily so that upon metamorphosis, the new pupae were moved to a separate dish and marked as Day 0. Different ages of pupae were determined primarily by the time from pupation, but also by the degree of eye pigment darkening.

Tissue Dissection and Fixation

All dissections were done in insect physiological saline (O’Shea and Adams, 1981) after chilling the insects on ice. The head capsules of beetles were removed from the body and the brains were partially dissected. This made the tiny

Developmental Neurobiology
brains more visible throughout the immunostaining procedure, and reduced the number of brains that were lost. Fixation took place in 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.2) for 1 h at room temperature. The tissue was then washed in PBS and stored at 4 °C in the dark until further processing.

Immunostaining and Fluorescent Labeling of F-Actin (Phalloidin Staining)

Before applying any antibodies, whole fixed brains were washed in 1% PBST (1% Triton X-100 in PBS) and then permeabilized in collagenase-dispase (Sigma-Aldrich, St. Louis, MO) for 20 min. The concentration of enzyme that was used differed according to age, as brains of younger beetles were damaged by the higher concentrations needed to permeabilize older tissue. For brains from late larval to new born adult beetles, the collagenase concentration used was 0.125 mg/mL. For adults up to 2-weeks old the concentration used was 0.25 mg/mL, and brains from all older beetles were treated with 0.5 mg/mL collagenase. After the enzyme treatment, all brains were washed several times in 1% PBST and then incubated in a blocking solution of 10% normal goat serum (NGS) in 1% PBST for at least 1 h.

Anti-DC0 primary antibody, a polyclonal antibody against the catalytic subunit of Drosophila melanogaster protein kinase A, was a gift provided by Dr. Daniel Kalderon. This antibody has been shown to have a high affinity for mushroom body intrinsic neurons in a range of insect species (Skoulakis et al., 1993; Farris, 2005a,b). In the present study anti-DC0 was diluted 1:1 in glycerol for storage, and a 1:500 concentration of this stock was used in 1% NGS in 1% PBST for staining. The following day, sections were washed with 1% PBST three times for 10 min each followed by incubation in a 1:200 concentration of goat anti-rabbit secondary antibody tagged with Texas Red (Invitrogen (Molecular Probes), Eugene, OR), also in 1% NGS solution in PBST. Alexa 488-conjugated phalloidin (Molecular Probes), which labels filamentous actin that is enriched in outgrowing Kenyon cell processes (Kurusu et al., 2002; Farris and Sinakevitch, 2003; Farris et al., 2004; Farris et al., 2005a), was applied at a 1:500 concentration at the same time as the secondary antibody. The brains were incubated in this solution overnight at room temperature. The next day sections were washed in PBST and cleared in 60% glycerol for half an hour, followed by incubation in 80% glycerol for one half hour. After mounting in 80% glycerol, brains were dissected free of the remnants of the head capsule using fine forceps, and viewed using an Olympus Fluoview 1000 confocal microscope within 48 h of coverslipping.

BrdU Treatments and Staining

Adult beetles were chilled on ice and a drop of insect saline containing 25 mg/mL 5'-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich, St. Louis, MO), a thymidine analog and marker of cellular mitotic activity, was placed on the mouthparts. As the beetles recovered from chilling, movements of their mouthparts caused them to ingest the solution. After BrdU treatment, beetles were placed in Petri dishes with food for 4 h, 8 h or overnight before dissection. In place of the enzyme treatment used prior to immunostaining, BrdU treated and dissected brains were immersed in 2 N HCl for twenty minutes. This served both to permeabilize the tissue and to denature the DNA to allow access to the anti-BrdU antibody. Monoclonal anti-BrdU was purchased from Becton-Dickinson (San Jose, CA) and used at a 1:200 concentration in 1% NGS in PBST. Anti-DC0 was also applied at a 1:500 concentration as a counterstain. Anti-BrdU was detected using a 1:200 solution of Texas Red conjugated goat anti-mouse secondary antibody in 1% NGS in PBST, and the anti-DC0 was detected using an Alexa 488 conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Following incubation in these secondary antibodies, processing and visualization of labeled tissue proceeded as described earlier.

Confocal Microscopy and Image Processing

All stained preparations were viewed on an Olympus Fluoview 1000 confocal microscope. Single 1.5 μm deep optical sections, or projections made from a small number of optical sections, were selected from stacks of each stained brain. These images were then processed as needed for brightness and contrast using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

RESULTS

Structure and Development of the Insect Mushroom Bodies with Reference to Drosophila melanogaster: A Review

Insect mushroom bodies are composed of intrinsic neurons, Kenyon cells, whose soma lay in the dorso-posterior region of the brain. Kenyon cell dendrites form a cup- or bulb-shaped structure called the calyx, which receives sensory afferents (primarily olfactory projection neurons from the antennal lobe; Ito et al., 1998; Gronenberg, 2001; Strausfeld et al., 2003). The axons of Kenyon cells travel anteroventrally through the pedunculus and bifurcate into the medial and vertical lobes, which are commonly thought of as an output region although they also receive input from multimodal protocerebral afferents (Ito et al., 1998; Li and Strausfeld, 1999; Strausfeld, 2002).

During development, four neuroblasts per hemisphere each produce at least three types of Kenyon cells, the γ, α/β′ and α/β cells (Ito et al., 1997;
Critenden et al., 1998; Lee et al., 1999). More recent studies suggest that there are several more Kenyon cell subpopulations in Drosophila based on dendritic morphology and afferent input (Strausfeld et al., 2003; Zhu et al., 2003; Lin et al., 2007). The first-born Kenyon cell subpopulation is produced from Stage 15 of embryonic development until the middle of the third larval instar and forms the medially projecting γ lobe of the adult mushroom bodies (Tettamanti et al., 1997; Armstrong et al., 1998; Lee et al., 1999; Noveen et al., 2000). Noveen et al. (2000) report that the mushroom body neuroblasts are present in the embryonic brain and actively dividing prior to Stage 15, however, the identity of these earliest progeny has yet to be determined. The second-born subpopulation of Kenyon cells form the α/β lobes with their bifurcated axons and are born in the short interval between the mid-third instar and the onset of puparium formation (Lee et al., 1999). The last-born subpopulation of Kenyon cells forms the α′/β′ lobes with their axons and is produced after puparium formation; neurogenesis ends shortly before eclosion and the neuroblasts do not persist in the adult brain (Ito and Hotta, 1992; Lee et al., 1999). The last-born Kenyon cells within the α′/β′ subpopulation, called the α′/β′ core neurons after the projection pattern of their axons in the core of the α′/β′ lobes, may also be differentiated morphologically and by distinct gene expression patterns (Kurusu et al., 2002; Lin et al., 2007).

During metamorphosis in holometabolous insects, much of the larval nervous system is remodeled through a combination of neuronal birth and death, and process degeneration and regrowth (Truman, 1990; Consoulas et al., 2000). In the brain, the antennal lobe is the primary olfactory processing center and is composed of densely packed glomeruli containing the synapses of olfactory receptor neurons from the antennae, inhibitory local interneurons and projection neurons that synapse onto Kenyon cell dendrites in the calyx (Stocker et al., 1990; Stocker, 1994). The antennal lobe undergoes a dramatic reorganization during metamorphosis during which the larval glomeruli are dissolved and reformed into the adult configuration and the connections of projection neurons in the calyx are similarly reshaped (Jefferis et al., 2002; Marin et al., 2005). Kenyon cell dendrites and axons are also pruned and re-established (Zhu et al., 2003; Marin et al., 2005), and the axons forming the larval γ lobe, which is composed of vertical and medial projecting components formed by the bifurcating axons of γ Kenyon cells, are engulfed by glia and regrow in the medial direction only (Armstrong et al., 1998; Lee et al., 1999). The α/β′ Kenyon cells that are born shortly before puparium formation serve as guidance cues for the re-extending γ axons (Zhu et al., 2003). Large-scale reorganization of the mushroom body lobes like that in Drosophila has also been observed in the wasp Polistes apachiensis, but in the honey bee Apis mellifera the γ Kenyon cell axons only undergo a slight pruning during metamorphosis (Farris et al., 2004).

**Structure of the Mushroom Bodies of Tribolium castaneum**

As in other insects, the mushroom bodies of Tribolium castaneum were composed of densely packed intrinsic neurons, the Kenyon cells. The Kenyon cell somata resided around a single calyx in each hemisphere. Figure 1(A) illustrates the structure of the calyx (Cx) in one hemisphere of the brain, and the location of the two large neuroblasts (Nb). The ovoid shape of the calyx is similar to that of Drosophila and is similarly composed of the dendrites of Kenyon cells. In contrast to Drosophila, the neuroblasts were clearly visible at all stages of development surveyed in the present account, from the late larva through adulthood. Phalloidin labeling, which has been demonstrated to reveal the ingrowing axons of newborn Kenyon cells in several insect species (Kurusu et al., 2002; Farris and Sinakevitch, 2003; Farris et al., 2004; Farris et al., 2005), showed two axon tracts entering the Tribolium calyx, one associated with each of the neuroblasts [Fig. 1(A), arrows]. Ventral to the calyx, Kenyon cell axons projected into the pedunculus [Pe, Fig 1(A)]. The vertical and medial lobes were formed by the vertical and medial branches of Kenyon cell axons [Fig. 1(B–E)]. Staining with the anti-DC0 antibody allowed the identification of five Kenyon cell types based on axon morphology and antibody affinity. Figure 1(B–E) show four optical sections from anterior to posterior through the mushroom body lobes of a single individual, and differently colored arrows indicate the five subpopulations of Kenyon cells. A summary of the Kenyon cell subpopulations observed is represented in the schematic in Figure 1(F), with different colors representing the arrows in Figure 1(B–E). The red “Ⅲ” cells, whose far anterior location suggests that they are among the first-born Kenyon cells (Farris and Sinakevitch, 2003), likely correspond to the Class III Kenyon cells that have been described in many insects, but not in Drosophila (Farris, 2005a,b). Orange, green, blue, and purple arrows indicate cell populations that, based on axon morphology and birth order (see below), are likely homologs of the γ, α/β′, α/β and α/β′ core Kenyon cells of Drosophila.
Development and Reorganization of the Tribolium Mushroom Bodies During Metamorphosis

The present study began with phalloidin labeling and anti-DC0 immunohistochemistry of the brain of the last instar feeding larva, which was identified as the largest larval instar that was observed moving freely through the flour medium. In these last instar larvae just two groups of Kenyon cells were observed in the lobes [Fig. 2(A)]. Phalloidin labeling revealed a tract of newborn Kenyon cell axons emerging from a small group of cell bodies immediately surrounding each of the two neuroblasts [Fig. 2(B), arrows]. The two tracts passed through the calyx and fused at the origin of the pedunculus to form an ingrowth core that continued into the vertical and medial branches of one lobe pair [arrows, Fig. 2(A–C)]. This lobe pair was termed γ, after their resemblance to the larval γ Kenyon cells of Drosophila. Kenyon cells making up a smaller, more anterior lobe pair that lacked a core of ingrowing axons were termed δ. As mushroom body neuroblasts produce Kenyon cell subpopulations sequentially (Lee et al., 1999) and no ingrowing axons were observed in the δ lobe, these Kenyon cells are likely born prior to the γ cells.

Clear glomeruli were also present in the antennal lobe of the last instar larvae [arrows, Fig. 2(D)], as were microglomeruli in the mushroom body calyx [arrowheads, Fig. 2(B)]. Glomeruli and microglomeruli are structures associated with synaptic associations in both the antennal lobe and the mushroom bodies.

Dramatic changes were observed in the structure of the antennal lobes and mushroom body calyces of non-feeding prepupae, which were identified as last instar larvae that were immobile save for rotational movements of the abdomen. The ingrowth core was now observed to contain axons of two Kenyon cell subpopulations in the vertical lobes [arrows, Fig. 2(E)], suggesting that the neuroblasts had begun producing a new subpopulation of Kenyon cells (termed δ′/β′ after those born in the late third instar Drosophila larva). The persistence of phalloidin labeling in the γ lobes, however, suggested that the outgrowth of γ Kenyon cell axons was not completed before neuroblast production of δ′/β′ neurons had begun. Interestingly, the medial lobe had a phalloidin labeled core only in the medial γ lobe, with no apparent medial δ′/β′ ingrowth at this stage [Fig. 2(G)]. This suggested that δ′/β′ axon outgrowth into the vertical lobes preceded branching into the medial lobe, the opposite of what has been observed in Drosophila (Tettamanti et al., 1997; Noveen et al., 2000; Kurusu et al., 2002).

In the prepupal mushroom body calyces and antennal lobes, all traces of glomerular structure had dissolved [Fig. 2(H,F)]. This is suggestive of synaptic reorganization of these two sequential components of the olfactory processing pathway, also reminiscent of Drosophila (Marin et al., 2005). In contrast to Drosophila, however, there was no apparent axon degeneration or pruning in the mushroom body lobes during the prepupal or pupal stages.

The appearance of the pupal mushroom bodies was similar to those of the prepupa for the first 2 days of the eight-day pupal stage [Fig. 3(A) inset]. At Day 3, phalloidin labeling of extending γ Kenyon cell axons disappeared from the vertical lobe so that only δ′/β′ axons were labeled [arrows, Fig. 3(A)]. In the medial lobe, axons of newborn δ′/β′ cells were now labeled along with those of γ Kenyon cells [arrows, Fig. 3(B)]. Again, this result suggests a delay in Kenyon cell branching into the medial lobe relative to outgrowth into the vertical lobe, as γ axon extension into the vertical lobe was completed prior to extension into the medial lobe. By the end of pupal Day 4, phalloidin labeling of γ Kenyon cell axons in the medial lobe had also disappeared and only labeling of δ′/β′ axons was observed [Fig. 3(C)].

The ingrowth of δ′/β′ Kenyon cells into the lobes continued through the remainder of the pupal stage (see Fig. 4). Glomeruli in the mushroom body calyces and antennal lobes gradually coalesced until these structures were indistinguishable from those of adults by pupal Day 8, which was the last day of the pupal stage under our rearing conditions at 28°C. Comparing the calyces of last instar larvae with those of late pupae, it appeared that the late pupal microglomeruli were smaller and finer in structure than were those of the larva, perhaps suggesting a larger number of synaptic connections in the mushroom body calyces of the developing adult.

Adult Neurogenesis in the Tribolium Mushroom Bodies

Newly eclosed adults could be differentiated from older adults by their lightly tanned cuticle [Fig. 5(A), newly
eclosed adult at top and 2-week-old adult below]. Both phalloidin staining and BrdU labeling revealed the two neuroblasts and their newborn Kenyon cell progeny in the adult mushroom bodies [Fig. 5(B1,B2)]. These neuroblasts were actively proliferating for the first 2 months of adult life. Beyond this age, however, BrdU incorporation and phalloidin labeling of newborn neurons became minimal or was totally lost [Fig. 5(C)]. At 88 days of age, only a small number of adults maintained active neuroblasts in the mushroom bodies (data not shown).
At adult eclosion, the neuroblasts began production of the last group of Kenyon cells, termed \( z/\beta \) [Fig. 5(C), blue arrow]. The axons of newborn Kenyon cells in the adult mushroom bodies formed an ingrowth core at the center of the \( z/\beta \) lobes [Fig. 5(C), purple arrow]. The \( z/\beta \) lobes steadily increased in size throughout adulthood as the axons of these new neurons entered via the ingrowth core [compare Fig. 5(C–E)]. The mushroom bodies as a whole appeared to increase in size as adult life progressed, perhaps reflecting the ramification of afferent and efferent processes within the mushroom body neuropil.

Interestingly, individual variation in mushroom body neuroblast number was observed in ~10% of the fifty larval, pupal and adult brains viewed for this study (see Fig. 6). A small number of these variants had only one rather than two mushroom body neuroblasts in one of the brain hemispheres [compare Fig. 6(A) with 6(B)]. When only one neuroblast was present, the mushroom body neuropil in that hemisphere appeared to be diminished in size. Most of the variants had three mushroom body neuroblasts in one hemisphere [Fig. 6(C)], with two of the neuroblasts more closely associated with one another, and with their progeny entering the mushroom body calyx via the same phalloidin-labeled tract. In these latter variants, the size of the mushroom bodies appeared to be more similar to that of wild type individuals.

**DISCUSSION**

**Comparing Mushroom Body Structure and Development in Tribolium castaneum with that of Drosophila melanogaster**

Mushroom bodies are protocerebral neuropils that are composed of one or more populations of intrinsic neurons, the Kenyon cells (Flögel, 1878; Strausfeld et al., 1995, 1998). The mushroom body calyx is made up of Kenyon cell dendrites that in most insects receive a preponderance of input from the antennal lobes. The lobes are composed of the axon-like processes of Kenyon cells and represent the primary outputs of information from the mushroom bodies. Kenyon cell supopulations are generated sequentially during embryonic, larval, pupal, and even adult development by the mushroom body neuroblasts (Ito and Hotta, 1992; Farris et al., 1999; Lee et al., 1999; Malaterre et al., 2002). This is the basic organizational groundplan upon which taxon-specific modifications in calyx and lobe morphology, and in the number and type of Kenyon cell subpopulations, have been evolutionarily acquired (Farris, 2005a,b).

The present study identifies five types of Kenyon cells in the mushroom bodies of the red flour beetle *Tribolium castaneum* that are sequentially generated during larval, pupal, and adult life. These Kenyon cell populations were identified primarily by the projection patterns of their axons in the lobes, as visualized using anti-DC0 immunohistochemistry and fluorescent phalloidin labeling. In *Tribolium*, Kenyon cells are typically produced by two neuroblasts in each brain hemisphere; mushroom body neurogenesis was continuous from the late larval stage (the earliest stage investigated in this study) until up to 88 days after adult eclosion. Neurogenesis was detected by fluorescent phalloidin labeling of the extending axons of newborn neurons, and by BrdU incorporation by the neuroblasts and their progeny.

Several aspects of the structure and development of the *Tribolium* mushroom bodies were very similar to that observed in the fruit fly *Drosophila melanogaster*. In both insects the mushroom bodies are composed of a single calyx, and medial and vertical

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**Figure 1** Structure and intrinsic neuron composition of the mushroom bodies in *Tribolium castaneum* adults. A: The calyx (Cx) is composed of the dendrites of Kenyon cells that are produced by two neuroblasts (Nb) in each brain hemisphere. The granular appearance of the calyx reflects the presence of microglomeruli, sites of synaptic interaction between afferent boutons and Kenyon cell dendrites (Yasuyama et al., 2002). Kenyon cell axons form the pedunculus (Pe) and lobes (B–E). Alexa 488-conjugated phalloidin (green) strongly labels the ingrowing axons of newborn Kenyon cells (arrows). Anti-DC0 staining of mature Kenyon cells and their processes is indicated in purple. The DC0 antibody is directed against the catalytic subunit of protein kinase A (PKA) of *Drosophila melanogaster* (Skoulakis et al., 1993). B–E: Anterior (B) to posterior (E) optical sections of the lobes labeled with anti-DC0 (green). Arrows indicate lobe subdivisions made up of different Kenyon cell populations. Red- \( \alpha \), orange- \( \gamma \), green- \( z/\beta \), blue- \( z/\beta \), purple- core. F: Schematic diagram of Kenyon cell populations in the *Tribolium* mushroom bodies, indicated using the same color scheme as in (B–E). Neuroblasts (Nb) are in bright green and are surrounded by the soma of newborn Kenyon cells (small purple circles). Newborn Kenyon cells extend their axons as two tracts into the calyx, which fuse into a single core in the pedunculus and lobes. Scale bars = 20 µm.

Developmental Neurobiology
Figure 2
lobes that are formed by the bifurcated axons of Kenyon cells that are subdivided into distinct lobe systems according to the Kenyon cell subpopulation supplying them. In *Drosophila* the γ Kenyon cells, which supply the vertical and medial γ lobes with their axons, are produced throughout larval life until a few hours before pupariation, at which time the neuroblasts transition completely to the generation of γ/β Kenyon cells that form the γ/β lobes with their bifurcated axons (Lee et al., 1999). The production of γ/β neurons ceases shortly after pupariation and gives way to the production of γ/β Kenyon cells that form the γ/β lobes, which is completed prior to adult eclosion in *Drosophila*. The very last born Kenyon cells form a core in the γ/β lobes of the *Drosophila* mushroom bodies and may be identified by the affinity of their axons to phalloidin labeling and by other molecular and morphological characteristics (Kurusu et al., 2002; Strausfeld et al., 2003; Lin et al., 2007). In *Tribolium castaneum* a very similar sequential pattern of Kenyon cell production is observed, albeit with an expansion of the γ/β neuron production period to encompass the duration of the pupal stage, thus delaying the onset of generation of γ/β neurons until adult eclosion. Unlike *Drosophila*, mushroom body neurogenesis in *Tribolium* persisted post-adult eclosion for as long as 88 days, with γ/β Kenyon cells being generated and their axons incorporated into the γ/β lobes via an ingrowth or γ/β core the entire time. Setting aside this developmental heterochrony when compared with *Drosophila*, the γ, γ/β, γ/β, and γ/β core Kenyon cells of *Tribolium* were observed to be very similar to those of *Drosophila* both in morphology and in birth order, and are likely to represent homologous cell populations.

A fifth Kenyon cell subpopulation, named δ, made up a pair of anterior lobes that appeared fully developed in the late *Tribolium* larva, indicating that δ Kenyon cells are born prior to the γ Kenyon cells whose phalloidin-labeled axons were entering the γ lobes at that stage. While no Kenyon cell population with a birthdate prior to that of the γ Kenyon cells has been definitively identified in *Drosophila* (but see Noveen et al., 2000), insects such as cockroaches, crickets and moths have an early-born cell population called Class III Kenyon cells (Malaterre et al., 2002; Farris and Strausfeld, 2003; Sjöholm et al., 2005). This subpopulation of Kenyon cells is the first born in development, prior to Class II Kenyon cells (the γ Kenyon cells of *Drosophila*), and appear to have a specialized function in processing gustatory input to the mushroom bodies (Weiss, 1981; Frambach and Schürmann, 2004; Farris, 2008). To further build the case for the homology of *Tribolium* Kenyon cell subpopulations with those of other insects, future studies must characterize their dendritic morphologies and the types and sources of their afferent inputs.

Neural circuits in the ventral nerve cord of holometabolous insects are extensively reorganized during metamorphosis, as the larval body plan is reshaped into that of the adult (Truman, 1990;Consoulas et al., 2000). In the brain, some neuropils are formed de novo during metamorphosis for processing input from adult-specific sensory structures such as the eyes (Monsma and Booker, 1996), whereas larval brain centers such as the antennal lobes are reorganized and new neurons are incorporated to form circuitry suited to the processing needs of adults (Jefferis et al., 2002; Marin et al., 2005). The *Drosophila* mushroom bodies are also reorganized during metamorphosis, a process in which the axons and dendrites of γ Kenyon cells that were produced in the larva are pruned by engulfing glia and reshaped into adult configurations (Armstrong et al., 1998; Lee et al., 1999; Watts et al., 2003; Awasaki and Ito, 2004; Watts et al., 2004). Notably, the bifurcated axons of larval γ neurons are pruned back to the pedunculus and then regrow unbranched in the

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**Figure 2** Mushroom bodies of the feeding last instar larva (A–D) and prepupa (E–F). A: Anti-DC0 immunostaining (purple) reveals two Kenyon cell populations, δ and γ, in the vertical lobes of the feeding last instar larva. Ingrowing axons form a thin tract in the γ lobe as revealed by phalloidin labeling (green, indicated by arrows). B: Phalloidin labels synaptic microglomeruli of the calyx (Cx, arrowheads) and extending axons of newborn Kenyon cells (arrows) generated by the mushroom body neuroblasts (Nb). C: Axons of newborn Kenyon cells (arrows) in the medial γ lobes of the feeding larva. D: Glomeruli in the antennal lobe of the feeding larva (AL; arrows). E: Phalloidin labels two tracts of ingrowing Kenyon cell axons in the pre pupal vertical lobe: one into γ (white arrows) and one into the newly forming γ/β lobe (black arrows). F: The prepupal calyx lacks definite microglomeruli and has a uniform consistency. G: In the prepupal medial lobe, ingrowing Kenyon cell axons (arrows) are observed only in the γ lobe; no β lobe is visible. F: Like the calyx, the prepupal antennal lobe has also lost its glomerular structure. Cc, central complex, Pe, pedunculus. Scale bars = 20 μm.
medial direction, so that the adult has only a medial γ lobe. The *Tribolium* mushroom bodies are also reshaped during metamorphosis, as microglomeruli formed by larval Kenyon cell dendrites in the calyx are dissolved during the prepupal stage and reformed gradually in the late pupal stage. One synapse before the calyx in the olfactory processing pathway, the antennal lobe glomeruli are reshaped following the same timeline. In contrast to *Drosophila*, however,

**Figure 3** Axon outgrowth into the mushroom body lobes of the early pupa. A inset: As in the prepupa, the pupal stage begins with two phalloidin labeled axon tracts (green, arrows) growing into the vertical γ and α'/β' lobes. Anti-DC0 immunostaining is indicated by purple labeling (A). By pupal Day 3, axon growth into the vertical γ lobe is completed and phalloidin labels a single tract in the α'/β' lobes of each mushroom body (arrows). B: In the Day 3 pupa, phalloidin labels two tracts of ingrowing axons in the medial lobes corresponding to the γ and α'/β' Kenyon cells. C: By pupal Day 4, phalloidin labels only a single tract of ingrowing axons of newborn α'/β' Kenyon cells. Scale bars = 20 μm.

**Figure 4** Mushroom bodies of the Day 8 pupa. A: Phalloidin labeling (green) reveals that microglomeruli have reformed in the calyx (Cx, arrows) and axogenesis of newborn Kenyon cells (arrowheads) is ongoing. B: Anti-DC0 immunostaining (purple) of the γ and α'/β' lobes, the latter of which are still being constructed by ingrowing axons (arrows). C: Reformed glomeruli (arrows) in the antennal lobe (AL). Pe, pedunculus. Scale bars = 20 μm.
Figure 5  Neurogenesis and continued growth of the mushroom body neuropil after adult eclosion. A: Newly emerged adults (top) have lightly sclerotized cuticle. The tanning process is completed by Day 3 of adult life (bottom). B1–3: Progression of neuroblast activity in the adult. B1: In newly eclosed adults, phalloidin (green) labels axons of newborn Kenyon cells associated with two neuroblasts in each hemisphere (arrowheads). Purple-anti-DC0 staining. B2: BrdU incorporation of neuroblasts and their progeny (arrowheads) in the newly eclosed adult. Green-anti-DC0 staining. B3: BrdU incorporation (arrowheads) is less pronounced in the older adult (44-days old in this example). Green-anti-DC0 staining. C: Production of α/β Kenyon cells (blue arrow) begins at adult eclosion as indicated by the location of the phalloidin labeled ingrowth core (purple arrow). D: Increased size of the α/β lobe (blue arrow) relative to α′/β′ (green) and γ (orange), due to continued production of α/β Kenyon cells, is evident in the 3-week-old adult. E: Overall growth of the mushroom body lobes is apparent in an 88-day-old adult, when compared with the lobes of newly eclosed adults (C). Cx- calyx. Scale bars = 20 μm.
no pruning could be observed in the *Tribolium* lobes using anti-DC0 immunohistochemistry and phalloidin labeling, and the γ Kenyon cells retain their bifurcated axons into adulthood. The degree of mushroom body metamorphosis thus seems to be variable according to species, as also evidenced by studies in the Hymenoptera in which the mushroom bodies of the honey bee *Apis mellifera* undergo just moderate pruning at the tips of γ axons, whereas the wasp *Polistes apachiiensis* displays massive pruning and regrowth of the entire lobe system similar to that observed in *Drosophila* (Farris et al., 2004).

Most holometabolous insect larvae lack compound eyes, meaning that these structures and the optic lobe neuropils that process visual information in the adult must be generated almost in their entirety during metamorphosis (Monsma and Booker, 1996; Friedrich, 2006). Hundreds of thousands of new neurons that will form the adult lamina, medulla and lobula are produced at this time by optic lobe neuroblasts in the optic anlagen adjoining the lateral protocerebrum. In the mushroom bodies of both *Drosophila* and *Tribolium* one Kenyon cell subpopulation, the α/β neurons, are produced during or after metamorphosis, and so like the optic lobe neurons these Kenyon cells must be serving adult-specific functions. In contrast to optic lobe neurons, however, it is not as clear what the adult specific functions of these α/β Kenyon cells may be. It has been hypothesized that Kenyon cells produced after adult eclosion in the moth *Ephestia kuniella* may be involved in processing information about sex pheromones, since in males the ability to respond to pheromone matures at the same time as the production and incorporation of adult Kenyon cells (Dufour and Gadenne, 2006). Although this appears to be a logical function for adult-specific neurons in an olfactory processing center, assigning definitive roles to these Kenyon cells awaits more detailed anatomical studies of their connections, in conjunction with physiological recordings of their outputs.

**Adult Neurogenesis and Plasticity of Tribolium Mushroom Bodies**

Neurogenesis in adult insect mushroom bodies appears to be relatively uncommon (Cayre et al., 1996), and thus cannot be a basis for neural plasticity in most species. Like vertebrates, neural plasticity in the insect mushroom bodies in response to environmental stimuli may thus take the form of neuronal process outgrowth, branching, and increased synaptogenesis (Diamond et al., 1972; Greenough et al.,
model systems and comparative studies, will be important for broadening our understanding of the mechanisms of neural plasticity, from an evolutionary perspective down to the cellular and molecular levels.

**Individual Variation in Neuroblast Number in the Tribolium Mushroom Bodies**

Although most *Tribolium* brains observed in this study displayed two mushroom body neuroblasts per hemisphere, a small number (10%) had either one or three neuroblasts (see Fig. 7). This variation typically occurred in just one hemisphere of the brain of each individual affected. In contrast, years of developmental studies in *Drosophila* have failed to turn up any variation from the four neuroblasts per hemisphere that is typical for this species (Ito and Hotta, 1992; Ito et al., 1997; J.S. deBelle, personal communication). If this variation in mushroom body neuroblast number in *Tribolium* is heritable, selective breeding may be utilized to directly test the impact of neuroblast number on mushroom body structure, afferent and efferent connections, and even behavioral output.

In conclusion, we have characterized mushroom body structure and development in a new genetic model system, the red flour beetle *Tribolium castaneum*. The presence of adult neurogenesis in this species makes it a promising new model system for studies of the cellular and molecular regulation of this process, and of the functions of adult specific neurons. *Tribolium* is thus unique among insect model systems in possessing adult neurogenesis in the mushroom bodies, quantifiable variation in neural precursor number, and being tractable for experiments utilizing genetic manipulations. Further studies of mushroom body development in this species will provide insight into interactions between behavioral and neural plasticity that span evolutionary to cellular and molecular levels of organization.

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**REFERENCES**


Developmental Neurobiology
Development of Tribolium Mushroom Bodies


