Development and evolution of the insect mushroom bodies: towards the understanding of conserved developmental mechanisms in a higher brain center

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Abstract

The insect mushroom bodies are prominent higher order neuropils consisting of thousands of approximately parallel projecting intrinsic neurons arising from the minute basophilic perikarya of globuli cells. Early studies described these structures as centers for intelligence and other higher functions; at present, the mushroom bodies are regarded as important models for the neural basis of learning and memory. The insect mushroom bodies share a similar general morphology, and the same basic sequence of developmental events is observed across a wide range of insect taxa. Globuli cell progenitors arise in the embryo and proliferate throughout the greater part of juvenile development. Discrete morphological and functional subpopulations of globuli cells (or Kenyon cells, as they are called in insects) are sequentially produced at distinct periods of development. Kenyon cell somata are arranged by age around the center of proliferation, as are their processes in the mushroom body neuropil. Other aspects of mushroom body development are more variable from species to species, such as the origin of specific Kenyon cell populations and neuropil substructures, as well as the timing and pace of the general developmental sequence.

1. Introduction

The mushroom bodies were first described in honeybees by Felix Dujardin (Dujardin, 1850). Later descriptions by Flögel (1876) established the defining characteristics of insect mushroom bodies: lobed structures in the supraesophageal ganglion consisting of the parallel fibers of hundreds or thousands of tiny basophilic perikarya called globuli cells. Mushroom body like structures have been identified in species of Annelida, Onychophora and many arthropod groups (Holmgren, 1916; reviewed in Strausfeld et al. (1998)).

Felix Dujardin (1850) suggested that the mushroom bodies were involved in ‘intelligent’ behavior due to their particular prominence in the brains of the social Hymenoptera. More recent studies support this suggestion, showing their involvement in context-dependent multimodal sensory integration (Schildberger, 1984; Li and Strausfeld, 1997, 1999), the prediction and monitoring of motor behavior (Mizunami et al., 1998b; Okada et al., 1999) and certain types of learning and memory (Zars et al., 2000; Pascual and Préat, 2001). Vowles (1964) was the first to show that mechanical lesions near the mushroom bodies of the ant perturbed performance in a maze learning paradigm based on olfactory discrimination. A possible role for the mushroom bodies in olfactory and other types of learning and memory led to the discovery of the first anatomical mushroom body mutants in the fruit fly (Heisenberg et al., 1985). The honeybee has also served as an important model organism in learning and memory studies, due to its rich repertoire of complex, learning-dependent behaviors in nature as well as its tractability in controlled learning and memory paradigms using the proboscis extension reflex (for review, Menzel, 2001).

Despite many anatomical and physiological studies, few general principles of mushroom body organization have been proposed to explain the structure and function of these centers. Developmental studies of the mushroom bodies...
have been performed in many insect species; however, such studies have tended to proceed more or less independently. An important exception is the early work of A.A. Panov, published in Russian and often overlooked today, which provides some of the first detailed accounts of mushroom body neurogenesis and comparative aspects of mushroom body development (Panov, 1957, 1966).

Comparative developmental studies have gained increasing popularity due to the insight that such an approach can provide about the evolution of specific structures.

Fig. 1. Anatomy of the insect mushroom bodies. (A) Frontal section of the mushroom body of one hemisphere of the brain in the honeybee *A. mellifera* at the level of the medial lobe (M), medial to the left. In this species the Kenyon cell somata (Kc) can be subdivided into three concentric subpopulations (oc, nc, ic) based on size and location about each calyx (C). Kenyon cells provide dendrites into the calyces and their axons continue into the short pedunculus (P), where they bifurcate to form the medial and vertical lobes. (B) Frontal section of the honeybee mushroom body at the level of the vertical lobe (V), which projects from the pedunculus towards the anterior surface of the brain. (C) Frontal section of the mushroom body of one hemisphere of the brain in the American cockroach *P. americana*. The same basic morphology is observed as in the honeybee, with Kenyon cells processes forming two calyces (only one visible) and bifurcating in the pedunculus to form a medial and vertical lobe. Unlike *A. mellifera*, the cockroach vertical lobe projects dorsally rather than anteriorly, and a distinct lobelet (L) made up of class III Kenyon cells is observed. (D) Sagittal section of the honeybee mushroom body, anterior to the left. Cc, central complex; oc, outer compact Kenyon cells; nc, non-compact Kenyon cells; ic, inner compact Kenyon cells. Scale bars = 100 μm.
Consequently, developmental studies of the mushroom bodies have again taken up the type of comparative approach used by Panov, combining conventional histological methods with newer immunohistochemical analyses to elucidate a variety of aspects of mushroom body development in widely divergent insect species. The results of such studies can be compared with those performed in genetically tractable insects in order to determine molecules and pathways involved in basic developmental processes such as axon outgrowth and branching. Results from studies of cricket, honeybees, cockroaches and flies reveal ontogenetic events that are highly conserved across taxa, suggesting fundamental organizational rules.

2. Anatomy of the insect mushroom bodies

The basic organization of the mushroom bodies is retained in all insects with the exception of the Archaeognatha (Fig. 1). Mushroom body intrinsic neurons, or Kenyon cells, are located in the dorso-posterior brain and are easily recognizable by their tightly packed, cytoplasm-poor soma. The number of Kenyon cells can range from thousands to hundreds of thousands depending on the species. Kenyon cell neurites project antero-ventrally into the brain, producing proximal to the cell body one or more dendritic arborizations in neopteran insects. The neuropil formed by these arborizations is termed the calyx. Although numerous Kenyon cell subtypes have been identified in the insects, most appear to fall into two broad categories: clawed (Class II) and spiny or otherwise (Class I). The cup- or bulb-shaped calyx receives olfactory afferents in most species, although in the Hymenoptera it also receives visual afferents. Each mushroom body may contain one or two calyces; in most insects with two calyces, the Kenyon cell populations and afferent input to each calyx appears to be equivalent (Gronenberg, 2001). In the Orthoptera (locusts, grasshoppers, katydids, crickets), however, each calyx comprises a distinct population of Kenyon cells and as such receives a separate set of afferents (Schürmann, 1973; Weiss, 1977, 1981; Malaterre et al., 2002).

Generally, Kenyon cell processes project along the inner surface of the calyx and at its center they funnel into the neck of the pedunculus. Several separate tracts of neurites can be visible in the most proximal region of the pedunculus, but these tracts typically fuse as their projection progresses antero-ventrally. Kenyon cell axons in the pedunculus can give rise to both presynaptic specializations (varicosities) and postsynaptic specializations (spines) and can receive afferent neurons (Li and Strausfeld, 1997, 1999; Strausfeld, 2002).

After projecting through the pedunculus, Kenyon cell axons typically branch, although class II Kenyon cells can provide exceptions as described below. Although conventionally referred to as the output region of the mushroom bodies, Kenyon cell axons in the lobes have swellings and spines and receive afferents as well as provide inputs onto the dendrites of efferent neurons (Li and Strausfeld, 1997). This is particularly evident in taxa lacking calyces, in which the lobes are necessarily the only part of the mushroom body to receive afferents (Strausfeld et al., 1998). Kenyon cell processes in the lobes are therefore not axons in the strictest sense, but for simplicity these ‘axons’ will be referred to as such in this account.

Most Kenyon cell axons bifurcate with the resulting branches projecting at approximately right angles to one another. This bifurcation results in vertical (dorsal, α) and medial (β) lobes that are typical of all insects. An exception is seen in vespid wasps, where mushroom bodies appear to have a single lobe due to the particularly convoluted trajectory of the ‘medial’ axons branches (Ehmer and Hoy, 2000). A more common exception is observed in clawed Kenyon cells, which in certain adult holometabolous insects provide just a single vertically or medially directed axon (Pearson, 1971; Lee et al., 1999; Strausfeld, 2002). In some insects, different populations of Kenyon cells may form several separate lobe systems that are designated by various combinations of characters. In the fruit fly Drosophila melanogaster Meigen (Diptera, Drosophilidae) three such lobe systems are present, and are referred to as α/β, α′/β′ and γ (Crittenden et al., 1998).

3. Early development and neurogenesis

3.1. Embryonic development of the mushroom bodies

Mushroom body development typically begins in the embryo. In hemimetabolous insects, in which immature nymphs behave in many ways like the adult insect, the mushroom bodies of the newly hatched nymph resemble tiny versions of those in the adult (Farris and Strausfeld, 2001; Malaterre et al., 2002). In the Holometabola, however, the appearance of the mushroom bodies at hatching is greatly variable from species to species. Newly hatched larval Diptera such as D. melanogaster and Phormia regina Meigen (Diptera, Calliphoridae) have well-defined mushroom bodies with a calyx, pedunculus and lobes (Gundersen and Larsen, 1978; Armstrong et al., 1998). The monarch butterfly Danaus plexippus plexippus L. (Lepidoptera, Danaidae), however, has at hatching only a small number of Kenyon cells with axons forming a thin pedunculus (Nordlander and Edwards, 1970); and the larva of the honeybee Apis mellifera L. (Hymenoptera, Apidae) has only progenitor cells at hatching and no identifiable Kenyon cells until the third or fourth instar (Panov, 1957; Farris et al., 1999).

Mushroom body progenitor cells (mushroom body neuroblasts; MBNBs) are unique in that they are smaller than other protocerebral neuroblasts, and may form discrete glial-delimited aggregates in the dorsal protocerebrum (Panov, 1957; Masson, 1970; Malun, 1998; Farris et al., 1999).
clusters per hemisphere in the first instar larva. 

et al. (1999) arising at the third larval instar. 

present in the first instar larva, with the second cluster 

blasts, at about 57% development (

Ganglion mother cells (GMCs) are first identified subsequent 

Periplaneta americana (Orthoptera, Gryllidae; often incorrectly cited as 

A. mellifera (Farris et al., 1999). In many species, each 

MBNB or MBNB cluster eventually resides within its own 

calyx as development proceeds. One partial exception to 

this rule occurs in the walkingstick Carausius morosus 

Brunner (Phasmatida, Phasmatidae), in which two embryonic 
calyces fuse into one but retain two separate MBNBs 

(Maltzacher, 1968). Although the adult A. mellifera mush-

room bodies are composed of two calyces, Malun (1998) 

reported that only a single MBNB cluster per hemisphere is 

present in the first instar larva, with the second cluster 

arising at the third larval instar. Panov (1957) and Farris 
et al. (1999), however, identified two distinct MBNB 

clusters per hemisphere in the first instar larva.

In the hemimetabolous insect Acheta domestica L. 
(Orthoptera, Gryllidae; often incorrectly cited as Acheta 
domesticus), MBNB clusters and associated cell bodies of 
ganglion mother cells (GMCs) are first identified subsequent 
to the appearance of the surrounding protocerebral neuro-

blasts, at about 57% development (Malaterre et al., 2002). In 

Periplaneta americana L. (Dictyoptera, Blattidae) the 
appearance of MBNBs occurs somewhat earlier, as early 
as 30–36% development (Salecker and Boeckh, 1995; 
Farris and Strausfeld, unpublished observations). It is likely, 
however, that the very first MBNBs arise significantly 
earlier than has been reported, but specific markers for these 
neuroblasts have not been developed in cricket and 
cockroach. The progenitors are therefore not recognizable 
until their numbers and those of their progeny are enough to 
form a distinct aggregate. Therefore the origin of MBNBs in 
hemimetabolous insects has yet to be determined.

In the holometabolous D. melanogaster, molecular 
markers for MBNBs have been identified and used to 
determine the exact origin of these progenitors during 
embryogenesis. The genes eyeless and dachshund are 
expressed in a proneural cluster of 10–12 neuroblasts per 
hemisphere, part of the Pe3 neuroblast group (Noveen et al., 
2000; Younossi-Hartenstein et al., 1996). This equivalence 
group gives rise to four MBNBs per hemisphere at 
embryonic stage 9 (Noveen et al., 2000). The MBNBs are 
among the first progenitors to delaminate in the developing 
brain, and begin producing progeny immediately. Interest-

ingly, these first progeny never express mushroom body 
markers and do not appear to contribute to these neuropils in 
a manner typical of Kenyon cells (Noveen et al., 2000). 
Production of Kenyon cells, as identified by their expression 
of specific markers, does not occur until around stage 15. 
This appears to contrast with the findings of Ito et al. (1997) 
that the MBNBs generate only mushroom body intrinsic 
neurons and glia; however, only MBNB progeny born after 
larval hatching were analyzed in this study. The identity and 
fate of the first MBNB progeny in the D. melanogaster 
embryo is unknown at present.

The basic sequence of embryonic mushroom body 
development is consistent between hemimetabolous and 
holometabolous insects. The onset of Kenyon cell pro-
duction is marked by the accumulation of minute, closely 
packed soma surrounding or ventral to each MBNB cluster. 
The neuropil of the embryonic mushroom bodies first 
appears as a thin bundle of fibers extending into the 
protocerebrum, which gradually thickens into a pedunculus 
(Tettamanti et al., 1997; Noveen et al., 2000; Malaterre et al., 
2002; Farris and Strausfeld, unpublished observations). In 

D. melanogaster the first Kenyon cell axons enter the 
protocerebrum along the fasciclin II expressing pioneer cell 
P41 (Noveen et al., 2000; Kurusu et al., 2002). The lobes 
arise from the pedunculus shortly afterwards. The medial 
lobe typically appears slightly before the vertical lobe 
(Tettamanti et al., 1997; Noveen et al., 2000; Kurusu et al., 
2002; Malaterre et al., 2002) indicating a delay in Kenyon 
cell branching during initial axonal outgrowth. The calyx is 
the last neuropil region to form, appearing late in 
embryogenesis or in the early nymph in the hemimetabolous 
insects (Afify, 1960, as cited in Edwards, 1969); Panov, 
1966; Farris and Strausfeld 2000; Kurusu et al. 2002; 
Malaterre et al. 2002), but typically after larval hatching in 
the holometabolous insects (Nordlander and Edwards, 1970; 
Tettamanti et al., 1997; Farris et al., 1999) (Fig. 3). As

Fig. 2. Developing mushroom bodies in the pupal brain of the ant Atta cephalotes L. (Hymenoptera, Formicidae). The mushroom body neuro-

blasts (Nb) form two large aggregates, each residing within a single 
developing calyx (C). Kenyon cell bodies (Kc) surround the neuroblasts. P, 
pedunculus; M, medial lobe. Scale bar = 50 μm.
growth continues in *P. americana* and *A. mellifera*, each MBNB cluster resides within the walls of each of the four calyces, eventually settling onto the ventral surface of the calyx for the remainder of mushroom body development (Farris and Strausfeld, 2001). In *A. domestica*, *D. plexippus plexippus* and *D. melanogaster* this organization is reversed, with the neuroblasts residing at the roof of the protocerebrum and separated from the more ventrally situated calyx by an ever-increasing volume of Kenyon cell bodies (Ito and Hotta, 1992; Malaterre et al., 2002). The relative location of MBNBs within the developing calyces appears to be characteristic of each insect order (S.M. Farris, unpublished observations).

The basic sequence of early developmental events described above is also followed in insects, such as *D. plexippus plexippus* and *A. mellifera*, in which MBNBs are present at larval hatching, but Kenyon cell production and process outgrowth is mostly or entirely post-embryonic (Farris et al., 1999). Two major differences in the general developmental pattern are evident in these larval derived mushroom bodies, perhaps as a result of the relative delay of onset. First, the number of MBNBs increases 10-fold or more through the larval instars as a result of symmetrical divisions (Panov, 1957; Nordlander and Edwards, 1970; Farris et al., 1999). In comparison, MBNB number in *D. melanogaster* is known to stay constant throughout development (Truman and Bate, 1988), and in *A. domestica* only a twofold increase in number is observed between hatching and adulthood (Cayre et al., 1996; Malaterre et al., 2002). In *D. plexippus plexippus* MBNB number increases from 3 to 30 per cluster (Nordlander and Edwards, 1970), while in *A. mellifera* an initial 25–45 MBNBs per cluster in the first instar larva generate a total of 2000 MBNBs in the brain of the late fifth instar (Farris et al., 1999). It is proposed in the case of *A. mellifera* that this enormous number of NBs is necessary to generate, in ten days of larval and pupal development, the estimated 170,000 Kenyon cells per hemisphere that make up the honeybee mushroom bodies, which are some of the largest described among the insects (Witthöft, 1967; Farris et al., 1999).

The second difference in developmental events in insects with larval derived mushroom bodies is that the formation of the medial and vertical lobes does not appear to be staggered. Nordlander and Edwards (1968, 1970) report only that the lobes of *D. plexippus plexippus* grow progressively thicker during development. The authors note, however, that the calyx is the last neuropil component to develop, exactly as is observed in embryonically derived mushroom bodies. In *A. mellifera* as well, no apparent disjunction in medial and vertical lobe formation is observed but again calyx formation is clearly delayed (Farris et al., 1999). Even in the fourth instar larva in which Kenyon cell production has just begun, both a medial and a vertical lobe are clearly present (Farris and Strausfeld, unpublished data). It is possible, however, that were larval brains examined at finer intervals during the critical stage in

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Fig. 3. Early stages of mushroom body development in hemimetabolous and holometabolous insects. Frontal sections. (A) In the early embryo of *P. americana* two neuroblast clusters (Nb) in the dorsal protocerebrum are surrounded by Kenyon cell bodies (Kc). Kenyon cell processes form a distinct pedunculus (P), but no calyx at this early stage. (B) The fifth instar larva of *A. mellifera* has a similar mushroom body morphology, in which the pedunculus is clearly visible beneath the neuroblast clusters, but the calyx has not yet formed. Scale bars A = 25 μm, B = 50 μm.
neuropil development, the medial and vertical lobes would be seen to arise at slightly staggered intervals as in the embryonically derived mushroom bodies.

3.2. Post-embryonic neurogenesis and its regulation

Whether generating Kenyon cells or additional neuroblasts, the MBNBs are continuously mitotically active throughout the majority of brain development (Panov, 1962; Truman and Bate, 1988). In D. melanogaster, the brain of the newly hatched larva contains only a few mitotically active progenitors, these being the optic Anlagen, the lateral neuroblast (which contributes to the antennal lobe) and the four MBNBs (Ito and Hotta, 1992; Truman and Bate, 1988). This scarcity of neurogenesis at larval hatching has allowed for relatively selective chemical ablation of the mushroom bodies by feeding first instar larvae doses of the DNA synthesis inhibitor hydroxyurea (deBelle and Heisenberg, 1994). In all holometabolous insects surveyed thus far the MBNBs do not enter a quiescent stage but appear to divide continuously for the duration of larval development (Nordlander and Edwards, 1970; Truman and Bate, 1988; Ito and Hotta, 1992; Datta, 1995; Farris et al., 1999). Mitotic activity continues until the mid- to late pupal stage (Nordlander and Edwards, 1970; Truman and Bate, 1988; Ito and Hotta, 1992; Farris et al., 1999; Ganeshina et al., 2000). In A. mellifera the MBNBs undergo programmed cell death in the mid-pupal stage and disappear before adult eclosion (Fahrbach et al., 1995; Farris et al., 1999; Ganeshina et al., 2000). The MBNBs also appear to be lost during the pupal stage in D. melanogaster, with scattered neurogenesis in the adult mushroom bodies attributed to the last divisions of persisting GMCs (Technau, 1984; Ito and Hotta, 1992). Persistence of MBNBs and adult neurogenesis in the mushroom bodies of holometabolous insects has been reported only in species of Coleoptera (Bieber and Fuldni, 1979; Cayre et al., 1996).

In hemimetabolous insects MBNB activity is also constant throughout the juvenile stages and adult neurogenesis is more widespread (Cayre et al., 1996; Farris and Strausfeld, 2001; Malaterre et al., 2002). MBNB activity in the adult brain does not always produce Kenyon cells, as newborn cells in the adult mushroom bodies of P. americana and Locusta migratoria L. (Orthoptera, Acrididae) express glial cell markers (Cayre et al., 1996). Members of the orthopteran family Gryllidae and the cockroach Diploptera punctata Eschscholtz (Dictyoptera, Blaberidae), however, continue adding Kenyon cells to the mushroom bodies during adult life (Cayre et al., 1994, 1996; Gu et al., 1999). In the case of A. domestica MBNB activity after adult eclosion is extensive, contributing up to 20% of the total Kenyon cell volume in 50 day old adults (Malaterre et al., 2002).

Adult neurogenesis in insect mushroom bodies is regulated by the actions of juvenile hormone (JH) and ecdysteroids, as are many other aspects of nervous system development. In A. domestica, in which neurogenesis in the mushroom bodies continues for the duration of adult life, mitotic activity of MBNBs decreases in response to naturally occurring and artificially induced increases in circulating ecdysone titers, and increases in the presence of JH (Cayre et al., 1994, 1997a, 2000b). Both hormones influence polyamine metabolism (Cayre et al., 1995, 1997a), and the polyamine putrescine has been shown to be an important mediator of the mitogenic effect of JH (Cayre et al., 1997b). Different polyamines have subsequently been proven to specifically induce neurogenesis (putrescine) or cell differentiation (spermine and spermidine) in cultured A. domestica Kenyon cells (Cayre et al., 2001).

In the blaberid cockroach D. punctata, the pattern of adult mushroom body neurogenesis and its hormonal control differs greatly from that of A. domestica. In D. punctata adults, mitotic activity does not continue indefinitely but rather declines gradually after adult eclosion and eventually ceases at about eight days of age (Gu et al., 1999). During this short period of mitotic activity in D. punctata JH has no apparent effect on neurogenesis in the mushroom bodies. The ecdyseroid 20-HE enhances proliferation, and 20-HE application in older insects can also prolong MBNB activity beyond eight days of age (Gu et al., 1999), a reverse effect from what is observed in A. domestica. The contradictory roles of JH and ecdyseroids in the two insect species may reflect the disparity in duration of MBNB activity after adult eclosion. For example, novel responses to JH and ecdysteroids may be necessary for the MBNBs of A. domestica, which continue to divide through multiple cycles of hormone fluctuations that are correlated with the reproductive cycle.

Adult neurogenesis in A. domestica is also regulated by interaction with the environment. In an invertebrate correlate of the classic ‘enriched environment’ studies in the rat (Volkmar and Greenough, 1972; Greenough and Volkmar, 1973), housing of A. domestica nymphs in complex environments was shown to increase Kenyon cell production by MBNBs in the newly eclosed adult (Scotto-Lomassese et al., 2000). The effect of environmental complexity on neurogenesis appears to be directly mediated by sensory input, as antennal ablations and removal of visual stimuli by painting the eyes removes the capacity for increased neurogenesis in enriched rearing conditions (Scotto-Lomassese et al., 2002). Environmental regulation of MBNB activity is likely to be an important component of mushroom body plasticity in A. domestica, but the sparse distribution of adult neurogenesis across the remaining insect orders indicates that this is not the case for most insects. In these insects mushroom body plasticity must instead be accomplished entirely by the modification of existing neurons. For example, increased mushroom body neuropil volume and Kenyon cell outgrowth in response to the acquisition of foraging experience has been observed in A. mellifera, in which adult neurogenesis is absent (Withers
et al., 1993; Durst et al., 1994; Fahrbach et al., 1995; Withers et al., 1995; Farris et al., 2001). Coss et al. (1980) also described morphological changes in Kenyon cell dendritic spines that appeared to be associated with foraging behavior. A subsequent paper by Brandon and Coss (1982) reported that these changes could be rapidly induced by a single orientation flight. The limitations of the experimental methods employed in these studies, however, prevent definitive conclusions about the effects of foraging on dendritic spine morphology. A later study on age- and experience-related changes in honeybee Kenyon cell dendrites failed to find any robust changes in spine morphology after the first day of adult life (Farris et al., 2001).

3.3. Generation and organization of Kenyon cell subpopulations

Neurogenesis patterns during development provide clues about the organization of Kenyon cells in the adult mushroom bodies. In the mushroom bodies of A. mellifera, three Kenyon cell types can be easily identified by the size and location of their cell bodies (Panov, 1957; Mobbs, 1982) (Fig. 1). They are arranged concentrically, such that one subpopulation with small cell bodies resides in the center of the calyx (inner compact), and the rest of the calyx is filled by large cell bodies (non-compact). Another subpopulation of Kenyon cells with small cell bodies lies outside of the calyx (outer compact; after the terminology of Kenyon cells with small cell bodies lies outside of the cell body region, even lying outside of the calyx cup in insects such as P. americana and again in A. mellifera (Farris et al., 1999; Farris and Strausfeld, 2001). Recent studies in D. melanogaster in which single cell and neuroblast clones were induced at different times in development have again confirmed that Kenyon cells born earlier in development occupy positions increasingly more distal to the MBNBs as development proceeds (Kurusu et al., 2002). This ordering of Kenyon cells about the calyx has therefore been observed in all insects surveyed so far, whether hemimetabolous or holometabolous. Birthdate dependent ordering of cell soma with respect to the calyx, generated by the passive movements of Kenyon cells away from the MBNBs, is a highly conserved aspect of mushroom body organization.

In A. mellifera there are at least three Kenyon cell subpopulations with characteristic cell body morphologies (Mobbs, 1982; Strausfeld, 2002). These concentric populations are an obvious indicator that the Kenyon cells are not an isomorphic pool of neurons. Kenyon cells further vary with respect to their dendritic and axonal morphology, neuropeptide contents and other aspects of gene expression (Mobbs, 1982; Kucharski et al., 1998; Farris et al., 1999; Strausfeld et al., 2000; Takeuchi et al., 2001; Strausfeld 2002). Analyses of the progeny of individual MBNBs in D. melanogaster show that all MBNBs contribute to the generation of each Kenyon cell subtype (Ito et al., 1997). As D. melanogaster possesses only four MBNBs per hemisphere, the mushroom bodies are thus composed of four identical ‘clonal units’, each containing all Kenyon cell subpopulations and each arising from a single neuroblast (Ito et al., 1997). This quadripartite structure of the mushroom bodies of D. melanogaster has also been revealed by the expression patterns of P[GAL4] lines exhibiting mushroom body labeling (Yang et al., 1995). In the calliphorid fly P. regina, in which the mushroom bodies are generated by five MBNBs, the resulting neuropil displays a five-fold structure (Gundersen and Larsen, 1978); however, immunohistochemical studies of another calliphorid fly, Phaenicia sericata Meigen (Diptera, Calliphoridae) reveal four-fold symmetry in the mushroom bodies (Sinakevitch and Strausfeld, 2002). MBNB number has not been determined in P. sericata; if four MBNBs are present as in D. melanogaster, a similar four-fold symmetry of the mushroom bodies may be expected. Further comparative studies will therefore be necessary to clarify the relationship between neuroblast number and neuropil organization in the Diptera.
Kenyon cell subpopulations are each born during a distinct developmental period. This is reflected by the organization of their cell bodies into discrete groups around the calyx. Due to their distinct cell body morphology, Kenyon cell subpopulations in *A. mellifera* suggest that the generation of one cell type begins only when the generation of the previous cell type is completed (Panov, 1957; Farris et al., 1999). For example, the onset of generation of non-compact cells is clearly marked by the appearance of their large cell bodies in the prepupal stage; treatment of prepupae with BrdU labels only this cell population (Farris et al., 1999). In *A. domestica*, all of the large Kenyon cells that supply the posterior calyx are born prior to hatching, as are the Class II and Class III Kenyon cells of *P. americana* (Panov, 1966; Farris and Strausfeld, 2000; Farris and Strausfeld, 2001; Malaterre et al., 2002; Farris and Strausfeld, 2003). Analysis of multicellular and single Kenyon cell clones generated at different time points in mushroom body development have confirmed the sequential generation of intrinsic neuron subtypes in *D. melanogaster* (Armstrong et al., 1998; Lee et al., 1999). Specifically, Kenyon cells that make up the γ lobe of the adult are generated from embryogenesis until the middle of the third instar. Those providing the α/β lobes are generated in the late larval stage, and those providing the α/β lobes appear after puparium formation. As these transitions coincide with the prepupal ecdysone peak and the pupariation event (also a period of high ecdysone), respectively, Lee et al. (1999) have suggested that cell type switching by neuroblasts may be under control of this hormone. The sequence of lobe neuropil formation is thus age-dependent as well, and represents another highly conserved characteristic of the developmental organization of the mushroom bodies (See ‘Process Outgrowth and Plasticity’ below). The early-generated Kenyon cells of the *D. melanogaster* γ lobe, also known as clawed or Class II Kenyon cells, have been identified in a number of insect species and are typically the first born mushroom body intrinsic neurons (Malzacher, 1968; Farris et al., 1999; Farris and Strausfeld, 2001; Malaterre et al., 2002). The later born Class I or spiny Kenyon cells appear to make up the remainder of the mushroom bodies. Each Kenyon cell subpopulation is thus defined not only by the shared morphology of its

Fig. 4. Kenyon cells born at different times in development demonstrating age-dependent concentricity in the adult honeybee mushroom bodies. Frontal sections of a single mushroom body calyx. (A) BrdU label applied to feeding fifth instar larva is localized in the outer compact cells (oc, arrows), indicating that these cells were born at the time of BrdU treatment. (B) BrdU applied to the D1 pupa marks the transition from production of non-compact (nc) to inner compact (ic) Kenyon cells (arrows). (C) BrdU labeling of the D5 pupa marks the last-born inner compact Kenyon cells at the center of the calyx (arrow). Scale bars = 50 μm.
neurons but by their time of generation during mushroom body development.

3.4. Conserved and divergent aspects of neurogenesis in the mushroom bodies

The basic progression of Kenyon cell generation during mushroom body development is conserved across a wide variety of insect taxa. Kenyon cells are produced by 2–4 aggregates of MBNBs, the number of which corresponds to the total number of calyces. Cell bodies are concentrically arranged by birthdate around the proliferative center, such that the earliest born cells are found at the outer edges of the calyx and the latest at the center, displacing the MBNBs in insects which lose these progenitors later in life. Distinct Kenyon cell types are produced during discrete blocks of time at specific periods in development, with the apparently ubiquitous clawed (γ) Kenyon cells typically being produced first.

An important difference in the general progress of neurogenesis between described species is the timing of specific developmental events. It is possible that these differences in the developmental timeline reflect the variety of early life histories and behavioral repertoires of the species studied (Farris et al., 1999; Farris and Strausfeld, 2001). In hemimetabolous insects the nymph must search for food and shelter immediately after hatching, thus necessitating the presence of functioning higher brain regions at an early stage in development. The embryonic stage is thus elongated and the brain resembles a smaller version of that of the adult at hatching. Holometabolous insect larvae, in contrast, typically hatch on or in their source of food and shelter and perform few non-feeding or defensive behaviors within the first few days. Such simple behaviors may not require the mushroom bodies; indeed, sensory centers such as the eyes and antennal lobes are often rudimentary in newly hatched larvae, and the necessity of a functioning higher sensory integration center is thus negligible at this time. Mushroom body development can thus be relatively incomplete at hatching and can be protracted into the larval and the even more behavior-poor pupal stage. This developmental delay is taken to its extreme in the social Hymenoptera, which are completely cared for by adult workers and correspondingly have in their early instars poorly developed brains in which Kenyon cell production does not begin until several days after hatching (Panov, 1957; Farris et al., 1999) (Fig. 5).

Other differences in neurogenesis patterns involve the number of MBNBs and the duration of their proliferative activity. Such variables are presumably tailored to the unique structural characteristics of the mushroom bodies of an individual insect, which may in turn be influenced by behavioral ecology. For example, the largest numbers of MBNBs are found in the social Hymenoptera, which have large mushroom bodies consisting of hundreds of thousands of Kenyon cells. Perhaps, as was initially proposed by Dujardin (1850), the behavioral demands of social behavior necessitate the development of a particularly complex sensory integration center. The persistence of MBNB activity in the imago of some insect species may similarly be influenced by life history, perhaps being most common in long-lived species that spend a significant portion of their lives in the adult stage. Future comparative studies of mushroom body neurogenesis will be necessary in order to determine how insect life history and neurogenesis in a complex brain region are correlated.
4. Process outgrowth and plasticity

4.1. Formation of the pedunculus and lobes

The axons forming the layered pedunculus and lobes transmute from a concentric to a laminar organization as they proceed distally from the cell soma. Kenyon cell neurites and axons are organized concentrically within the calyx and/or proximal pedunculus, but typically unfold into a flat laminar organization in the distal pedunculus and lobes. The point at which this concentric to laminar transition occurs varies from species to species. In *P. americana* and *A. mellifera* the transition to laminae occurs almost immediately beneath the calyx (Strausfeld and Li, 1999; Strausfeld 2002), while in *D. melanogaster* the concentric organization unfolds further distal, forming three or more individual lobe systems that are arranged anterior to posterior as are laminae in larger insects (Strausfeld et al., 2003). In *A. domestica* and *Schistocerca gregaria* Forskal (Orthoptera, Acrididae) the axons of the vertical lobe remain concentrically organized, while those in the medial lobe unfold into a more typical laminar arrangement (O’Shea et al., 1998; Malaterre et al., 2002).

Annuli and laminae in the pedunculus and lobes are added sequentially during mushroom body development. Annuli are arranged by decreasing age from the outside of the neuropil inwards (Yang et al., 1995; Tettamanti et al., 1997; Armstrong et al., 1998; Kurusu et al., 2002; Malaterre et al., 2002), while laminae are arranged from anterior (oldest) to posterior (newest; Farris and Strausfeld, 2001; Farris and Strausfeld, unpublished data). The axons of newborn Kenyon cells enter the pedunculus and lobes via a...
discrete tract and are phenotypically quite distinct from mature Kenyon cells. This ‘core’ is found in the center of concentric neuropils, and is shifted gradually more posterior as the concentric arrangement unfolds into laminae in the distal pedunculus and lobes. In laminar neuropils, the core forms the posteriormost ‘ingrowth’ lamina (Farris and Strausfeld, 2001; Kurusu et al., 2002; Strausfeld et al., 2003)

In P. americana, the anteriormost and earliest identified lamina in the lobes (termed the γ layer) is composed of the branched axons of the embryonically produced clawed Kenyon cells (Strausfeld and Li, 1999; Farris and Strausfeld, 2001; Sinakevitch et al., 2001). Additional laminae are added to the posterior edge of the lobe one by one throughout nymphal development via the ingrowth lamina (Farris and Strausfeld, 2001; Sinakevitch et al., 2001) (Fig. 6). In D. melanogaster, the axons of the early-born clawed Kenyon cells make up the outer sheath of the concentric pedunculus, with progressively younger annuli added internally due to the ingrowth of axons through the centrally-located core (Armstrong et al., 1998; Verkhrusha et al., 2001; Kurusu et al., 2002). Further distal, the pedunculus unfolds into three major lobe systems (γ, α/β′ and α/β) that are arranged by birthdate from anterior to posterior (Crittenden et al., 1998; Lee et al., 1999). The γ lobe occupies the most anterior position exactly as does the γ layer in the laminar medial and vertical lobes of larger insects such as A. mellifera and P. americana (Strausfeld and Li, 1999; Strausfeld 2002).

Kenyon cells with axons in the ingrowth core or lamina are distinct from those forming older laminae, likely due to their immature nature. The axons occupying the core are characteristically delicate, and in P. americana are decorated with numerous growth cone-tipped filaments (Tschau and Heisenberg, 1982; Farris and Strausfeld, 2001; Sinakevitch et al., 2001; Kurusu et al., 2002). Newborn Kenyon cells do not express most markers common to mature Kenyon cells (Armstrong et al., 1998; Crittenden et al., 1998; Farris and Strausfeld, 2001; Sinakevitch et al., 2001). The extending axons, however, contain large amounts of f-actin and therefore have a high affinity for the mushroom toxin phalloidin (Kurusu et al., 2002). In the D. melanogaster calyx, four tracts are observed, one from each neuroblast clonal group; these four tracts fuse into one core as they progress into the pedunculus and lobes. As would be expected, application of this non-species specific label to the developing mushroom bodies of P. americana and other insects invariably identifies Kenyon cell axons making up a discrete ingrowth lamina or core (Fig. 7(C), (D) and (G)). As in D. melanogaster, a number of smaller tracts are typically seen to exit the calyces, fusing distally into a single tract as they progress deeper into the pedunculus. Exceptions to the fusing of core fibers into a single tract occur in some Orthoptera and Coleoptera. In coleopteran species, the core fibers from each calyx remain as two separate tracts throughout the pedunculus and lobes (Bretscheider, 1914; Larsson et al., 2002). In the mushroom bodies of S. gregaria the core splits into six separate tracts in the vertical lobe and forms a broad ingrowth lamina in the posterior medial lobe (O’Shea et al., 1998; S.M. Farris and I. Sinakevitch, unpublished observations). Regardless of the specific morphology, the core region of each species occupies the same relative region of the pedunculus and lobes throughout development, therefore providing the basis for an age-dependent organization of axons in the mushroom body lobes of a wide variety of insect taxa.

4.2. Unusual characteristics of core Kenyon cells

Newborn Kenyon cells are therefore distinct from mature Kenyon cells, but there is some evidence that they are already taking on complex functions at this early stage in maturation, and perhaps contributing to the organization of other neurons. In P. americana, many of the axons in the ingrowth lamina produce a dense system of collaterals that extend roughly perpendicular to the long axis of the medial and vertical lobes (Farris and Strausfeld, 2001) (Fig. 7(A)–(C)). These collaterals are only produced by Kenyon cells with axons in the ingrowth lamina, and are readily visualized with phalloidin as are the main axons. Ingrowth lamina collaterals apparently identical to those in P. americana can be observed in other members of the Blattaria and in basal Isoptera, and a dense network of fibers reminiscent of the dictyopteran collateral system is observed to surround the core in A. domestica (S.M. Farris, unpublished observations). In the holometabolous insects, core fiber collaterals are particularly evident in the medial lobe of A. mellifera, and minute collaterals have been observed in D. melanogaster (Farris and Strausfeld, 2003). These structures therefore appear to represent a common morphological characteristic of newborn Kenyon cells. The function of collaterals is unknown, although they have been proposed to serve as guidance cues for lobe extrinsic neurons, which must constantly reorganize their processes in order to accommodate Kenyon cells added throughout nymphal development in hemimetabolous insects. The collaterals, which extend from the ingrowth lamina to the anterior surface of the lobes where extrinsic neurons enter, may provide a scaffold along which these neurons can grow and make contact with newly added Kenyon cell axons (Farris and Strausfeld, 2001). Whether collaterals perform a similar function in holometabolous insects, in which mushroom body development occurs over a much shorter time span, remains to be determined.

Although axons of the ingrowth lamina or core are typically characterized by an apparent lack of expression of most mushroom body markers, they do show a high affinity for the putative neurotransmitter glutamate (Sinakevitch et al., 2001) (Fig. 7(B), (E), (F) and (H)). In P. americana, the ingrowth lamina and collateral system is strongly labeled by anti-glutamate antibody; such labeling is not observed in any other Kenyon cell population. Anti-glutamate immunoreactivity is also observed in core fibers
of the holometabolous insects *A. mellifera* and *D. melanogaster*, (Strausfeld et al., 2003) indicating that this is a widespread characteristic of newborn Kenyon cells. Interestingly, in *A. domestica*, glutamate immunoreactivity is found in a population of Kenyon cells forming the posterior calyx (Schürmann et al., 2000) as well as in fibers making up only the outer ring of the core. Unlike the other insects surveyed, the centralmost part of the core in *A. domestica* shows no affinity for anti-glutamate antibody.

Due to the immature nature of its constituent axons, the ingrowth lamina or core consists of a constantly revolving population of neurons. Axons extending from newborn Kenyon cells into the core are soon pushed into the periphery by still younger fibers. The production of collaterals and glutamate seems, therefore, to be a transient stage in the maturation of Kenyon cells. Such a dramatic change in phenotype may be considered a transdifferentiation-like event, similar to that seen in the radial glia of the mammalian brain (Chanas-Sacre et al., 2000). The distinct morphology and putative neurotransmitter profile observed in ingrowth lamina and core Kenyon cells indicates that they serve a unique function prior to their final differentiation into the more typical morphology of Kenyon cells occupying the remainder of the lobes. Further investigations into the immature phenotype of Kenyon cells will be necessary to determine whether these cells are serving as transient guidance cues for extrinsic neurons or are playing other roles required for their proper integration into the mushroom body circuitry.

Studies of *D. melanogaster* mutants with axon branching defects in the mushroom bodies provide evidence that some Kenyon cells may perform guidance functions, at least for other intrinsic neurons. The mushroom bodies of flies with mutant copies of the *Dscam* gene (the *D. melanogaster* homologue of the mammalian Down syndrome cell adhesion molecule gene) show a variety of axonal branching and guidance defects, often leading to the loss of the medial or vertical lobe (Wang et al., 2002). Individual mutant neurons produce supernumerary axonal branches that often fail to project into both lobes. The morphology of wild type Kenyon cells in mosaic mushroom bodies depends on the time in development when the mutant phenotype is revealed in a subset of Kenyon cells. When homozygous mutant clones are induced before the onset of *αβ* Kenyon cell production, loss of the medial or vertical lobe is often observed. In these insects, the wild-type *αβ* neurons produced later in development by non-recombinant neuroblasts continue to follow the mutant projection pattern. A similar mutant phenotype and cell non-autonomous effect was observed after induction of Rac GTPase (Ng et al., 2002) and irto (Awasaki et al., 2000) homozygous mutant neuroblast clones. It thus appears that Kenyon cells in the mushroom bodies of *D. melanogaster* play a significant role in regulating proper branching and outgrowth in subsequently produced intrinsic neurons.

### 4.3. Formation of the calyces

Many authors have noted that development of the calyx lags behind that of the lobes in most insects, indicating that Kenyon cells produce axons first, and then dendrites. Calyces are entirely absent in basal insects like the Odonata (dragonflies and damselflies) and in those with secondarily rudimentary antennae and antennal lobes such as the aquatic dytiscid beetles (Strausfeld et al., 1998). In these insects, afferent input necessarily enters the pedunculus and lobes directly. The late addition of calyces in development together with their absence in basal insects has led to speculation that these neuropils are relatively recent additions to the insect mushroom bodies (Strausfeld et al., 1998). Expression patterns of P[GAL4] lines, which exhibit either 4-fold (corresponding to each neuroblast) or 2-fold symmetry, indicate that two of the MBNBs are more closely associated to each other (Yang et al., 1995). In the pedunculus, the four fiber tracts exiting the calyx initially fuse into two tracts, and finally into a single axon bundle (Ito et al., 1997; Strausfeld et al., 2003). The single calyx of insects such as *D. melanogaster* is therefore believed to represent the fusion of two calyces, each derived from two neuroblasts (Ito et al., 1997). It has also been proposed, however, that most double calyces are derived from the duplication of a structure similar to the single anterior calyx of the Orthoptera, which is made up of the clawed and spiny Kenyon cells commonly observed in most insect species. 

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Fig. 7. Morphological and immunochemical characteristics of ingrowing Kenyon cell axons across insect taxa. The ingrowth lamina or core is indicated by an arrow in all panels. (A)–(C) are sagittal sections, (D)–(H) are frontal sections. (A) In the adult cockroach (*P. americana*), Golgi impregnated sagittal sections through the medial lobe reveal axons of the ingrowth lamina and the associated collateral system reaching across the width of the lobe. (B) The ingrowth lamina of the adult cockroach exhibits glutamate-like immunoreactivity as does the collateral system. (C) Ingrowth lamina axons and collaterals in the cockroach nymph also show a strong affinity for phalloidin (green), which labels f-actin. Antibodies directed against the catalytic subunit of the adhesion molecule gene) show a variety of axonal branching and guidance defects, often leading to the loss of the medial or vertical lobe (Wang et al., 2002). Individual mutant neurons produce supernumerary axonal branches that often fail to project into both lobes. The morphology of wild type Kenyon cells in mosaic mushroom bodies depends on the time in development when the mutant phenotype is revealed in a subset of Kenyon cells. When homozygous mutant clones are induced before the onset of *αβ* Kenyon cell production, loss of the medial or vertical lobe is often observed. In these insects, the wild-type *αβ* neurons produced later in development by non-recombinant neuroblasts continue to follow the mutant projection pattern. A similar mutant phenotype and cell non-autonomous effect was observed after induction of Rac GTPase (Ng et al., 2002) and irto (Awasaki et al., 2000) homozygous mutant neuroblast clones. It thus appears that Kenyon cells in the mushroom bodies of *D. melanogaster* play a significant role in regulating proper branching and outgrowth in subsequently produced intrinsic neurons.
Weiss, 1981). Again, further comparative studies of calyx anatomy and development in a wider range of insect species will be necessary to determine with certainty the origins of this neuropil.

Calycal development has been studied in relatively less detail than lobe development. In D. melanogaster, little has been reported aside from the fact that the neuropil steadily increases in size until pupariation, at which point massive degeneration and subsequent regrowth into the adult structure occurs (Lee et al., 1999). Like the cell bodies themselves, the calyces are divided into four equivalent units, each unit being supplied by the Kenyon cells produced by a single neuroblast (Yang et al., 1995). Golgi impregnation studies have revealed several distinct dendritic morphologies among Kenyon cells of the dipteran species D. melanogaster and Musca domestica L. (Diptera, Muscidae; Strausfeld, 1976; Strausfeld et al., 2003). The developmental organization of specific subtypes of Kenyon cell dendrites in D. melanogaster has not been described to date.

The development of subdivisions of the calyces and their constituent Kenyon cells has been best described in A. mellifera. In this insect the calyces, like the cell somata, can be divided morphologically into three main subdivisions, the lip, collar and basal ring (Mobbs, 1982). Although sequentially arranged from dorsal to ventral in the calyx, these three regions are not produced in their entirety strictly by birth-date as are the cell soma and lobe regions. The calyx is first visible in the prepupal honeybee. Panov (1957) reported that the basal ring forms first in development, in contrast to Farris et al. (1999) who reported that this neuropil region differentiates last, during the third to fourth day of the pupal stage (D3–D4). In his account, Panov describes the early born outer compact Kenyon cells as providing dendrites only to the basal ring, which may have led him to conclude that the initial rudiments of the calyx present in the prepupa must correspond to this region. It has subsequently been shown, however, that outer compact Kenyon cells are in fact clawed Kenyon cells, which in A. mellifera will eventually contribute to all three regions of the calyx (Strausfeld, 2002). A similar organization is observed in D. melanogaster (Yang et al., 1995). The dendrites of subsequently born non-compact (spiny) cells are therefore not stacked on top of the basal ring as proposed by Panov, but rather are integrated within the early calyx. Both accounts agree that the lip and collar region are first distinguishable at D2–D3 of the pupal stage and appear to arise simultaneously (Panov, 1957; Farris et al., 1999) (Fig. 8). The late formation of the basal ring coincides with the birth of the inner compact cells whose dendrites, along with those of the outer compact cells, make up this neuropil region (Farris et al., 1999; Strausfeld, 2002). Olfactory projection neurons, the primary afferents to the lip region of the calyx, are already present in the dorsal calyx by the first day of the pupal stage (Schröter and Malun, 2000). The differentiation of calycal regions according to afferent innervation patterns may therefore occur even before

Fig. 8. Successive stages of calyx development in the honeybee pupa. Frontal sections, dorsal to the top. (A) The calyces (C) are visible in the D1 pupa as balls of neuropil residing below each neuroblast aggregate (Nb). All three Kenyon cell populations (oc, nc, ic) can be identified from this stage onwards. (B) Increased calyx growth and decreased size of the neuroblast aggregate is apparent in the D2 pupa. The division between the lip and collar regions of the calyces (arrow) are first visible at this stage. Glial cells entering the ventral calyx may indicate the onset of differentiation of the basal ring (arrowheads). (C) The calyces clearly begin to resemble those of the adult, with distinct lip (arrow) and collar regions, in the D3 pupa. Growth of this neuropil is accompanied by a steady decline in size of the neuroblast aggregates, whose constituent cells undergo programmed cell death by D5–D6. Glial cells outline the developing basal ring region (arrowheads). Ic, inner compact Kenyon cells; nc, non-compact Kenyon cells; oc, outer compact Kenyon cells. Scale bars = 50 μm.
pupation, and must be mediated by the clawed Kenyon cells whose dendrites are the sole constituents of the prepupal calyx (Panov, 1957).

Prior to adult eclosion, the dendritic branches of spiny Kenyon cells in the lip and collar region of the A. mellifera mushroom bodies are decorated with many short, filamentous fibers (Farris et al., 2001). In the adult these structures are replaced by stout spines, indicating that the filaments may be immature spines that obtain their mature morphology only after adult eclosion.

In P. americana, most Kenyon cells produce several dendritic arborizations along their entire dorsal–ventral axis, rather than being confined to a discrete calycal sub-region as in A. mellifera. Exact correlations between cell birthdate and the positions of individual dendritic arbors are not immediately evident, although the last-born Kenyon cells do tend to provide arborizations to the more ventral regions of the calyx (Mizunami et al., 1998a; Farris and Strausfeld, 2001). The dendrite-producing neurites, however, are contained within discrete age dependent layers in the parallel fiber containing inner calyx wall (zona interna of Weiss, 1974). Kenyon cells whose cell bodies are ventral in the calyx and whose axons make up the most posterior laminae in the lobes (and are thus the most recently born) send their neurites along the innermost margin of the zona interna (Mizunami et al., 1998a; Sinakevitch et al., 2001). Neurites occupy progressively deeper positions in the zona interna as their age increases, and the neurites of clawed Kenyon cells are so deep as to pass directly through the synaptic neuropil of the calyx (Mizunami et al., 1998a; Strausfeld and Li, 1999). A newly discovered class of Kenyon cells in the cockroach, termed Class III, perpetuate this trend to its extreme by projecting along the outer surface of the calyces before entering the pedunculus (Farris and Strausfeld, 2003). As expected, these cells appear to be the first produced mushroom body intrinsic neurons in the P. americana embryo.

In A. domestica, the unusual calyx configuration of the adult has a correspondingly unique developmental story. Unlike the double calyces of most insects which appear to be made up of equivalent groups of Kenyon cells, the anterior and posterior (or accessory) calyces of the Orthoptera are formed from different intrinsic neuron subtypes (Panov, 1966; Schürmann, 1973; Weiss, 1981; Malaterre et al., 2002). The posterior calyx arises before the anterior calyx in embryonic development (Panov, 1966; Malaterre et al., 2002). The first formed posterior calyx appears to be composed of a population of Kenyon cells that, based on morphological similarities, may be homologous to the embryonically-produced Class III Kenyon cells of some Dictyoptera (Schürmann, 1973; Farris and Strausfeld, 2003). This structure also contains clawed Kenyon cells in A. domestica (Schürmann, 1973), although Weiss (1981) reported that these cells contribute instead to the anterior calyx in the acridid Orthoptera. The anterior calyx is also composed of dendrites of spiny Kenyon cells, and grows increasing larger through nymphal and adult development due to continued production of these Kenyon cells by the MBNBs (Malaterre et al., 2002).

4.4. Metamorphic plasticity of clawed Kenyon cells

Massive reorganization of the nervous system is characteristic of metamorphosis, and has been extensively described in the ventral nerve cord. As in other regions of the nervous system, metamorphosis of the mushroom bodies consists of restructuring of larval processes combined with the generation of adult-specific neurons (reviewed in Consoulas et al., 2000). Metamorphic restructuring of the mushroom bodies of the dipteran brain was noted in P. regina (Gundersen and Larsen, 1978), but was first described in detail in D. melanogaster (Technau and Heisenberg, 1982). By counting axon profiles in cross-sections of the pedunculus, the authors observed a 40% decrease in the number of axons within 12 h after puparium formation, followed by an increase in axon number beyond that observed prior to metamorphosis (Fig. 9). A bundle of extremely thin fibers forming a tract through the center of the pedunculus was observed to persist throughout the period of degeneration. This tract was frequently absent in mushroom bodies deranged (mbd) and mushroom body

![Fig. 9. Selective labeling of clawed Kenyon cells reveals reorganization during metamorphosis in D. melanogaster (from Lee et al. 1999).](image)
defect (mud) mutants, in which the lobes were malformed only in the adult. It was therefore proposed that the thin fiber tract could provide guidance cues for the fibers that repopulate the pedunculus and lobes in the adult.

The identity of the reorganizing and persisting Kenyon cell populations proved somewhat elusive. The first hint as to the result of axon reorganization was provided when Yang et al. (1995) determined that clawed Kenyon cells produced unbranched axons in the medial $\gamma$ lobe of the adult. Armstrong et al. (1998) subsequently discovered that these neurons are produced early in development, and affirmed that the larval mushroom bodies contain only a single vertical/medial lobe pair. Further evidence from enhancer trap lines that labeled both of the larval lobes and the adult $\gamma$ lobe led to the conclusion that these structures are composed of the same population of Kenyon cells (Armstrong et al., 1998). After axon degeneration, the adult $\gamma$ lobe was seen to arise first. The $\alpha/\beta$ lobes were the last to appear, indicating that their constituent Kenyon cells were adult-specific (Armstrong et al., 1998; Lee et al., 1999). Using the MARCM technique to label small subpopulations of cells born at specific times in development (Lee and Luo, 1999), the degeneration and reorganization of larval clawed Kenyon cells into the adult $\gamma$ lobe and the pupal origin of the neurons making up the adult $\alpha/\beta$ lobes was confirmed (Lee et al., 1999). The $\alpha/\beta$ lobes first identified by Crittenden et al. (1998) are made up of neurons that are born after the clawed Kenyon cells but before puparium formation and do not undergo reorganization (Lee et al., 1999). The persistent thin fiber tract described by Technau and Heisenberg (1982) is therefore likely composed of the axons of $\alpha/\beta$ neurons (Kurusu et al., 2002).

Reorganization of clawed Kenyon cells in the mushroom bodies of D. melanogaster occurs during a period of increasing ecdysteroid titers in the pupa (Kraft et al., 1998). The ecdysone receptor (EcR), particularly the EcR-B1 isoform, is highly expressed in the clawed Kenyon cells of the larval and early pupal mushroom bodies (Truman et al., 1994; Lee et al., 2000a). Cultured Kenyon cells isolated at this time respond to 20-hydroxyecdysone treatment with increased neurite outgrowth and branching (Kraft et al., 1998). The EcR protein forms a heterodimer with the ultraspiracle (usp) gene product in order to regulate gene expression (Yao et al., 1992, 1993). Clawed Kenyon cells of EcR and usp mutants fail to degenerate and retain the branched larval morphology into adulthood (Lee et al., 2000a). Interestingly, usp mutations generated in $\gamma$ neurons in vivo appear to disrupt neither initial axonal outgrowth nor continued growth throughout development. These experiments indicate that ecdysone likely plays an important role in the regulation of degeneration of clawed Kenyon cell axons, but its role in process outgrowth in vivo is less clear.

Reorganization of clawed Kenyon cells has thus far been reported only in D. melanogaster, so the question is whether metamorphic restructuring of the mushroom bodies is a common occurrence in holometabolous insects, or whether it is a unique characteristic of a highly derived species. Evidence for clawed Kenyon cell reorganization in other holometabolous insects is at the present indirect and relatively weak. The mushroom bodies of the moth Sphinx ligustri L. (Lepidoptera, Sphingidae) contains clawed Kenyon cells with unbranched axons that project medially as in D. melanogaster (Pearson, 1971). Both a medial and a vertical lobe is observed in larval Lepidoptera (Hanström, 1925; Nordlander and Edwards, 1968, 1970). Given the early origin of clawed Kenyon cells in all insects, it is perhaps possible that these neurons bifurcate in the larva and reorganize during metamorphosis into the unbranched adult form. In A. mellifera the picture is more complicated as only some clawed Kenyon cells are unbranched, the axons projecting into the vertical rather than the medial component of the $\gamma$ layer (Strausfeld, 2002). The developmental history of this subset of clawed Kenyon cells is at present unclear. The remaining branched clawed Kenyon cells of the adult A. mellifera have tiny medial axons forming a short medial $\gamma$ layer in the adult that appears to have persisted unchanged from the larval stage (Farris, Abrams and Strausfeld, unpublished observations). As these examples indicate, definitive evidence for metamorphic restructuring of clawed Kenyon cells exists only for D. melanogaster at present. A great deal of further study will be necessary before any conclusions on the extent of mushroom body reorganization in non-dipteran holometabolous insects can be made.

4.5. Conserved and divergent aspects of process outgrowth in the mushroom bodies

As with patterns of neurogenesis, there is much common ground in the developmental processes of neuropil formation across the insects. Although the dendritic neuropil of the calyces is not strictly arranged by birthdate, the neurites providing the arborization are, and this age-dependent ordering is maintained into the pedunculus and lobes. The calyces are established by the dendrites of clawed Kenyon cells, which represent the entire neuropil, and are subsequently filled in with the processes of later born spiny neurons. Whether concentric or laminar, axons of newborn Kenyon cells enter the lobes via a discrete tract, such that axons located progressively further from this core have correspondingly earlier birthdates. Lobes and layers made up of the early-born clawed Kenyon cells ($\gamma$) are constructed first. Newborn Kenyon cells with axons in the core display unusual phenotypes, such as the production of collaterals and glutamate immunoreactivity, indicating that they are functioning in some unknown, transient role prior to differentiating into mature Kenyon cells. A role for Kenyon cells as guidance cues for extrinsic and intrinsic neurons during development has been proposed, but remains as yet unproven. Finally, the presence of unbranched clawed Kenyon cells in the adult stage of holometabolous insects...
hint at a widely occurring process of metamorphic reorganization of these neurons.

No evidence of massive clawed Kenyon cell reorganization is observed in hemimetabolous insects, perhaps due to the absence of complete metamorphosis. In *P. americana* and *A. domestica*, clawed Kenyon cells maintain their medial and vertical branches throughout life (Schürmann, 1973; Strausfeld and Li, 1999). The drastic reorganization of the axonal projection pattern of clawed Kenyon cells in the holometabolous insects indicates that they likely have different targets and perform different functions in the larval and adult mushroom bodies (Technau and Heisenberg, 1982; Armstrong et al., 1998; Lee et al., 1999). Perhaps such reorganization is not necessary in the hemimetabolous insects, in which juvenile and adult behaviors are not as vastly divergent as those of holometabolous larvae and adults.

### 5. Genetic control of mushroom body development

Genetic studies in *D. melanogaster* have led to the discovery of a number of genes that guide mushroom body development. The first mushroom body structural mutants proved to be deficient in learning and memory, thus providing some of the first solid evidence for mushroom body functioning in these processes. Subsequent screens have isolated a variety of mutants in all aspects of mushroom body development. The majority of mutants described exhibit some manner of disruption of process outgrowth and branching, perhaps because the mutant phenotypes tend to be particularly severe and are therefore easily identified.

#### 5.1. Genes involved in neurogenesis

Two of the earliest described mushroom body structural mutants have since proved to have defects in neurogenesis. The *mushroom body defect* (*mbd*) and *mushroom bodies miniature* (*mbm*) mutants produce too many and too few Kenyon cells, respectively. In *mbd* mutants, up to 25 MBNBs are found in each brain hemisphere, rather than four in wild type brains, leading to a large increase in Kenyon cell number (Technau and Heisenberg, 1982; Prokop and Technau, 1994). The *mbm* gene encodes a coiled-coil protein containing conserved actin-binding motifs (Guan et al., 2000). The *mbm* phenotype is sex-specific, with adult females having a tiny portion of the wild type number of Kenyon cells, while males have normal or even larger mushroom bodies (Heisenberg et al., 1985). The female phenotype first manifests in the late larval stage. Another structural mutant that was identified in these initial screens, *mushroom bodies deranged* (*mbd*), has been reported to have decreased Kenyon cell number in the adult (Tettamanti et al., 1997), although no neurogenesis defect was noted in the original accounts (Technau and Heisenberg, 1982).

Other neurogenesis mutants typically exhibit smaller mushroom bodies resulting from a reduction in Kenyon cell number. Aside from being critically important for development of the compound eye, the ‘master gene’ *eyeless* (*ey*) is also expressed in mushroom body primordia and intrinsic neurons. Both hypomorphic and overexpression mutants display a decrease in Kenyon cell number (Callaerts et al., 2000; Noveen et al., 2000).

The *enok* and *rho A* genes, when mutant, produce immediate and severe defects in neurogenesis that arrest mushroom body development entirely. Only the γ lobe is produced in *enok* mutants due to MBNB arrest shortly after larval hatching (Scott et al., 2001). Induction of mutant clones later in development, however, leads to a similar gradual cessation of MBNB proliferation, so this gene is not specifically involved in clawed Kenyon cell production. *Enok* has been shown to encode a histone acetyltransferase; thus the observed mutant phenotype observed in the rapidly dividing MBNBs. The *rhoA* gene is involved in cytokinesis, and causes a similar rapid arrest of MBNB activity after induction of mutant clones (Lee et al., 2000b).

#### 5.2. Genes involved in process outgrowth and branching

The early neuroanatomical mutant screens turned up a number of genes that appeared to affect axon pathfinding, particularly during metamorphic reorganization. Along with increased neurogenesis in the larval stage, the mushroom bodies of *mbd* mutant flies exhibit a complete disruption of the adult lobes (Heisenberg, 1980). Degenerating axons in the early pupa are apparently unable to reenter the protocerebral neuropil to form the adult lobes, and instead form a large disorganized mass around the calyx. The *mushroom bodies deranged* (*mbd*) mutant displays a similar phenotype (Heisenberg et al., 1985; Heisenberg, 1989). The α/β core fibers are often missing in the early pupae of both of these mutants, providing additional evidence that these Kenyon cells guide the γ and α/β axons into the lobes during metamorphosis (Technau and Heisenberg, 1982).

*Ey* mutants display a wide variety of neuropil defects, which are highly variable depending on the particular mutant allele and genetic background. The *ey<sup>3D</sup>* and *ey<sup>DIDA</sup>* hypomorphic mutant alleles lead to a gross reduction in neuropil size in homozygous adults (Callaerts et al., 2000), while the *ey<sup>2</sup>* and *ey<sup>K</sup>* hypomorphic alleles cause few apparent adult defects but generate fused, reduced or absent lobes in larvae (Noveen et al., 2000). Interestingly, overexpression of *ey* in these flies also causes lobe fusion and reduction. Null *ey* mutant alleles *ey<sup>15.71</sup>* and *ey<sup>C7;20</sup>* cause minor defects in homozygous larvae, but Kenyon cell development appears to be arrested during the pupal reorganization so that adult neuropils are completely ablated (Kurusu et al., 2000).

*Dachshund* (*dac*), another regulatory gene primarily
known for its role in eye development, is expressed in mature Kenyon cells throughout development (Kurusu et al., 2000; Martini et al., 2000). Null mutant homozygotes have misdirected or missing vertical lobes in the larval mushroom bodies and disorganized medial lobes in the pupa and adult, with individual stray fibers observed throughout.

Although ey and dac act synergistically during eye development, studies using hypomorphic ey mutants initially failed to support such a relationship in the mushroom bodies. Experiments using ey null mutants, however, indicate that ey and dac work together in the mushroom bodies as they do in the eye. Dac heterozygotes were shown to enhance the abnormal phenotype of ey homozygous null mutants, and null double mutants showed a strong degeneration of the lobe neuropil in the adult (Kurusu et al., 2000).

Fasciclin II (fasII), a cell adhesion molecule best studied for its important role in axon fasculation in the embryonic nerve cord, is expressed in γ and α/β Kenyon cells of the D. melanogaster mushroom bodies (Cheng et al., 2001). As with ey mutants, phenotypes vary between the two mutant studies published to date. Fas II null and hypomorph mutants display no obvious adult phenotype (Cheng et al., 2001), while hypomorph mutant larvae often have reduced vertical lobes and fused medial lobes (Kurusu et al., 2002). In those larvae in which both lobes are reduced the calyces are often enlarged, indicating pathfinding defects that cause axons to bunch up around the calyx as in mud and mbd mutants. As might be expected in neurons defective for proteins involved in fascilitation, the axons of fasII null mushroom body clones do not respect the concentric arrangement of the pedunculus and lobes. Rather than entering the lobes through the core region, fasII mutant axons apparently penetrate the lobes at random and are thus irregularly distributed with no relation to birthdate (Kurusu et al., 2002). Overexpression also leads to disruption of the core in the developing mushroom bodies.

Other genes that appear to play roles in axon guidance in the mushroom bodies, particularly during reorganization, are members of the Rho and Rac GTPase cascades (Awasaki et al., 2000; Ng et al., 2002) and the D. melanogaster homologue of the human gene for Down syndrome cell adhesion molecule, DSCAM (Wang et al., 2002). Induction of trio (a member of the Rho GTPase cascade) and Rac GTPase mutant clones causes disruption of later born lobe systems in the adult, indicating non-cell autonomous guidance effects on wild-type neurons. Trio mutants have shortened lobes indicating axon stalling, as well as individual misguided fibers leaving the calyx at random trajectories (Awasaki et al., 2000). Interestingly, clones containing mutant Rho GTPase, which is part of the same chemical cascade as trio, do not have lobe defects but rather overgrown dendrites resulting in large calyces (Lee et al., 2000b). The successive mutation of three Rac GTPases (Rac1, Rac2 and Mtl) leads to defects in branching (loss of a single lobe), guidance (axons form a ball around calyx as in mud and mbd), and finally growth (axons do not extend past pedunculus) (Ng et al., 2002). DSCAM mutant γ Kenyon cells have no noticeable defects, but α/β* and α/β mutant cells have supernumerary branches that are often directed in only the vertical or medial direction (Wang et al., 2002). In another instance of apparent guidance roles for α/β* axons, mutant clones induced immediately before the production of α/β* neurons often exhibit mutant projection patterns (both axon branches extending into a single lobe) of wild-type α/β neurons as well. In mutants in which only a non-doubled lobe is present wild-type neurons extend a single unbranched axon into this lobe, indicating that DSCAM also regulates axon branching.

As mentioned previously, ecdysteroid hormones play an important role in γ Kenyon cell reorganization during metamorphosis. This is underscored by the dramatic phenotype of adult flies mutant for the ecdysone receptor isofrom B1 (EcR) or ultraspiracle (usp), the gene products of which act as a heterodimer to effect ecdysteroid-regulated gene transcription (Lee et al., 2000a). Adult mutant flies show no reorganization of γ, with the constituent clawed Kenyon cells branching into a medial and a vertical component as in the larva. EcR-B1 is expressed only in γ Kenyon cells and its effects appear to be completely cell-autonomous, indicating direct effects of ecdysteroids on clawed Kenyon cells. Interestingly, several known EcR/USP gene targets had no noticeable effects on reorganization when mutant; perhaps another pathway is involved in mushroom body neuronal remodeling.

A large class of mushroom body structural mutants also have more widespread effects on the development of midline neuropils such as the central complex. These mutants manifest phenotypically as a fusion of the medial lobes of the two mushroom bodies across the midline. Medial lobe fusion phenotypes typically involve the β and β' lobes, and in severe cases the γ lobe as well. One such gene, beta lobes fused (bef), was described in the early mutant screen that turned up mud, mhm and mbd (Heisenberg, 1980); since that time many more medial lobe fusion mutants have been added to the list. The genes ciboulot, brainstorming, split central complex, alpha lobes absent, brainwashing, fused lobes and castor all display central complex disruption and variable degrees of medial lobe fusion when mutant (Boquet et al., 2000a,b; Hitier et al., 2001). Missing vertical lobes are also seen in more severe cases, and the mutant alpha lobes absent has been of special use in functional studies, as mutants are shown to be deficient in long-term memory (Pascual and Prétat, 2001). These genes are not always expressed in the mushroom bodies, but rather in midline cells such as the glia of the transient interhemispheric fiber ring (TIFR) (Simon et al., 1998; Boquet et al., 2000b), indicating that the functional gene products may be part of a pathway generating repulsive signals preventing Kenyon cells from crossing the midline.

The best-characterized medial lobe fusion gene is linotte
(lio, also known as *derailed*), which encodes a receptor tyrosine kinase and was initially isolated in a screen for learning and memory mutants (Dura et al., 1993, 1995). The phenotype is particularly severe, with the α and sometimes the α′ lobes reduced or absent and the β and β′ and perhaps γ lobes fused across the midline. Lio was initially thought to be expressed only in glial cells of the TIFR, but a *lio*-lacZ reporter gene produces Kenyon cell staining as well (Moreau-Fauvarque et al., 1998; Simon et al., 1998). In either case, expression of *lio* is not observed in the adult, indicating that *lio* plays a role only in the development of mushroom body neurons.

Fig. 10. Schematic diagram of a developing mushroom body of *P. americana*, sagittal view, illustrating both general and specific aspects of insect mushroom body development. Neuroblasts (MbNb) are located centrally with respect to each calyx (C) of the insect mushroom bodies. Cell bodies within and around the calyx are arranged in an age gradient. Class I and class II Kenyon cells, with their characteristic locations and process organizations, appear to be common to the mushroom bodies of the majority of insect taxa. In all cases, class II cells are among the first born intrinsic neurons and their cell bodies are located at the outer periphery of the cell body region. Class I cells make up the remainder of the Kenyon cell population and their cell bodies fill the calyx cup. Kenyon cell axons travel through the pedunculus (P) and bifurcate into the medial (M) and vertical (V) lobes, where they are also arranged by age. Class II axons form the anterior γ layer (or the separate γ lobe in *D. melanogaster*) while class I axons occupy progressively more posterior laminae, layers or lobes as neurogenesis proceeds. Newborn class I cells form with their axons the core of the pedunculus and lobes and are distinct in both their morphology (often producing collaterals) and gene expression patterns. The existence of class III cells outside of the Dictyoptera has not been confirmed thus far. In *P. americana* these cells are born even before the class II cells and form a separate accessory calyx. Their axons occupy the most anterior laminae of the cockroach lobes, and in the vertical lobe form a looping structure termed the lobelet. Kenyon cells homologous to dictyopteran class III cells may form the posterior/accessory calyx system in the Orthoptera.
5.3. Genes and mushroom body development

The study of the molecular control of mushroom body development is in its infancy, with a few well-described genes and little knowledge of how they function to effect proper axon organization. Further comparisons with well-studied pathways from other systems, such as those regulated by ecdysteroids or ey and dac, should fill in some of these gaps in time. In-depth investigation of individual aspects of mushroom body development, such as axon branching or reorganization, will be helpful in the search for suites of genes that are involved in the regulation of these specific processes. Such widely-conserved developmental events are likely to share homologous genes with other insects and arthropods, and perhaps even vertebrates.

6. Conclusions

Comparative and molecular analyses of mushroom body development have provided important information about the formation of this complex brain region. Although species-specific differences exist, a clear, coherent picture of the basic events in insect mushroom body development and the subsequent organization of these brain structures is beginning to be realized (Fig. 10). Fundamental principles of neurogenesis and cellular organization are highly conserved, and may be compared to the birthdate-dependent ordering of layered brain structures in the mammalian brain such as the cerebral cortex. Continued investigation of these conserved events at the cellular and molecular level are likely to uncover evolutionarily ancient genes and pathways that are fundamentally important for the construction of complex nervous systems. Comparative studies of less universal aspects of development, such as Kenyon cell reorganization during metamorphosis, will give insight into the evolution of the insect mushroom bodies. Finally, the diversity of behavioral ecologies within closely related insect groups provides an excellent basis for studies of correlations between life history and brain development, and how these factors interact to effect observed trends in brain evolution.

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