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REVIEW

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Behavioral Development in the Honey Bee: Toward the Study of Learning Under Natural Conditions

Introduction

The adult worker honey bee is a sophisticated learner in a small package. To a certain extent, the same may be said of many insects. But the striking tendency of the social insects—the ants, bees, wasps, and termites—to organize social life on the basis of age polyethism (division of labor by worker age) results in a relatively prolonged period of adult behavioral development (Wilson 1971; Hölldobler and Wilson 1990). In colonies of the European honey bee, *Apis mellifera*, young adult workers rear the next generation and store food; middle-aged workers maintain the physical structure of the hive; and the oldest workers forage (Winston 1987; Robinson 1992; Moritz and Southwick 1993). These different tasks require different skills and provide different opportunities for skill improvement (learning).

How best can one study the neural correlates of learning in the honey bee? The honey bee can be (and has been, with fruitful results) tested in well-defined learning paradigms in the laboratory and then probed by electrophysiological, anatomical, biochemical, and molecular means (for review, see Menzel 1990; see also Hammer and Menzel 1995). This approach takes advantage of the many economies and conveniences of using arthropod nervous systems for the experimental study of neural function but denies the bee's rich social life.

The honey bee can also be studied within its own social and ecological context. With this approach, one focuses on the cellular mechanisms supporting the naturally occurring changes in behavior that accompany behavioral maturation and the accumulation of experience in a free-living animal. A long tradition of honey bee ethology, in particular, studies of foraging behavior (Lindauer 1961; von Frisch 1967; Seeley 1985), has provided insights into the relationship of the sensory and learning capacities of bees as they relate to floral stimuli (for review, see Menzel 1985). To date, however, even the most "natural" studies of neural mechanisms in honey bees have by and large lacked a developmental context.

Behavioral development is widespread in the animal kingdom. As individuals age, their responses to the environment change predictably. Often, an individual's behavioral responses increase in complexity and involve learning and memory. A child refines its ability to reach for objects in its visual field (Thelen and Corbetta 1994): a worker bee shifts from hive activities to foraging and becomes efficient at searching for

pollen and nectar (Dukas and Visscher 1994). As suggested by these examples, improvement in skill at a particular task is often a major component of behavioral development.

Behavioral development can be viewed as being genetically programmed and, in many cases, is hormonally mediated. Is this learning? In many cases, the two phenomena are extremely difficult to separate. Consider the classic example from the work of Marler and Konishi on song development in male white-crowned sparrows (for review, see Gould and Marler 1987). A white-crowned sparrow reared in isolation sings only a general species song as an adult (that is, the "inherited" song of that species is performed under certain endocrine conditions). The normal adult behavior, however, is to sing a specific, learned local dialect of the species song during the appropriate season; the learning occurs when the sparrow is ~3 months of age. In this case, there can be behavioral maturation without learning, but during normal behavior maturation learning is an essential part of the process, and both are required to produce the normal, species-typical behavior. It is therefore possible and necessary to ask whether learning and behavioral development share common brain mechanisms and neuroanatomical substrates.

Advances have been made recently in understanding the molecular basis of learning in *Drosophila melanogaster.* Continued progress in this area is likely to be rapid and substantial (Davis 1993; Dezazzo and Tully 1995; Feany and Quinn 1995). Generalizations from these data are, at the molecular level, applicable to other animals, including humans, but fruit flies offer a narrow behavioral repertoire. Much work, for example, is based on laboratory studies of mass-screened fruit flies tested in situations that, with a few exceptions (notably, mating: see Hall 1986, 1994) would be unlikely to occur in nature. Thus, these studies are behavioral only in a limited sense; they are typically devoid of social, ecological, endocrine, or developmental contexts. We propose that a missing element in research on the mechanisms of animal learning can be supplied by study of free-living, relatively long-lived, social insects that, as adults, undergo an extensive program of hormone-mediated behavioral development. We believe that a molecular genetic analysis of learning, combined with an equally rich analysis of behavior, will provide increased predictive power concerning the role of homologous gene products in complex behavior.

In our current research we have begun to delineate the hormonal control of adult behavioral development in honey bees (Robinson et al. 1989; Huang et al. 1991, 1994; Robinson 1992). We have also discovered recently that there are changes in brain structure associated with behavioral development in honey bees (Withers et al. 1993). These changes, which have been replicated in our own laboratory and elsewhere (Durst et al. 1994; Withers et al. 1995), occur in the mushroom bodies or corpora pedunculata of the protocerebrum. The mushroom bodies have been strongly implicated as centers for insect learning and formation of long-term memories (e.g., Erber al. 1980; Technau 1984; Heisenberg et al. 1985; Davis 1993; Debelle and Heisenberg 1994). Our findings provide a starting point for a program of research that combines field studies of behavioral development with laboratory analyses of brain structure.

Recent reviews have dealt with molecular mechanisms of olfactory

learning in fruit flies and electrophysiological analyses of olfactory learning in honey bees (Davis 1993; Hammer and Menzel 1995). The present review complements these analyses by focusing on the opportunities provided by the study of behavioral development in honey bees. We have specifically addressed the following questions: What do honey bees learn as they undergo normal behavioral maturation? What is the evidence that the volume changes that occur within the bee brain during this developmental period are associated with behavioral plasticity? What is the evidence that changes in volume of a particular brain region are associated with changes in learning-based behavior in any animal? Finally, we present a model of neural plasticity and behavioral development in honey bees.

Behavioral Development in Honey Bees and its Endocrine Control

The honey bee society consists of reproductives—a single long-lived queen and anywhere from zero to several thousand male drones—and tens of thousands of female workers that typically do not reproduce but rather perform all tasks related to colony maintenance and growth. Therefore, the behavioral development that we describe here is not linked to the attainment of reproductive ability. Newly emerged worker bees toil in the nest for the first 2 to 3 weeks of their lives, progressing through a succession of nest maintenance and brood-related tasks: cell cleaning, brood and queen care ("nursing"), food storage, comb construction, removal of corpses, and guarding the nest entrance. During this 3-week period, they also take short flights away from the hive but do not yet visit flowers. This sequence of age-related changes in task performance culminates with a switch to foraging for nectar and pollen relatively late in life, typically at 21–24 days. Behavioral transitions within the hive are not absolute and involve age-dependent changes in the relative frequency with which various activities are performed (Seeley 1982; Robinson 1987). In contrast, the shift from hive activities to foraging is more dramatic; once a bee becomes a forager, she performs few, if any, tasks in the hive (Winston 1987).

The exact timing of these behavioral transitions is not fixed. Individuals can respond to changing colony needs by accelerating, delaying, or reversing their behavioral development (Robinson 1992). The rate of behavioral development is determined by weather, nutritional status, and age structure of the colony. Genetic predisposition also plays a role. Africanized ("killer") bees, for example, forage at an earlier age than the European honey bees found in most of North America (Winston 1992). Similar genetic variation exists within North American honey bees, as well (for review, see Robinson 1992; Giray and Robinson 1994). Among these factors, colony age structure appears to be the most important factor determining the transition from hive activities to foraging (Huang and Robinson 1992). If a colony is deficient in older, foraging bees, young bees compress the period of time devoted to nest activities and become precocious foragers. Similarly, foragers will revert to nest activities in the absence of younger colony members. Bees therefore exhibit a stereotyped pattern of behavioral development that is susceptible to environmental influences.

There is good evidence that juvenile hormone, the sesquiterpenoid insect developmental hormone produced by the corpora allata glands, is involved in the control of age-related division of labor in worker honey bees (Robinson 1992). Blood levels of juvenile hormone increase

as the worker bee ages (Fluri et al. 1982; Robinson 1987). Low titers are associated with behavior in the nest such as nursing during the first 1–3 weeks of adult life, whereas a higher titer at ~3 weeks of age accompanies foraging. Treatment with juvenile hormone and juvenile hormone analogs induces precocious foraging (Jaycox et al. 1974; Jaycox 1976; Robinson 1985, 1987; Sasagawa et al. 1989).

Changes in juvenile hormone titers accompany plasticity in behavioral development. Precocious foragers have a precociously high juvenile hormone titer, overaged nurse bees have a lower titer than their foraging sisters of the same age, and bees that revert from foraging to nursing show a drop in juvenile hormone (Robinson et al. 1989, 1992). Recently, it has been shown that young bees respond specifically to a shortage of foragers with both an increase in juvenile hormone production and precocious foraging (Huang and Robinson 1992). These changes have been detected both by means of in vitro biosynthetic assays of juvenile hormone production by isolated corpora allata (Huang et al. 1991) and by determination of juvenile hormone titers in blood samples taken from individual honey bees using the technique of radioimmunoassay (Hunnicutt et al. 1989; Huang et al. 1994). Changes in the environment that affect endocrine functions are probably mediated through neurosecretory cells in the brain, as these cells are known to control the activity of the corpora allata in other insects (Riddiford 1985). Neural control of the corpora allata is thus similar in many respects to neuroendocrine control of the pituitary in vertebrates (e.g., Khan 1988; Woodhead et al. 1989).

Learning During Behavioral Maturation in Honey Bees

There is no doubt that a worker honey bee learns much about her environment during her 35- to 60-day adult life. Foraging outside the hive for food, water, and propolis (plant resins used in hive construction) involves, at a bare minimum, learning the appearance and location of the nest, learning to navigate in both the outbound and homeward directions, and learning to extract food efficiently from different types of flowers.

Initial learning of the location of the nest site occurs when bees that are not yet foraging take short flights that permit a reconnaissance of their home base (Becker 1958; Vollbehr 1975). Nest location is apparently referenced to landmarks and to celestial cues (Dyer and Gould 1983; Dickinson 1994). These short orientation or "play" flights, which were first described by Rösch as typically occurring on the eighth day of adult life (Rösch 1925, cited in Winston 1987), are readily observed during the first week of adult life in our colonies in Illinois. It is not known yet how much experience is required to guarantee that a foraging bee can find its way home, although this topic is currently being studied. Several days of flight experience may be required, for example, for bees to compute solar position with accuracy (Lindauer 1959; Dyer and Dickinson 1994). Nest location learning is not an imprinting or critical period phenomenon, as older bees have no difficulty learning the location of a new nest site after swarming has occurred (Robinson and Dyer 1993), and a change in the appearance of the hive entrance causes only minimal disruption of behavior. There is evidence that even when foragers learn the location of a new nest site, they still remember the location of their former home (Robinson and Dyer 1993); also, bees that

have established a new home after swarming return reliably to sites at which they foraged previously (Dyer 1993).

Learning the location of the nest site permits the bee to forage. Like other generalist pollinators, bees can handle a variety of pollen and nectar sources and do so with gains in efficiency as they become more experienced (Heinrich 1979; Winston 1987; Dukas and Visscher 1994). Compelling evidence from the closely related bumblebee demonstrates that experienced foragers make the best choices among the available resources (i.e., they do not visit flowers that are less rewarding in terms of pollen and nectar when high reward flowers are available); they take less time than inexperienced bees to extract nectar and pollen; and they handle complicated flowers with ease (Heinrich 1979).

Honey bees learn associations based on olfactory and visual cues to forage efficiently (Cartwright and Collett 1983). They show their ability to form lasting memories by coming to feeding tables provisioned by humans (e.g., von Frisch 1967) and by successful discriminations of rewarding from nonrewarding artificial feeders (Waller 1972; Waddington and Gottlieb 1990). Experienced foragers also appear to communicate more precisely the location of a food source via dances (Schweiger 1958, cited in von Frisch 1967). Conditioning has also been demonstrated repeatedly in laboratory experiments (e.g., Bitterman et al. 1983); this literature recently has been reviewed extensively (Menzel 1990) and will not be covered here. Finally, bees are capable of forming memories linked to time of day. For example, they can learn an association between time of day and sites at which foraging will be rewarded (von Frisch 1967).

We can summarize by saying that (1) foraging worker bees have been demonstrated to orient themselves in space with reference to the location of their nest, (2) they learn both the geographic and temporal resource pattern of their locality, and (3) they also learn to work with many different types of flowers. But bees do not typically begin to forage until they are at least 21 days old. Young adult bees take orientation flights during their preforaging phase, but what do they learn inside the hive?

A hive has an internal organization of brood-rearing and food storage areas that offers a spatial learning task to young bees, but there is no direct evidence that such learning occurs. Bees outside of the hive can learn discriminations of surface textures and can also perceive substrate vibrations as well as airborne sounds (Kirchner et al. 1991), yet there is again no direct evidence of learning associated with these sensory modalities during a worker bee's normal development within the hive. Comb building is a cooperative activity involving bees that secrete wax, bees that chew wax, and bees that make comb out of wax (Hepburn 1986), but there is no evidence that this is a learned behavior. [There is, however, some evidence suggesting learning by *Polistes fuscatus* paper wasps during nest construction (see Downing 1992).] It is possible to study colonies of bees housed in observation hives with clear glass sides, but despite this methodology the occurrence and extent of learning associated with hive task performance remains a mystery.

Although opportunities for visual learning are limited within the dark hive, laboratory studies have demonstrated that bees >2 days old are capable of learning olfactory discriminations (Visscher 1983; Menzel 1990). One use to which this olfactory capability is clearly put is kin

recognition. Kin recognition is an essential element of sociality and is especially critical for animals that, like worker honey bees, do not typically produce offspring themselves (Hamilton 1964). But for kin selection to be a major force in evolution, animals must be able to distinguish kin from non-kin. Honey bees are capable of such kin recognition, and learning is clearly involved (for review, see Breed et al. 1994). Worker honey bees learn to recognize the odor of their own queen and will kill a foreign queen unless they experience a queenless interlude long enough to enable them to "forget" their original queen (Boch and Morse 1979; Winston 1987; Breed 1991). Bees can also discriminate unrelated from related workers based on perception of both innate and acquired odors. Although this has been demonstrated in laboratory studies of small groups of bees, the ability to make such discriminations is displayed naturally by guard bees that defend the hive entrance (Breed et al. 1988). By learning certain identification odors on their own or nestmates' bodies, bees appear to form a recognition template that guides their behavior toward conspecifics (see Pfennig and Sherman 1995). Similar template formation is a key component of song learning and intraspecific recognition in birds (for review, see Marler 1981; Williams 1990; Konishi 1994).

Honey bees have learned other associations in the laboratory (for a comprehensive review, see Menzel 1990) but these results do not alter our appreciation of the worker bee as a specialist in navigation by landmarks and celestial cues, flower handling, and recognition of kin on the basis of olfactory discrimination. One other aspect of honey bee life, however, deserves mention in this context. Bees communicate information about floral resources and nest sites by means of stereotypical movements referred to as dances, which are performed within the hive on the surface of the comb (von Frisch 1967; Winston 1987). Performance of the dance reflects the formation of memories about the spatial location and quality of resources. Use of dances to guide subsequent foraging reflects the formation of memories of the information contained in other bees' dances. Although there is currently no evidence that the mushroom bodies play a critical role in the performance, decoding, and use of dances, we predict that such a relationship is not only likely but will perhaps provide one of the richest areas of future research on brain and behavior relationships in honey bees.

Changes in the Mushroom Bodies Associated with Behavioral Development in Honey Bees

Our integrated behavioral and anatomical studies have revealed an association between regional brain volume and behavioral development in worker honey bees (Withers et al. 1993; Withers et al. 1995). The most notable change detected is an increase in the volume of the neuropil of the mushroom bodies that is associated with foraging behavior.

The mushroom bodies comprise a highly organized set of neuropils (the calyces and the α and β lobes) associated with an intrinsic population of Kenyon cells (see Fig. 1). These small (4–7 μm), densely packed neurons (~340,000 total in the honey bee brain; Witthöft 1967) have characteristic dendritic arborizations in the calyces and send bifurcating axons via the pedunculus to form neuropilar columns of parallel fiber bundles: The α and β lobes (for review, see Schürmann 1987). Postsynaptic extrinsic output fibers in the lobes connect the
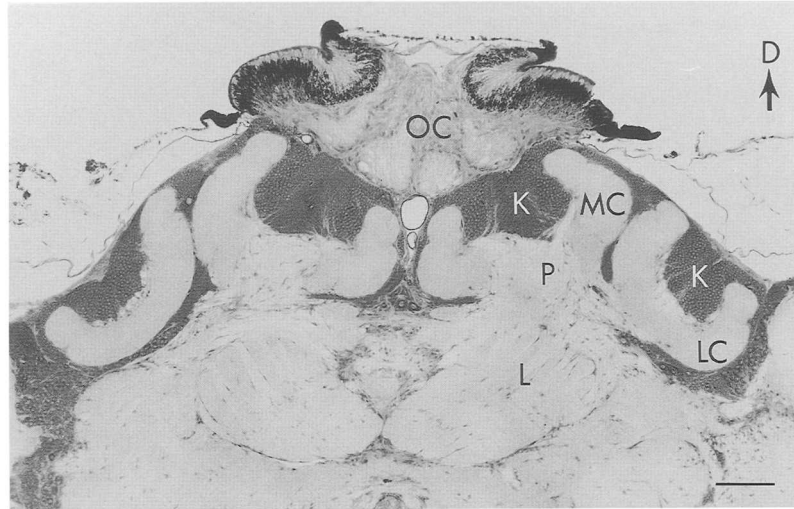
Figure 1: Transverse section through the brain of a worker honey bee, showing the mushroom bodies. Brain was fixed in alcoholic Bouin's fixative, embedded in paraffin, sectioned at 10 μm, stained with Luxol fast blue, and counterstained with cresyl violet. Paired calyces are located on both the right and left side of the brain. Scale bar = 100 μm. (D) Dorsal; (K) Kenyon cell body region; (L) lobes; (LC) lateral calyx; (MC) medial calyx; (OC) ocelli; (P) pedunculus.

different compartments of the mushroom bodies and project to other regions of both the ipsilateral and contralateral protocerebrum (Rybak and Menzel 1993). The Kenyon cells may be divided into several subpopulations (on the basis of peripheral vs. central location in the calyces or on the basis of immunocytochemical staining patterns: e.g., see Schäfer et al. 1988), but the functional implications of differences between these subpopulations are unknown. According to Strausfeld (1976), the Kenyon cells of insects may represent the most densely packed population of neurons in any brain region of any animal.

The calyces of the mushroom bodies receive both olfactory and visual input (Mobbs 1982) and may also receive inputs from mechanosensory centers (Mizunami et al. 1993). The calyces of the bee are composed of three distinct compartments: the lip, which receives primarily olfactory input; the collar, which receives primarily visual input; and the basal ring, which receives input from both visual and olfactory centers.

The mushroom bodies do not represent the unique projection field of any brain pathway: for example, olfactory projections from the antennal lobes are also sent directly to the lateral protocerebrum. The additional level of processing through the Kenyon cells provided by the mushroom bodies has been assumed, on the basis of good experimental evidence, to reflect the role of these structures in insect olfactory learning and memory. Important functional and anatomical features relevant to such a role include an octopaminergic innervation arising in the ventral unpaired median (VUM) cells of the subesophageal ganglion that mediates olfactory learning in the honey bee (Hammer 1993) and the ability of the Kenyon cells of the locust to respond as oscillating ensembles when presented with olfactory stimuli (Laurent and Naraghi 1994). A recent and important set of lesion experiments in the

cockroach *Periplaneta americana* suggests, however, that an equally critical role of the mushroom bodies may be participation in the formation of spatial memories (Mizunami et al. 1993). This is consistent with the suggestive (but by no means definitive) findings of Vowles (1964) and Bernstein and Bernstein (1969) for the wood ant *Formica rufa.* Possible functional roles of the mushroom bodies in arthropods have been reviewed recently by Erber et al. (1987) and Strausfeld et al. (1995).

In our first look at behavioral development and structural plasticity in the worker honey bee brain, we collected bees designated by direct behavioral observation as nurse bees (workers seen feeding larvae in cells) and foragers (workers captured returning to the hive with pollen in their pollen baskets). The brains of these bees were fixed, frozen, and cryosectioned at 10-μm intervals (details are given in Withers et al. 1993). The Cavalieri method of volume estimation, which is based on systematic random sampling from a complete set of serial sections through the structure of interest, was combined with an efficient point-counting strategy for determination of cross-sectional area (Gundersen et al. 1988; Michel and Cruz-Orive 1988). Volumes of all major regions of the brain were compared with those of 1-day-old bees from the same colony. Most regions exhibited stability over the period of behavioral development from 1 day old to foraging. Only two regions displayed distinctive patterns of volume change. First, the volume
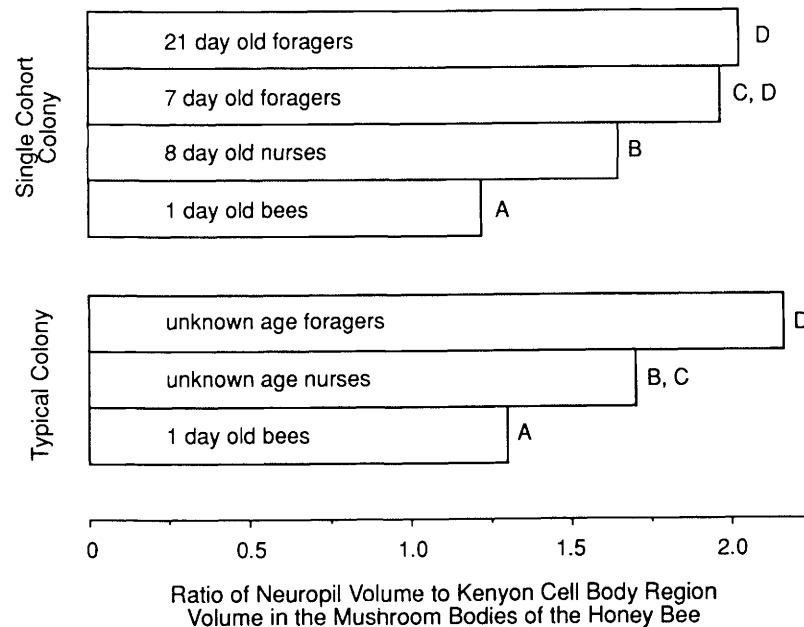

Figure 2: Changes in the relative volumes of the compartments of the mushroom bodies during behavioral development in the honey bee, expressed as the mean ratio of neuropil volume to Kenyon cell body region volume. The Cavalieri method was used for volume estimation. See text and Withers et al. (1993) for details. The *lower* set of bars shows the ratios for a large colony with a normal age distribution. The *upper* set of bars shows the ratios for a single cohort colony. Each group consisted of four to six bees. Bars marked with different letters differed significantly from each other ($P < 0.05$; Student Newman-Keuls test).

occupied by the olfactory glomeruli was larger in nurse bees than in 1-day-old bees and foragers. Second, the mushroom bodies underwent a striking internal reorganization. Although their total volume remained stable, the volume of their neuropil was increased significantly in foragers (Fig. 2). A decrease in the volume occupied by the cell bodies of the Kenyon cells occurred in parallel with the increase in neuropil volume. Because of the important association of the mushroom bodies with insect learning, we have currently chosen to focus exclusively on the mushroom bodies.

The observed differences between nurse bees and foragers in this first experiment may have been a consequence of aging or behavioral state. Foragers were almost certainly at least 7–10 days older than nurses because they were sampled from a colony with a typical, mixed-age structure. To uncouple these two factors, we used manipulations of colony demographics to produce workers that foraged "precociously" (at 7 days of age), at the same time that other bees were acting as normal-aged nurses. Forager status, rather than age, was associated with increased volume of the mushroom body neuropil (Withers et al. 1993). This result has been confirmed by a group working independently with another population of honey bees (Durst et al. 1994). This general pattern of results has also been replicated in our own laboratory using a variety of histological methods, over the course of several field seasons.

These findings suggested that the reorganization of the mushroom bodies was in some way associated with the transition from within-hive to forager status. We were therefore interested in determining whether this plasticity is *experience-dependent,* that is, a consequence of foraging experience or, rather, *experience-expectant,* occurring in anticipation of the demands of this phase of the bee's life (Black and Greenough 1986). Earlier studies demonstrating an increase in juvenile hormone titers in association with the onset of foraging suggested the possibility of an endocrine-based, experience-expectant mechanism. To test these alternatives, we treated 1-day-old adult bees with a juvenile hormone analog (methoprene) to give them a forager-like endocrine profile but did not permit them to fly (Withers et al. 1995). "Big-back" bees were produced by gluing a plastic disc to the dorsal thorax of worker bees on

Figure 3:   Workers within the hive. Arrowhead points to a big-back bee to illustrate the disk that is glued onto the thorax on the first day of adult life. Big-back bees move freely within the hive, but cannot pass through the hive entrance (see Withers et al. 1995).

the first day of adult life (Fig. 3). A slight modification of the hive entrance permitted the other workers in the colony to forage as usual, whereas the big-back bees could not exit the hive. Volume changes within the mushroom bodies, comparable in magnitude to those seen in our first studies, were observed in the big-back bees. Big-back bees did not differ from bees of the same age permitted to forage freely. These results demonstrate that the reorganization of the mushroom bodies is not dependent on foraging experience but leave open the question of any additional role for experience in shaping this region of the bee brain.

The findings of the big-back bee study are consistent with a role for hormonal mediation of this neural plasticity. The neural mechanisms that link changes in production of juvenile hormone to changes in behavior are currently not known in insects. During insect metamorphosis, juvenile hormone modulates the developmental effects of ecdysteroids on neural circuitry and behavior (for review, see Weeks and Levine 1990). In adult crickets, juvenile hormone is required for neurogenesis in the mushroom bodies (Cayre et al. 1994). But neither of these mechanisms appears to account for the changes in brain structure that accompany behavioral maturation in the honey bee. Ecdysteroids are not involved in the regulation of honey bee adult behavioral development (Robinson et al. 1992), and as discussed in the following section, neurogenesis cannot explain the changes observed in the mushroom bodies (Fahrbach et al. 1995c).

In a more recent study, we used a finer grained temporal and behavioral analysis to explore the relationship of changes in volume of the neuropil of the mushroom bodies to changes in worker bee behavior (Fahrbach et al. 1995a). Recognizing the bias imposed by a focus on foraging, we stocked a large, unmanipulated colony with 500 number-tagged bees. The entrance to this hive was observed continuously by a team of trained observers so that the complete flight history of every tagged bee could be known. The entire hive entrance was covered by a sheet of Plexiglas that forced the bees to walk into and out of the hive so that their number tags could be read. Orientation flights were defined as short duration flights (5 min or less) during which the bee turns to face the nest entrance (Winston 1987). Foragers were identified as they returned to the entrance with pollen loads. Collections of tagged bees were made on the occasion of the first flight away from the hive (first orientation flight), fifth flight away from the hive (fifth orientation flight), first pollen-foraging trip, after 1 week of pollen foraging, and after 2 weeks of pollen foraging. The results replicated our earlier studies but also suggested that the trend toward increased volume mushroom body neuropil could be detected as early as the first orientation flight (Fig. 4). These results are reminiscent of those of Coss et al. (1980) and Brandon and Coss (1982), who reported changes in dendritic spine shape of Kenyon cells in association with early flight experience and foraging activity. However, it is not easy to generalize from these Golgi studies because the results were based on a small number of cells sampled from a population that is now known to be heterogeneous (Yang et al. 1995). In retrospect, the possibility that mushroom body reorganization actually begins quite early in the bee's adult life was hinted at in data from nurse bees in our first experiments (Withers et al. 1993), but the larger sample sizes and more extensive behavioral monitoring the study of Fahrbach et al. (1995a) now make
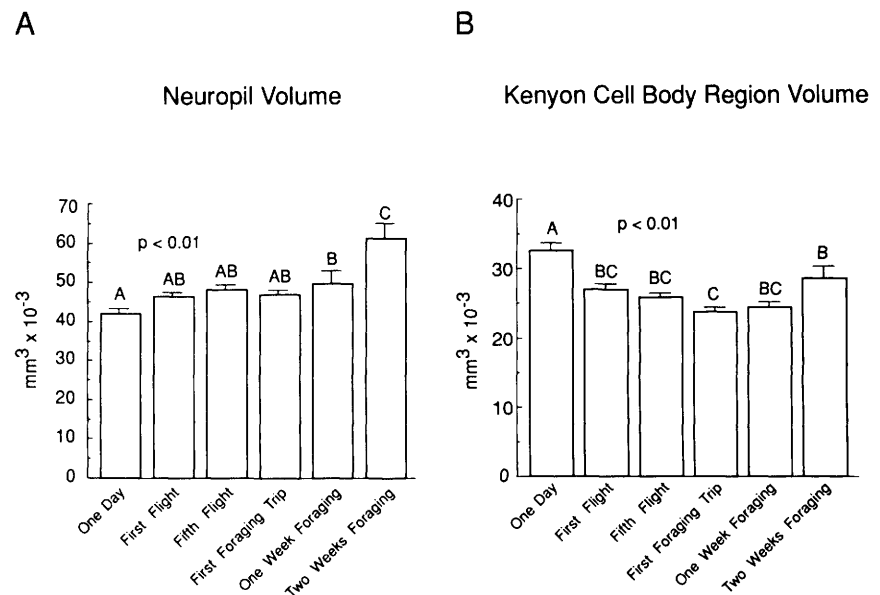
A   **B**

Figure 4: Volume estimates for the two compartments of the mushroom bodies in honey bees from a large colony undergoing normal behavioral development. Bees were number tagged at 1 day of age and all subsequent flight activity was recorded. Bees were collected at the hive entrance upon return from a flight. "Foragers" were defined as bees returning to the hive entrance with a pollen load. Each group consisted of 6–15 bees. Brains were fixed in alcoholic Bouin's fixative, embedded in paraffin, sectioned at 10 μm, and stained as in Fahrbach et al. (1995b). The Cavalieri method was used to estimate the volume occupied by the cell bodies of the Kenyon cells and the volume of the neuropil associated with the mushroom bodies (calyces, pedunculus, and lobes). Analysis of variance was followed by post hoc comparisons using the Student Newman–Keuls test. Bars marked with different letters differed significantly from each other ($P < 0.01$). (A) Volume of the neuropil of the mushroom bodies, by worker flight experience. (B) Volume of the Kenyon cell body region of the mushroom bodies, by worker flight experience.

this point obvious. We have also shown that queen bees, which do not forage but which do take orientation flights (so that they can find their way home after mating) undergo a reorganization of the mushroom bodies comparable to that seen in workers and exhibit these changes in the first week of life (Fahrbach et al. 1995b).

Despite our initial formulation of an association of changes in the neuropil volume with foraging, the data currently available suggest that a link between neuropil expansion and learning the location of the nest site via orientation flights [spatial learning or place memory in the terminology of Mizunami et al. (1993)] is more likely. This new hypothesis explains the results with precocious foragers, big-back bees, and queens. It is also supported by the findings of Durst et al. (1994), showing that the collar regions of the calyces (the region that receives visual inputs) are increased in volume in foragers.

It is also possible that changes within the mushroom bodies coupled with behavioral maturation are related to aspects of flight that do not involve learning but are mediated through the mushroom bodies.

However, mushroom bodies do not seem to be required for flight. Flies with abnormal mushroom bodies as a result of mutation or chemical ablation are still capable of flight (Debelle and Heisenberg 1994), but it is not known whether bees with lesioned mushroom bodies fly. Also, no effect of age was found in a detailed analysis of the conditioned proboscis extension response in worker bees 8–64 days of age (Bhagavan et al. 1994), suggesting that maturational changes in the volume of the mushroom bodies are not important for this well-studied form of learning.

Regional Volume Increases in Brains

MECHANISMS

How can the volume of a brain region increase? The possibilities are rather limited (although they may occur in complex combinations). A region may become larger because more neurons are present. Studies using bromodeoxyuridine as a marker for mitosis, however, have produced no evidence for neurogenesis in the adult bee brain (Fahrbach et al. 1995c). A region may also become larger because the somata and/or processes of a set of neurons become larger. Another possibility is that the same number of same-sized neurons or neuronal processes are present, but they are less dense, pushed farther apart by an increase in the number or size of the nonneuronal elements present. Because the somata of the intrinsic neurons of the mushroom bodies are segregated from their arborizations according to the common arthropod plan, we can be certain that expansion of the neuropil reflects changes that impact synapses: an increase in process size or number, a change in the associated glial population, or some combination of these three. A form of Kenyon cell plasticity has already been identified in *Drosophila* by Heisenberg and colleagues (1995). These researchers have shown that the number of axons in the peduncle of the mushroom bodies of *Drosophila* varies in response to the conditions under which the flies are reared (Technau 1984; Heisenberg et al. 1995). Although these studies were initially performed by counting axonal profiles in electron micrographs, Heisenberg et al. (1995) have recently reported that total volume of the calycal region also reflects rearing conditions in adult flies. These results suggest that the changes seen in the honey bee reflect changes in the processes of the Kenyon cells.

MEANING

It is not known whether a honey bee requires an expansion of mushroom body neuropil volume over the volume present on the first day of life to mature and become a forager, or whether changes must occur in the queen bee's brain if she is to mate and begin her life as an egg layer. The meaning of the changes in fiber number/calycal volume in the fly brain is also unknown. Why are volume changes within the brain of interest to neurobiologists? The hope that changes in anatomy might be related to changes in patterns of electrical activity and they, in turn, to changes in behavior, sustains this enterprise.

Do increases in the size of a brain region reflect increased processing capacity? Under the widely accepted "bigger is better" hypothesis, the bigger the brain region devoted to that function, the better the behavior performance. *Better* is defined as performance of the behavior with

greater efficiency or fewer errors or, in the case of behavior evaluated in a natural context, greater fitness. Implicit acceptance of this hypothesis actually lies at the root of the long-standing fascination with the mushroom bodies of insects. Biologists beginning with Dujardin in 1850 noted that bees, and other social insects thought to be the most behaviorally complex and socially advanced, had larger mushroom bodies, especially the calyces (Howse 1975; Strausfeld et al. 1995).

Four different examples of volume differences within animal brains that are strongly correlated with the performance of learned behavior will be reviewed briefly here and considered as possible examples of mechanisms also shared by honey bees. They were selected to emphasize the point that regional brain volume *matters* in terms of learning and memory. These examples also illustrate that increases in the size of a specific brain region involve diverse control agents (hormones, experience) and cellular mechanisms (addition of synapses, prevention of neuronal death).

SONGBIRD BRAINS

Bird song arises from a joint process of development and learning, as discussed above (for review, see Marler 1981; Williams 1990; Konishi 1994). Some species learn their song during a specific period early in life (e.g., zebra finches), whereas others learn song anew with each period of reproductive activity (e.g., canaries). The regions within the brain that control song (typically referred to as nuclei in vertebrates) have been described in detail (Nottebohm et al. 1976, 1982). Three types of regional brain volume/function relationships have been described: between species, within species, and within the lives of individual birds.

One of the potent inducements to study the song system as an example of hormone-mediated neural plasticity lies in the power of the comparative approach. Many birds sing, and the songs they produce are species specific and extraordinarily diverse (DeVoogd et al. 1993). Even in free-living birds, song is an easily recorded behavior susceptible to rigorous quantitative analysis. Between-species comparisons suggest strongly that volume matters for song learning. A comparative study of song complexity and brain nuclear volume in 41 species of song birds revealed a significant positive correlation between the volume of the nucleus high vocal center of the neostriatum (HVC; formerly referred to as hyperstriatum ventralis pars caudalis) and the size of the song repertoire (DeVoogd et al. 1993; see also Canady et al. 1984). The relationships between regional brain volume and behavior, however, are not always entirely predictable: The same study revealed no correlation of HVC volume with number of discernible syllable types produced per song bout.

The second correlation between brain structure and behavior arises from the fact that within species of songbirds, the sexes often differ in the quality and quantity of song they produce. This difference in behavior is reflected in differences in the organization of the brain song centers, with males having significantly larger song nuclei in species in which only males sing, such as the canary and the zebra finch. There is evidence from numerous studies that exposure to steroids, particularly estradiol, before the period of song learning plays a role in creating the sexual dimorphisms of the song nuclei (for review, see Williams 1990).

The third powerful correlation between regional brain volume and

learning arises from the seasonality of bird song. Many birds sing only at certain times of the year, as a result of seasonal changes in plasma levels of androgens. The demonstration that the volume of two song nuclei, HVC and the nucleus robustus of the archstriatum (RA), undergo major changes in volume between autumn and spring in the male canary showed that changes in volume can be directly correlated with seasonal changes in the performance of song (DeVoogd and Nottebohm 1981; Nottebohm 1981). Analyses of the cellular changes resulting in changes in total regional volume suggested that changes in processes of intrinsic neurons (specifically, increases in dendritic length) led to changes in synaptic density in HVC (DeVoogd and Nottebohm 1981) and RA (Canady et al. 1988). More recent studies have uncovered a relationship between seasonal changes in singing, testosterone production, and neuronal number in HVC (Kirn et al. 1994; Nottebohm et al. 1994; Rasika et al. 1994). Gonadal steroids thus coordinate changes in the structure of the brain with reproductive status. The steroid-induced volume changes appear to occur regardless of the bird's experience but have important consequences for the bird's subsequent performance.

The bird song system thus presents us with evidence for developmental plasticity in three contexts: (1) during behavior maturation within individuals (which may occur once or repeatedly during the life of an individual); (2) between sexes; and (3) an evolutionary structure–function relationship for a specific region of the brain that holds across numerous species.

EXPERIENCE-DEPENDENT PLASTICITY IN RODENT CORTEX

The changes in regional brain volumes observed in bird brains might be described as experience-expectant. In contrast, increases in the thickness or volume of rodent cortex that occur as a result of rearing in a complex environment (and, presumably, as a result of learning about that complex environment) are, by definition, dependent upon the animal's experience (Bennett et al. 1964; Rosenzweig et al. 1972; Greenough and Volkmar 1973). In a large series of anatomical studies, the cellular changes that result in a measurable change in cortical volume have been described. These studies have revealed that nearly every functional aspect of the cortex is changed by experience: capillaries, glial cells, and synapses (Sirevaag and Greenough 1987, 1991; Black et al. 1990). The association of learning with a larger number of synapses per neuron is especially clear and is similar to changes seen in gastropod mollusks as they learn (Bailey and Chen 1989; Bailey and Kandel 1993).

What are the behavioral consequences of these changes in rat cortex? A recent review demonstrates that there is a general association of rearing in a complex environment with superior performance on various learning tasks, when behavior is compared with that of rodents reared in less "interesting" environments (Greenough and Black 1992). This relationship, however, has not been the focus of recent research in this area, which has concentrated more on delineating the cellular changes produced by different environments and the molecular mechanisms that produce these changes. Additionally, the broad scale participation of the cortex in multiple aspects of the animal's behavior makes such an analysis more difficult, than, for example, study of the relationship of song nuclei volume to song production. The studies with rodent cortex, however, are extremely important in that, taken together with studies on

changes induced in the molluscan nervous system by learning, they suggest that change in the number of synaptic connections is the major structural change in the brain produced by experience (Bailey and Kandel 1993).

FOOD-STORING BIRDS AND THE HIPPOCAMPUS

When faced with more pieces of durable food than can be consumed at once, some birds hide those items and return to them at later times, in the process demonstrating memory for the location of the hidden food (Krebs 1990; Shettleworth 1990). Damage to the hippocampus blocks retrieval of stored items, presumably because the birds cannot remember where they are (Sherry and Vaccarino 1989). Many different species of birds store food, but other, often closely related, species do not. In general, the hippocampus of food-storing species has a greater volume than the hippocampus of nonstorers, again suggesting that regional brain volume matters for learning (Krebs et al. 1989; Sherry et al. 1989; Hampton et al. 1995).

The coincidence of increase in regional brain volume and behavioral maturation has been studied extensively in the marsh tit *Parus palustris*. As in the case of the song nuclei or the rat cortex, the increase in hippocampal volume occurs relatively late in development (Healy and Krebs 1993; Healy et al. 1994). It is not seen in younger birds or untrained birds of the same species and was only observed when marsh tits had the opportunity to engage in a learning task that has some of the attributes of their naturally occurring behavior (Clayton 1995). Furthermore, a comparable volume increase did not occur in a nonstoring species (the blue tit) that received the same training, suggesting that this form of hippocampal plasticity is species specific. One of the mechanisms by which experience manipulates hippocampal volume in marsh tits appears to be through experience-dependent regulation of neuronal number (Clayton and Krebs 1994). The hippocampal volumes of control birds not permitted to store food (learn and remember) actually became smaller during the course of this experiment. Interestingly, interactions between experience-dependent and experience-expectant mechanisms may be complex. A study of neurogenesis in the hippocampal complex of free-living black-capped chickadees showed that there was a peak of neuronal addition to this structure in the autumn (Barnea and Nottebohm 1994) This is immediately prior to the time when the chickadees begin a winter pattern of behavior involving food storing and therefore likely precedes the time when the rate of spatial memory formation is increased.

SPATIAL LEARNING, HIPPOCAMPUS, AND STEROIDS IN VOLES

Recent studies of spatial learning in rodents of the genus *Microtus* once again demonstrate that regional brain volume can be linked to learning performance. Males of a polygnous species, the meadow vole *Microtus pennsylvanicus*, range over a wider territory during the breeding season and perform better on laboratory spatial learning tests than female meadow voles (Gaulin and FitzGerald 1986, 1989). These behavioral differences are correlated with a sex difference in hippocampal size that favors males (Jacobs et al. 1990). Monogamous species of vole lack both the behavioral and the brain sex differences. The performance of individual voles may be tied to endocrine status, which in turn predicts

the volume of the hippocampus. There is evidence that male meadow voles do not differ significantly from females on spatial learning tasks until the voles reach reproductive maturity (Galea et al. 1994).

The female meadow vole also appears to be subject to hormone-mediated changes in behavior and hippocampal volume. In experiments in which female meadow voles were tested in the Morris water maze, a spatial learning task, low physiological levels of estrogen were associated with better spatial learning than high physiological levels (Galea et al. 1995). These changes, which mirror male-induced fluctuations in circulating levels of gonadal steroids (Cohen-Parsons and Carter 1987), may be a result of hormone-mediated changes in hippocampal volume. They imply that the relationship between regional brain volume and performance of a learned behavior is not an unusual characteristic of the song nuclei and the hippocampus of birds or certain regions of the vertebrate cortex. It is especially intriguing that the situation in voles does not inevitably appear to follow the hypothesis that bigger is better. Whereas the wide-ranging males of the polygnous species have a larger hippocampus than the less mobile females, high levels of estrogen among females are associated both with larger hippocampal volume and with poorer performance in the water maze (Galea 1994; Galea et al. 1995).

SUMMARY: THE MEANING OF REGIONAL VOLUME CHANGES WITHIN THE BRAIN

These examples show that the volume of specific brain regions involved in learning can vary in adult animals in ways that are correlated with and possibly causal to changes in performance on learning tasks. But even in these carefully selected, well-studied examples the mechanisms linking brain volume and subsequent behavioral performance are only dimly discernible. For example, some populations of male song sparrows are nonmigratory. They sing and defend territories on a year-round basis. Despite this behavioral stability, there is significant seasonal variation in the volume of the song nucleus HVC (Smith et al. 1995). The number of song types produced also does not change seasonally, leaving open the question of how changes in HVC volume are related to behavior.

Present data suggest that the volume changes described in the bee brain could reflect the operation of both hormonal (experience-expectant, as in the song nuclei of the bird brain) and learning-based (experience-dependent, as in the case of the rodent cortex or the avian hippocampus) mechanisms. The link of juvenile hormone to behavioral plasticity in the worker honey bee suggests that this sesquiterpenoid may also be responsible for structural changes in the brain, which may need to be coordinated with other physiological changes that allow the bee to switch from working in the hive to foraging. Determination of the developmental times at which hormone exposure is necessary (or possibly essential) for reorganization of the mushroom bodies will be a critical focus of future investigations. Yet, a role for experience is suggested by findings that more experienced foragers have larger mushroom body neuropils (Fahrbach et al. 1995a) and that overaged nurse bees have larger mushroom body neuropils than normal-aged nurse bees (Withers et al. 1995). Changes in the volume of *Drosophila* mushroom bodies that are apparently related to rearing conditions also imply that experience can alter the volume of this brain region, although the factors related to this plasticity remain a matter for

speculation (Heisenberg et al. 1995). It is of great interest that the *Drosophila* learning mutants *dunce* and *rutabaga* do not show experience-mediated plasticity in the number of Kenyon cell fibers (Balling et al. 1987). One possible interpretation of these data is that deficits in cAMP-mediated pathways that reduce the ability of mutant flies to learn may in turn reduce experience-dependent proliferation or stabilization of synapses.

Mushroom Bodies and Behavioral Development in Honey Bees: A Model

Our current hypotheses on the expansion of the neuropil of the mushroom bodies are based on the observations that older bees, foraging bees, and bees with high levels of juvenile hormone all have a relatively greater volume of neuropil than younger bees, nurse bees, and bees with lower levels of juvenile hormone. Given that neuronal somata and processes are not intermingled in the calyces, and that little or no neurogenesis occurs in the mushroom bodies (Fahrbach et al. 1995c), we assume that the increase in volume reflects changes in synaptic structures, but this remains to be determined empirically. We propose that, in part, this volume change results from the addition of synapses to the calyces of the mushroom bodies of the adult bee, independent of experience and endocrine status. This steady accumulation of synapses could be referred to as "ontogenetic synapse addition".

This baseline rate of synapse addition could be increased by experience and juvenile hormone in one of two ways. Experience (either the social and physical environs of the hive, or the world outside the hive) or exposure to a level of juvenile hormone above a certain threshold, or both, could lead either to a greater rate of synapse formation or to a greater degree of stabilization (less loss) of newly formed synapses. Modulation of the neuropil by juvenile hormone would not necessarily require the very high levels of juvenile hormone typical of the foraging bee; this effect could instead have a significantly lower threshold. High levels of juvenile hormone may be associated with high levels of foraging activity (increased behavior) rather than with greater effects on the structure on the brain. The dissociations of age, experience, and juvenile hormone needed to test these hypotheses in honey bees are now all experimentally feasible. The current evidence supports a major role for juvenile hormone with a possible contribution of visual experience (Durst et al. 1994; Withers et al. 1995). Exposure to olfactory/tactile/auditory stimuli of the hive would, in the absence of raised levels of juvenile hormone or visual experience outside the hive, support only a basal rate of synapse addition.

One scenario is that changes in the brain (possibly mediated by juvenile hormone) that precede orientation flights are paired with experience to produce the final configuration of the mushroom bodies, and that it is this combined action that determines the bee's ability to perform well on tasks that demand learning such as foraging. More experienced foragers have a larger mushroom body neuropil volume than new foragers (Fig. 4); experienced foragers also forage more efficiently than new foragers (Dukas and Visscher 1994).

Ultrastructural comparisons of the mushroom body neuropil from the brains of foragers and younger bees are necessary to determine which elements within that neuropil are changing. These studies can soon be coupled with molecular genetic analyses of neural plasticity. New

techniques for the study of differential gene expression under different endocrine and behavioral states are for the first time being applied to the honey bee brain and will provide new insights. Substantial genetic variability exists for honey bee behavior (for review, see Page and Robinson 1991; Moritz and Southwick 1993), including learning abilities (Brandeis 1988; Bhagavan et al. 1994). Molecular genetic analyses of brain and behavior in honey bees will likely be strengthened as soon as methods for genetic transformation of insects other than *Drosophila* become available (Robertson 1993; Hoy 1995).

Is the increase in the neuropil of the mushroom bodies is associated with spatial learning, particularly that associated with learning the location of the nest during orientation flights? This hypothesis does not rule out the possibility of a role for the mushroom bodies in other aspects of worker foraging behavior, but it does reflect the fact that neuropil expansion has been detected in queen as well as in worker bees, as noted above. Clearly, a detailed exploration of the relationship between volume changes and improvements in behavioral performance is necessary.

Conclusion

Past and present studies of the honey bee have maintained distinctions between "learning" (what bees do when presented with a conditioned stimulus/unconditioned stimulus pair under controlled conditions) and the "behavioral maturation" that supports age polyethism. Although behavioral maturation is clearly not a synonym for learning, behavioral maturation is the context in which the learning of the bee (kin recognition, nest site location, improvement in skill at getting resources from flowers) naturally occurs. It is clear that laboratory studies of bee learning have been designed to reflect the abilities of bees in their ecological niche as social, fixed-site nest dwellers that forage as individuals but return home to share resources (Menzel 1985). This synthesis of laboratory and field studies can now be taken one step further. Our purpose in writing this review has been to highlight the plasticity inherent in honey bee maturation, to recognize the convergence of separate lines of research on the mushroom bodies, and to suggest that the three processes of learning, behavioral maturation, and developmental plasticity share common brain mechanisms in the honey bee.

Acknowledgments

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