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Recent Advances in Behavioral (Epi)Genetics in Eusocial Insects

Comzit Opachaloemphan[1], **Hua Yan**[1,3,4], **Alexandra Leibholz**[2], **Claude Desplan**[2], and **Danny Reinberg**[1,4]

[1]Department of Biochemistry and Molecular Pharmacology, New York University School of Medicine, New York, NY 10016, USA

[2]Department of Biology, New York University, New York, NY 10003, USA

[3]Department of Biology, University of Florida, Gainesville, Florida 32611, USA

[4]Howard Hughes Medical Institute, New York University School of Medicine, New York, NY 10016, USA

Abstract

Eusocial insects live in societies in which distinct family members serve specific roles in maintaining the colony and advancing the reproductive ability of a few select individuals. Given the genetic similarity of all colony members, the diversity of morphologies and behaviors is surprising. Social communication relies on pheromones and olfaction, as shown by mutants of orco, the universal odorant receptor coreceptor, and through electrophysiological analysis of neuronal responses to pheromones. Additionally, neurohormonal factors and epigenetic regulators play a key role in caste-specific behavior, such as foraging and caste switching. These studies start to allow an understanding of the molecular mechanisms underlying social behavior and provide a technological foundation for future studies of eusocial insects. In this review, we highlight recent findings in eusocial insects that advance our understanding of genetic and epigenetic regulations of social behavior and provide perspectives on future studies using cutting-edge technologies.

Keywords

social behavior; neuroplasticity; caste development; odorant receptor; critical periods; epigenetics

1. INTRODUCTION

Many animals live in cooperative societies that confer specific advantages in increasing group fitness. While these animal societies maintain a traditional reliance on individual reproductive success, eusocial insects live in colonies, where the fitness of the colony greatly outweighs individual fitness. Individuals in a colony are divided into castes, which exhibit unique and complex caste-specific behaviors. The vast majority of individuals in a colony is reproductively inactive and serves one or a few fertile female queen(s). Although all workers in a colony are closely related siblings, they have widely different behaviors that are adaptive and responsive to changing circumstances.

Colonies can be viewed as superorganisms (49). Each caste fulfills a specific role that contributes to the functioning of the whole colony but cannot survive on its own. The queen

is the “ovary” of the colony, as the only (or one of very few) reproductively active member(s) of the colony. Nurses are reproductive accessories, feeding and caring for the queen, larvae, and pupae. Foragers are the “muscle” and fulfill multiple functions, including defending the colony and gathering food. Stable colonies have a strictly maintained social distribution of roles. However, individuals in several eusocial species have the remarkable ability to switch between castes, and in exceptional cases, this caste transition can extend the life span of the colony past the death of the queen or other members of the colony. When various castes act in a coordinated manner, the result is a wildly successful species, which is showcased by the often-quoted fact that the biomass of all ants on Earth is equivalent to that of humans (48).

Multicellular organisms with disrupted intercellular communication suffer severe consequences owing to a lack of coordination between cells and organs (12). Similarly, intracolony communication is paramount for coordination of actions and resource sharing. Ants have two main routes of communication: chemosensory and tactile. Chemosensory communication is the most essential, as it dictates caste status and colony affiliation (138). As such, it has long been assumed that olfaction plays an important role in determining social deference and dominance (72, 118). Accordingly, given the reliance of communication on chemical signals, the number of odorant receptors (ORs) in ants is dramatically expanded compared with that of solitary insects such as fruit flies and mosquitoes, indicating the considerable importance of the olfactory system in eusocial insects (147, 148). Recently, functional analysis of the universal odorant receptor coreceptor (Orco) was performed in ants to test the role of their chemosensory system, providing major clues into the mechanisms by which environmental signals influence social behavior via olfaction (130, 143).

Since 2010, several research and review papers have highlighted the rich diversity of behaviors in eusocial species and revealed the underlying molecular mechanisms controlling social behaviors. We provide an expanded understanding of subsequent recent successes in genetic manipulation, pharmacological inhibition, and RNA interference in eusocial insects and discuss what this reveals about the cellular and molecular regulatory mechanisms controlling behavior and behavioral transitions. Additionally, we discuss the implications of achieving genetic manipulation in eusocial insects, a stated goal of previous papers (144). We argue that, owing to the rich repertoire of individual behaviors, social interactions, and strong neurobehavioral plasticity during development and in adults, as well as the current genetic and epigenetic tools available, eusocial insects provide a fascinating model that allows researchers to study the interactions of molecular, neural, and synaptic controls of behavior.

2. COMPLEX SOCIALITY IN EUSOCIAL INSECTS

Eusociality is the highest level of social organization in an animal colony and is broadly defined by its division of labor including distinct reproductive individuals (22). Eusociality is postulated to have arisen in hymenopterans more than 100 million years ago (37, 95), which has allowed for a rich diversity of intra- and intercolony dynamics to develop. In this section, we discuss several unique caste systems, whose determination can be either rigid or

flexible, and the range of social interactions (which are antecedent to colony fitness) between members of a single colony and between different colonies.

Rigid caste determination is irreversible and happens during prepupal development (8, 48, 136). This is the case in the carpenter ant *Camponotus floridanus,* in which colony reproduction depends exclusively on a single queen (called a monogynous structure) determined irreversibly during early development. Additionally, worker subcastes in *C. floridanus*, termed majors and minors owing to their differing sizes, are determined irreversibly in late larval development (Figure 1a). Leaf-cutter ants are another example of a rigid caste species, as worker size, which is optimized for specified caste-associated duties, is established during larval development (139). When castes have distinctive alternate morphologies, as in the case of drastic size differences, the species is said to be polyphenic (87). Worker subcaste polyphenism is a characteristic associated with rigid caste societies; however, polyphenisms between queens and workers exist in both rigid and flexible caste systems. Rigid determination allows for multiple physically optimized castes or subcastes, but adults in these species are limited by an inability to adapt immediately to situational demands.

Harpegnathos saltator is considered an extremely flexible caste system; these ants can transition from a worker to a pseudoqueen (gamergate) and revert back to a worker (C. Opachaloemphan, unpublished data) (144). This adaptation allows *Harpegnathos* colonies to survive queen death (provided colony members still remain), unlike the rigid caste species discussed above. Although initial establishment of worker versus queen castes occurs during development, transition and reversion processes can occur in adulthood and are accompanied by physiological and behavioral changes, such as increased ovary size and reduced optic lobe volume in the gamergate and loss of foraging behavior in the workers (42). Varying degrees of caste flexibility are evident, as some species display adult plasticity only between certain castes (Figure 2). Although adult honeybee workers (*Apis mellifera*) cannot become queens, they do display age polyethism, behavioral change that is characteristic of eusocial insects: In a stable colony, a young honeybee worker is tasked with nursing the young and cleaning inside the hive, while an older worker (>3 weeks) forages for food. This division of labor establishes a caste ratio, a ratio of the number of workers within each subcaste (50, 100). In colony instability, caste ratios may shift to suboptimal levels (e.g., not enough foragers or nurses). When this occurs in honeybees, older foragers can transition into nurses to compensate and vice versa, and this transition is accompanied by a shift in the DNA methylation profiles of brain tissue (47).

Rigid division of labor in a massive colony necessitates extensive social interactions to ensure collaboration. Interactions can be simple, as in the case of tandem running (repeated abdominal and hind-leg tapping used to lead nestmates to a new destination) (34), or complex, as in the case of leaf-cutter ants, who are able to form sustainable agricultural farming societies. Leaf-cutter workers collaboratively forage for, clean, reserve, and culture leaves to grow fungi (83). Another interesting example of highly organized cooperation is in *Eciton hamatum*, an army ant, which has learned to create an ant bridge in the face of an obstacle blocking its path. This allows the ants to travel and transport food efficiently in unfamiliar situations (99). These behaviors showcase the rich diversity of social interactions

within a colony. Furthermore, colonies can also interact with one another in a multitude of ways.

Intercolony social dynamics of ants are not well studied, although an astounding array of intercolony interactive behaviors has been reported. *Polyergus lucidus*, or slave-making ants, practice intercolony parasitism; these ants survive by invading the nests of other ant species, mimicking the chemical profile of the queen (effectively stealing her identity), and using this newfound social position to manipulate the enslaved ants into serving the counterfeit queen (19). When *Camponotus* ants, which have a precise nestmate recognition system, encounter another colony, they engage in immediate warfare (14, 82, 91). In contrast, *Harpegnathos* colonies often accept a nonnestmate worker from a different colony (9).

Colonies have a diverse set of complex hierarchical structures that fit the specific needs of the environment. Diversity in structures is paired with diversity in caste plasticity, routes of communication, complex coordinated behavior, and intercolony interactions. The vast range of social dynamics regulating colony coordination fosters an interesting comparison of caste-specific behaviors and distinct neurodevelopment from individuals with closely related genetic traits.

3. DEVELOPMENT OF SOCIAL BRAINS IN EUSOCIAL INSECTS

3.1. Developmental Trajectories

As described above, an ant colony acts as a superorganism: To maintain its homeostasis, members of the somatic lineage (wingless workers) stay in the colony and perform tasks such as nursing, foraging, and defending, while members of the germline lineage (newborn virgin queens and males, which bear wings) normally leave the nest to mate and found new colonies or die (in the case of males). Differential behaviors between castes are important for both colony survival and expansion of the species.

Earlier studies identified temporal critical periods (see Section 3.3) when individuals have heightened sensitivity to exogenous hormonal treatment: The response window for queen versus worker differentiation occurs in late embryos or early-instar larvae, whereas subcaste differentiation normally occurs in late-instar larvae (136). How is development in separate trajectories differentially regulated, thereby giving rise to distinct neural anatomies and behaviors? What hormones, signaling pathways, transcription factors (TFs), and chromatin factors are involved? Earlier studies on wing development in queens versus workers shed light on the differential mechanisms underlying tissue fate (1). The era for tackling the genetic and epigenetic mechanisms underlying neuronal differentiation and neural circuit formation has now arrived.

3.2. Peripheral Neuron Development

Eusocial insects rely primarily on chemosensory communication to organize social cooperation. Chemosensory receptor neurons, which underwent positive selection during social evolution, play a crucial role in animal communication (43, 147). Here we compare the development of vertebrate and invertebrate olfactory systems and discuss the importance of olfactory plasticity in establishing social communication.

3.2.1. Odorant receptors.—Chemosensory receptors include ORs, gustatory receptors (GRs), and ionotropic receptors (IRs) (56). Whereas both vertebrate and insect ORs comprise 7-transmembrane domains, vertebrate ORs are G-protein-coupled receptors, and insect ORs form heteromultimers with Orco, an obligate common coreceptor (6, 61). Together, ORs and Orco form odorant-gated ion channels. Interestingly, there are only 60 *Or*genes in *Drosophila*. By contrast, the number of *Or* genes increases in hymenopteran insects, with the largest numbers (300–500) in ants, thus strongly suggesting that the olfactory system plays an important role in social communication (148). Notably, a major *Or* gene subfamily that encodes 9-exon ORs is dramatically expanded in hymenopteran insects, with the most dramatic expansion in ants. Indeed, 9-exon ORs play an important, but not exclusive, role in sensing cuticular hydrocarbon (CHC) pheromones, as evidenced by functional analyses using the *Drosophila* system discussed in Section 4.1. Both vertebrates and invertebrates follow a general one neuron–one receptor rule: Each odorant receptor neuron (ORN) expresses only a single type of OR, with axons of ORNs that express the same OR converging to the same glomerulus located in the olfactory bulbs in vertebrates or the antennal lobes (ALs) in invertebrates, thereby forming highly organized glomerular maps of the olfactory world (6, 61).

3.2.2. Odorant receptor neuron development and axon targeting.—Insect ORNs are located mainly in the antennae and maxillary pulp. Dendrites of ORNs are held in hair-like structures called sensilla. Different types of sensilla (e.g., basiconic, trichoid, and coeloconic) hold various combinations of ORN types and numbers. A *Drosophila* antenna contains a total of 410 sensilla, with each sensillum containing 1–4 ORNs; therefore, a *Drosophila* antenna normally has ~1,000 ORNs (65). These numbers are dramatically expanded in ants: A *Camponotus* worker contains a total of ~7,500 sensilla, and basiconic sensilla can have up to 130 ORNs (86). Thus, the total number of ORNs in *Camponotus* might reach 60,000!

Insect ORNs project axons into glomeruli and form synapses with projection neurons that project further into the mushroom bodies (MBs) and the lateral horns. Consistent with the expansion of ORs and the increased number of ORNs, the number of glomeruli is also dramatically expanded from 60 in *Drosophila* to 250–500 in female ants (35, 130, 143, 149).

In mice, genetic inactivation of ORNs (e.g., via kir2.1) leads to the loss of these neurons (145), although whether they die or convert to a different type of neuron is not clear. In *Drosophila*, loss of Orco that also inactivates ORNs does not affect the neuroanatomy of ORNs and glomeruli during development (66). However, it leads to the degeneration of axons and morphological changes in glomeruli after eclosion (16). In contrast, *orco* mutations in two ant species, *H. saltator* and *Ooceraea biroi*, dramatically reduce the number of ORNs and glomeruli. Strikingly, only ~20% of glomeruli remain in mutant workers (130, 143) (Figure 3a) in these two ant species that separated 120 million years ago, suggesting that the function of Orco in regulating neural development is conserved in ants and perhaps in all hymenopteran eusocial insects whose *Or* genes were expanded in evolution. Although dramatically reduced, the number of glomeruli remaining in *Harpegnathos* mutant workers is 62, more than the number of ORN subtypes that express one of the other chemosensory receptors, 17 GRs and 23 IRs (148). This finding suggests that a small number of OR-

expressing ORNs are Orco independent and survive. Reliance on Orco for ORN development is not limited to female *Harpegnathos*: Wild-type male AL has only 79 glomeruli (fewer than one-third compared with females) and displays a striking reduction in *orco* mutants—only 31 glomeruli remain in mutant male ants (143).

Developmental defects in *Harpegnathos* occur at the pupal stage, suggesting a critical period after ORNs are differentiated (143). Further investigation is required to elucidate the temporal window of this critical period. Importantly, it is not yet clear whether the activity required for the survival of ORNs is spontaneous (independent of odorant sensory input) or induced by odorants (as ant pupae are likely exposed to nest odorants). If the latter is true, the selection of neurons may depend on the early social environment, as only the neurons responsive to nest odorants survive, which may explain the discrimination between nestmates and nonnestmates observed in ant species such as *C. floridanus* (14, 82, 91).

3.2.3. Regulation of odorant receptor expression and odorant receptor neuron development.—A group of TFs controls the development of olfactory neurons in both vertebrates and invertebrates (3, 98). In mice, *Or* genes are expressed in a stochastic manner through a process that remains to be understood (81). Two conserved *cis*-regulatory sites are required in every *Or*promoter: a homeodomain site bound by the TFs Lhx2 and Emx2 and an O/E (Olf1/early B cell factor)-like site. Before a given ORN expresses a specific *Or*,*Or* loci are marked with heterochromatic histone modifications: H3K9 and H4K20 trimethylations (77, 81). Only the selected *Or* genes are derepressed by the transcriptional regulators, including the TFs Lhx2 and Emx2. This leads to expression of a single *Or* gene, and presence of the corresponding OR protein suppresses expression of all other *Or* genes via a negative-feedback loop that involves the unfolded protein response pathway and other factors, such as the histone demethylase LSD1, that ultimately reestablish global gene silencing via formation of densely compacted foci (24). Reversing formation of these foci by overexpression of the lamin B receptor results in coexpression of many ORs in a single ORN (17).

In *Drosophila*, ORs are expressed in neurons derived from a sensory organ precursor (SOP) that develops into a sensillum. Differentiation of an SOP is regulated by the combined expression of patterning factors, including Lozenge (Lz), Amos, and Atonal, which control the fate of sensilla. Atonal lineage develops into coeloconic sensilla, many of which contain IRs and not ORs. Amos specifies basiconic sensilla when coexpressing high levels of Lz but leads to the development of trichoid sensilla when coexpressing low levels of Lz. Within sensilla, further differentiation of an SOP into neurons depends on the activity of Notch signaling. In the first round of division, the initial SOP divides into two daughter cells: PIIa and PIIb. PIIb is Notch-off, and PIIa is Notch-on. Two more rounds of division follow, generating the sensillum as well as ORNs derived from PIIb and glia derived from PIIa (3). Because each round relies on the same Notch-on/off signaling, it is generally assumed that the chromatin state is crucial in selecting the final OR expressed. Indeed, the transcriptional corepressors Hamlet (a homolog of Prdm16) and C-terminal-binding protein specifically increase H3K27 trimethylation and decrease H3K4 trimethylation on Notch target loci, thereby erasing their inherited Notch-on status and enabling subsequent responses to Notch signaling (27). At the later stage of ORN maturation, seven TFs (Acj6, zf30c, Sim, Xbp1,

Fer1, E93, and Onecut) regulate the selection of an *Or* gene (3, 54, 55). In *Drosophila*, only two more rounds of division from the PII precursor cells generate one to four neuron(s) in each sensillum. However, given the dramatic expansion in ants, at least seven to eight more rounds must be required to generate the necessary number of ORNs, which, with an increased number of SOPs, might explain why there are ~60-fold more ORNs in an ant antenna than in *Drosophila*. How TFs and chromatin modifications achieve this requires further investigation.

3.2.4. Regulation of axon targeting of odorant receptor neurons.—Proper axon targeting requires expression of different adhesion molecules. In adult mice, ORNs undergo continuous turnover (5). How do young postnatal ORNs target the correct glomeruli? There appear to be two stages of ORN axon targeting: one for ORNs born within a temporal window before postnatal day 7 (critical period) and the other for ORNs born later (75, 131). In the critical period, ORNs do not follow the track of existing ORNs but instead use their own targeting adhesion molecules—such as Robo, neuropilins, semaphorins, Kirrels, and ephrins (104)—to find their correct glomerulus targets. How each class of ORNs targets a specific glomerulus remains unknown. In contrast, later-born ORNs follow the track of existing ORNs that express the same OR via homotypic interactions, which may also use guidance molecules such as Kirrels. As such, the olfactory/glomerular map may be established during the critical period in mice.

Similar to early ORN axon targeting in mice, a wide range of adhesion molecules—such as Robo, Dscam, N-cadherin, Semaphorin-1a, and Teneurins (3)—regulate the projection of ORN axons to specific glomeruli in *Drosophila*. However, whereas the presence of an OR controls adhesion molecule expression and axon targeting via G-protein signaling in mice, ORs have no influence on axon targeting in *Drosophila*.

As discussed above, mutations in *orco* lead to loss of ORNs and glomeruli in ants. However, Orco-independent ORNs survive in mutant ants. It is not yet clear whether and how ORs or ORN activity regulates adhesion molecule expression and axon targeting in Orco-independent versus Orco-dependent ORNs (which disappear in *orco* mutant ants). Intriguing questions include the following: (*a*) Do Orco-dependent (unlike Orco-independent) ORNs rely on Orco or proper OR activity to express adhesion molecules, thereby targeting correct glomeruli, which is essential for their survival? (*b*) As the volume of glomeruli increases in *orco* mutant ants (143) (Figure 3a), how do survivor neurons, i.e., Orco-independent ORNs, project their axons to these larger glomeruli? These questions merit further investigation.

3.3. Development of Central Nervous System

In the *Drosophila* central brain, approximately 30,000 neurons, including peripheral and central neurons, are generated from a pool of 100 neuroblasts. Each neuroblast expresses a specific set of TFs and divides asymmetrically to produce a distinct group of neurons (i.e., a lineage) (106). During embryonic stages, neuroblasts generate a primary set of neurons and glia, which serves as a scaffold for later development. At larval stages, neuroblasts proliferate and produce differentiated neurons, generating secondary lineages. During this second phase of neuronal development, axons project along the primary scaffold, generating

a progressively more specialized neural network. After metamorphosis, the newly emerged adult brain contains mature neural circuits (122). Identifying the developmental pathway and specific circuitry in eusocial insects is required to provide fundamental insights on the neuroanatomical and neurophysiological basis of caste-associated behaviors.

3.3.1. Epigenetic regulation of caste development and behavior in eusocial insects.—Since most individuals in eusocial insects are highly related genetically, caste determination is controlled mainly by environmental cues rather than genetics. This more evolutionarily viable alternative to genetic determination provides the opportunity for adaptation: For example, caste ratios, a ratio of worker subcastes, can be adjusted to increase colony survival in the face of new demands (100, 101, 140). Differential trajectories governing caste determination are established by gene expression patterns induced by multiple interacting factors, including temperature and larval nutrition (10, 58, 127).

Specialized feeding controls queen versus worker fate in the honeybee *A. mellifera,* such that early feeding of larvae with royal jelly nutritionally determines the queen (58). Royal jelly contains up to 5% histone deacetylase inhibitor (HDACi) and perhaps has an effect on the early larval chromatin state (121). Interestingly, Sir2, an HDAC, is also nutritionally responsive in honeybees (92); higher Sir2 levels in *Caenorhabditis elegans*and mice are correlated with extended life span, which is also a feature of honeybee queens (105, 129). Whereas the exact downstream targets of royal jelly HDACi are not yet known, histone modifications are essential mediators of important caste determinations in *C. floridanus*. *Camponotus*shows distinct patterns of histone H3K27 acetylation between castes (116). The resulting differential gene expression in *Camponotus* occurs in genes related to neural development and olfactory learning (115, 116).

Besides histone modifications, DNA methylation, which is associated with honeybee queen larval development (63), appears to be another important caste determinant and has been extensively investigated in honeybees. Differing DNA methylation profiles of the brains of workers and future queens can be observed as early as 96 h posthatch. Some differentially methylated genes affect expression of metabolically associated pathways (33). However, most DNA methylation is enriched in gene bodies of actively transcribed genes and correlates with moderately and highly expressed genes, such as housekeeping genes (8, 142). In the honeybee, this regulatory methylation pattern was functionally analyzed by knocking down *DNA methyltransferase 3 (Dnmt3*), a gene encoding an enzyme that catalyzes DNA methylation (63). *Dnmt3*-knockdown bees display reduced genome-wide DNA methylation levels and altered RNA splicing patterns, suggesting the functional role of DNA methylation in honeybee queen caste determination (63, 69) (Figure 1c).

The role of DNA methylation in caste determination is further supported by studies in other eusocial insects such as the termite *Zootermopsis nevadensis*, in which queen and worker larvae have significantly different methylation patterns (39). However, additional confirmation of the association between caste determination and DNA methylation in eusocial insects is needed, as the limited number of replicates in the DNA methylome studies was recently called into question (71). Nevertheless, DNA methylation and histone

modifications both show key roles in caste development and social behavior in eusocial insects (63, 115, 116).

3.3.2. Juvenile hormone and critical periods.—Juvenile hormone (JH) is one of the main insect hormones broadly regulating insect development and reproduction (44, 88). It plays a major conserved role in early caste determination; functionally, JH regulates queen development in ants (1, 88, 94), wasps (4, 7), stingless bees (22a, 45), bumble bees (88, 113a), and honeybees (25a, 88) (Figure 1b,c). JH is synthesized from the corpora allatum, an endocrine gland in the brain (32), and positively correlates with insulin signaling in honeybees (21), thus suggesting a connection with the specialized feeding of queen-destined larvae.

Interestingly, JH is associated with differentiation of the soldier caste in the termite *Hodotermopsis sjostedti* (20, 78). The application of a JH analog (methoprene) before molting induces upregulation of several genes in the central nervous system (CNS) of soldiers (52). These genes may contribute to the enlargement of the mandibular motor neurons that is observed in workers. Upregulation occurs only during the transitional period and does not continue throughout adulthood, indicating that high JH levels coincide with the enlargement period (51). JH, however, is effective only during certain critical periods.

Long assumed to exist, critical periods in development represent intervals when an organism is particularly sensitive to environmental stimuli (46). Enhanced sensitivity to particular regulatory hormones defines critical periods in caste development (88, 136). Injection of JH is an invaluable tool for identifying the time frame of these sensitive periods in different species (Figure 1b). *Harpegnathos* larvae are responsive to the use of JH to induce queen development when the hormone is introduced in the third- and fourth-instar larvae, but not before (94). In contrast, the fire ant *Solenopsis invicta* is responsive only in the embryonic and early-instar larval phases (94, 99a). Even more interesting is that ants with dimorphic (polyphenic) subcastes appear to have more than one critical period; early treatment with a JH analog in *Pheidole* ants triggers queen development, whereas late treatment (during the last instar phase) triggers the development of major workers (88, 97, 136, 137). While the branching point for polyphenic development occurs at the larval stages, certain drug treatments of dimorphic adults may alter behavior, but not morphology, at crucial sensitive periods. In *C. floridanus*, treatments that affect epigenetic marks induce behavioral changes only in young adult workers (discussed in Section 4.2.2).

Identifying windows of heightened sensitivity is important in understanding exactly when upstream caste-determining effectors are acting. Once a temporal window is established, a spatial map of gene- and chromatin-level activities can be elucidated, giving valuable information on the often-irreversible caste-determining steps that regulate behavior.

4. FUNCTION OF THE SOCIAL BRAIN IN EUSOCIAL INSECTS

The advanced repertoire of cooperative social behaviors that eusocial insects display necessitates a sophisticated peripheral nervous system as well as CNS to achieve collective fitness. In this section, we summarize the current understanding of how peripheral and

central neurons in eusocial insects respond to and process social cues and how these signals regulate social behavior, social status, and reproduction. Importantly, we highlight progress in the investigation of epigenetic regulation of neural functions and social behavior.

4.1. Function of Peripheral Neurons

As described above, pheromones and general odorants trigger behavioral responses (68) through chemosensory receptors in insects. There are up to 130 ORNs in each ant sensillum, which makes electrophysiological analysis of chemoresponses challenging. Nevertheless, single-sensillum recording on *Harpegnathos* indicates that reproductive gamergates have an overall reduced response to a wide range of long-chain CHCs compared to workers (38), perhaps explaining their lack of repression by the queen pheromone.

Furthermore, *Or* genes underwent dramatic expansion during the evolution of hymenopteran eusocial insects. *Or* genes belonging to the 9-exon family experienced the most dramatic expansion and account for one-third of all *Or*genes in ants but are not represented in *Drosophila* (148), although other families are also expanded, indicating the importance of olfactory sensing in social communication. Interestingly, 9-exon ORNs are enriched in basiconic sensilla and specifically target T6 glomeruli in the AL (80, 149), leading to the hypothesis that the 9-exon OR family particularly mediates CHC sensing. To test this hypothesis, two groups (93, 119) used transgenic *Drosophila*that express a single ant OR in an otherwise empty ORN and analyzed the responses of *Harpegnathos* 9-exon and non-9-exon ORs to social CHC pheromones versus general odors. They identified two *H. saltator* ORs (HsOR263, HsOR271) that mediate the response to the queen pheromone (Figure 3b). Importantly, they found that both 9-exon and non-9-exon ORs respond to CHCs and general odors, suggesting that the spatial segregation of ORNs and glomeruli does not translate to functional specificity. Therefore, amplification of several OR subfamilies increases the number of ORs for CHC detection, likely allowing ants to distinguish subtle differences of CHC profiles among individuals inside or between social groups (23). Indeed, *C. floridanus* ants can discriminate between closely related hydrocarbons, allowing them to cooperate with nestmates that display the same pheromones but attack nonnestmates with slightly different pheromone cocktails (111).

Available *orco* mutant ants (see Section 3.2.2) enable behavioral analysis of their defective social communication (130, 143). The dramatically reduced number of ORNs in *orco* mutant ants disrupts their responses to general odorants (Figure 3a). In addition, *orco* mutant ants appear to be unable to sense brood, sex, and trail pheromones, among others. As a consequence, they display a lack of social interactions, abnormal social behaviors, and reduced fitness. Because mutant ants have intact GRs, IRs, and other chemosensory receptors, expanded ORs likely play a critical role in regulating social communication and social behavior. Interestingly, although HsOR263 and HsOR271 are strongly responsive to the queen pheromone, loss of this OR function in *orco* mutant ants is not sufficient to allow them to become gamergates in the presence of a queen (143). This is likely because becoming a gamergate requires positive signals by other pheromones, rather than simply the lack of repression by queen pheromones. Alternatively, chemosensory receptors beyond the OR family might mediate queen pheromone perception.

4.2. Function of Central Neurons

Eusocial insects can display dramatic adult plasticity in regulating caste transition (see Section 2): For example, nurses can switch to foragers in honeybees, and workers to gamergates in *Harpegnathos*. How genetic and epigenetic processes regulate the functionality and plasticity of the mature brain has become a major field of inquiry for researchers of eusocial insects. Mechanisms of regulation, including DNA methylation, histone modifications, and noncoding RNAs, are responsible for regulating and, importantly, maintaining gene expression patterns. These changes can result in massive shifts in neuronal organization and behavioral phenotypes (8, 47, 74, 115, 116). The growing number of studies in animals and humans emphasizes the importance of this field, revealing that epigenetic alterations in the CNS result in neurological disorders (57, 135). For example, loss of function of methyl-CpG-binding protein 2 (MECP2) leads to symptoms characteristic of Rett syndrome, a neurological disorder characterized by loss of language acquisition and intellectual impairment (2).

4.2.1. Central nervous system.—Animal behaviors are inherently responsive to external stimuli. Stimuli are first sensed and transmitted to the CNS, where they are processed and integrated, and the regulatory response is enacted. Eusocial insects have a unique sensitivity to a wide variety of stimuli, such as infrared light (112), polarized ultraviolet light (36, 133), magnetism (107), and a vast range of odors, all of which contribute to the behavior of colony members (68). Given the essential role that the CNS plays in behavioral regulation, it is no surprise that differing brain morphologies exist between individuals with vastly distinct social roles. Surprisingly, however, this morphological shift can occur in an adult insect within its lifetime and is sometimes reversible.

To understand morphological brain changes in eusocial insects, deep knowledge of the *Drosophila* nervous system is important, as it shares common organization and function (28). The ALs and MBs are highly developed brain regions in both species and are similarly responsible for olfaction and learning/processing. Accordingly, well-established *Drosophila* neurodevelopmental tools and insights can serve as useful models in studying hymenopteran brain development. The MBs in invertebrates, which may be analogous to the hippocampi in vertebrates (126), coordinate responses to external stimuli. They have been implicated in behavioral responses and the consolidation and retrieval of memory (79).

Both ALs and MBs are highly altered in individuals with very different social behaviors. The noneusocial but well-studied desert locust provides evidence of the fluidity of these brain regions in varying social settings. The desert locust has two different life phases: solitary and gregarious. Population density catalyzes the transition between phases: An isolated condition yields a solitary-phase insect, and a highly populated condition yields a gregarious swarm-forming locust. The gregarious locust engages in migratory behavior, but the solitary locust does not; this distinction is correlated with dramatic differences in their brain morphology. Compared with solitary locusts, gregarious locusts have a considerably larger overall brain, larger absolute volume in MBs, and reduced AL proportions to midbrain (89). Broader diet preferences and more extensive foraging strategies may explain the larger

MBs in gregarious locusts, whereas higher sensitivity to food odorants and more limited food preferences may explain the relatively larger ALs in solitary locusts (25, 89).

Sociality has a pronounced effect on MB development, as evidenced in *Megalopta genalis*, the sweat bee whose queen can switch from being solitary to social (110). The social queen has more developed MBs than its solitary counterpart (120). This is likely due to the reliance of social communication on olfactory processing. Even within a lifetime, behavior has a pronounced effect on brain morphology: In the honeybee, organization of the MBs of a newly emerged queen is very plastic and responsive to early experiences, such as mating and leaving the hive (29). In worker castes of *A. mellifera*, *C. floridanus*, and *Cataglyphis bicolor* (a desert ant), foraging induces a direct increase in MB neuropil size (28a, 41, 64, 141). Caste-specific behaviors can require particular neural functions, as in the genus *Pheidole*, where dimorphic worker subcastes have dramatically different brain organizations and relative volumes, particularly in the MBs (84).

Given the predominance of age polyethism in eusocial insects, age and experience likely play a role in the morphological shifts of brain mass. As expected, experience-based memory formation in eusocial species can alter neuronal reorganization and increase the density of synaptic complexes in the MBs (30, 108, 124). For example, in honeybees, the density of microglomeruli (a synaptic complex formed by axonal ends of projection neurons and dendritic branches of Kenyon cells) in the MBs increases while the volume remains constant as the insect ages (31). This is observed in all regions of the MBs, which include both visual and olfactory processing areas. Specifically, MB density increases as foraging behavior increases, likely owing to a heightened need for visual processing, spatial orientation, and memory, all of which are necessary for successful foraging (26, 53, 64, 124, 125).

Eusocial insects have a stark division of labor between castes that defines each caste's specialized function in the colony. Importantly, this behavioral exclusivity is independent of the original genomic content of the organism, leading to mysterious questions of how epigenetics shapes neuroplasticity and enables complex social interactions. Given naturally occurring morphological and behavioral plasticity, environmentally induced neuroplasticity and neurodevelopmental trajectories can be readily studied and manipulated in eusocial insects.

4.2.2. Functional analysis of behavior in eusocial insects.—The carpenter ant *C. floridanus* has no naturally occurring behavioral switch between its two distinct worker subcastes, minors and majors. Both types of workers have highly specific behaviors and morphologies that are irreversible. Nevertheless, a recent study (115) showed that the specific foraging behavior of a subcaste can be reprogramed pharmaceutically in early adulthood by changing histone acetylation levels in the brain and altering the expression levels of genes involved in specifying neuronal activity. Young major brains were injected with an epigenetic drug that disrupts the removal of histone acetylation (HDACi), resulting in major workers displaying minor worker–specific behaviors (Figure 2a). These results were confirmed through knockdown of the HDAC *Rpd3* in majors. Conversely, young major brains coinjected with histone acetyltransferase inhibitors display reduced foraging activity,

suggesting the dependence of minor and major worker behaviors on histone acetylation (115). Interestingly, this study also identified an epigenetic window of sensitivity (critical period) in the young brain, which shows a heightened early responsiveness to histone-modifying inhibitors that decreases sharply with age (115).

A similar decrease in plasticity is speculated to occur in *Harpegnathos*; compared with old workers (foragers), young workers (nurses) have a heightened ability to become gamergates. The neuropeptide corazonin stimulates foraging behavior (40) (Figure 2b). Likewise, in other eusocial insects including ants, termites, and honeybees, nonreproductive foraging workers have high expression levels of *corazonin*, and nonforaging individuals have low expression (59, 90, 128). Corazonin is thus considered a putative key neural regulator of complex social behavior. Further analysis (40) confirmed its functional role: Injection of synthetic corazonin peptide into *Harpegnathos* brains induced hunting behavior and suppressed egg-laying ability. Additionally, knockdown of *corazonin receptor* gene by RNAi conferred the opposite behavioral modifications (40). The study displays a stark example of a behavioral switch in eusocial insects that is regulated by a simple gene encoding a neuropeptide.

Behavioral changes may occur as a result of cascades leading to general pathway inhibition or upregulation. Transcriptomic analysis in honeybees has revealed a molecular basis for aggressive worker-associated behaviors: Decreased mitochondrial activity in the brain correlates with increased aggressive behaviors (15). In accordance, pharmaceutical-based inhibition of oxidative phosphorylation significantly promotes aggression in honeybees (70). In the mature adult stage, females in stable eusocial insect societies have well-established caste affinities: What set of genes (genetic tool kit) regulates their behaviors? Functional analyses discussed in this section provide a basis for understanding the genetic and epigenetic mechanisms controlling caste-specific behaviors, such as aggression in honeybees and foraging in ants.

5. DEVELOPMENT OF GENOMIC, GENETIC, AND EPIGENETIC TOOLS IN EUSOCIAL INSECTS

Defining characteristics of model organisms include ease of genetic manipulation and availability of experimental technologies. To establish eusocial insects as model organisms, the development and use of a wide array of technologies are paramount. In this section, we provide an overview of recent technological advances developed in and applicable to eusocial insects.

5.1. Traditional Transcriptomics

In recent years, comparative genomic approaches have been widely integrated into eusocial insect studies to identify the genes responsible for organizing societies, analyze the evolution of sociality from solitariness, and address the common molecular mechanisms underlying social communication and behaviors between humans and animals (114). The main goal has been to grasp how the genomes of eusocial insects program their complex social behaviors yet allow the insects to retain plasticity and adaptability to environmental cues. A growing

number of genomes in eusocial insects including bees, ants, wasps, and termites have been sequenced to date; however, the relatively low quality of many draft genomes in nonmodel organisms impedes sophisticated genome-wide analyses. Two ant genomes (*C. floridanus* and *H. saltator*) have recently been improved through the generation of long genome reads, which provide long, contiguous, and accurate assemblies as well as comprehensive protein coding and long noncoding RNA annotations (113). High-quality genome assemblies have allowed scientists to apply bioinformatic approaches to eusocial insects, thus opening the door to more sophisticated studies and functional analyses.

Nowadays, most studies attempting to identify a key gene controlling caste development, behavior, or aging rely heavily on prerequisite transcriptomic data. The genes identified are often based on whole-insect or whole-tissue transcriptomes that compare bulk gene expression profiles among castes. Owing to the heterogeneity of these tissue samples, gene expression measurements provide average information from multiple cell populations, resulting in imprecise outcomes.

In the past few years, rapid advances in high-throughput technology have enabled highly sensitive approaches toward quantifying gene profiles from single cells. Drop-seq and 10x Genomics, approaches for collecting messenger RNA (mRNA) transcripts simultaneously from multiple cells and barcoding each cell individually in a distinctive droplet, have become critical for studying transcriptomes in complex tissues (62, 76, 146). These valuable tools use transcriptomic data to identify key genes in specific cells of highly complex and heterogeneous tissues such as the brain. However, these single-cell transcriptomic approaches do not provide spatial information pertaining to neural circuitry.

5.2. Spatial Transcriptomics and Chromatin-State Analysis

Once a key gene has been identified, its spatiotemporal expression patterns must be confirmed. This can be achieved by performing serial hybridizations or multiplexing barcoding probes (73). Single-molecule fluorescent in situ hybridization (FISH) can produce patterns that are quantitative and sensitive enough to count individual mRNA molecules, thus potentially mapping the spatial distribution of expression in tissue and pinpointing functional circuitry. An alternative approach to FISH is in situ sequencing, which uses a spotted-barcoded microarray to capture mRNA from tissue and sequence the spatially barcoded transcripts (123). This alternative has the advantage of not requiring prerequisite sequence knowledge, but its resolution is limited by the spot size.

Recent rapid advancements in experimental tools are not limited to gene expression measurement. Tools to detect chromatin state through chromatin accessibility (ATAC-seq) (13), chromatin structure (Hi-C) (85), and histone modifications (ChIP-seq) (11, 103, 117) are becoming sensitive enough to detect single-cell inputs. Given that DNA methylation and histone modifications have been implicated in regulating caste differentiation, these low-input genomic tools are useful for determining the spatial patterns of chromatin modifications.

5.3. Genetic Manipulation

Genomic methods can likely specify an area where epigenetic modifications responsible for caste determination occur, but using them to track cells through development would be difficult, time consuming, and expensive. Clonal lineage analysis is a powerful tool for studying neuronal lineage development in *Drosophila* (106). This method requires the introduction of a label into early cells, which can be accomplished using established CRISPR technology in ants. This technique can both precisely label cells and manipulate gene expression levels, allowing for visual tracking of neurodevelopment. As such, it would be possible to answer several pressing questions about developmental trajectories and the propagation of epigenetic signals.

Development of genetic tools in eusocial insects is considered essential in neuroepigenetics (144). Recently, mutant and transgenic honeybees have been derived using CRISPR and a piggyBac transposon, respectively (60, 109), thus providing proof of principle for genetic manipulation and germline transformation of eusocial insects. Also recently and for the first time, two groups (130, 143) performed functional analyses using two species of *orco* mutant ants generated by CRISPR technology. They demonstrated the essential function of ORs in regulating social communication, behavior, and neural development (130, 143).

We envision that the next steps of genetic analysis in eusocial insects will harness the immense array of genetic technologies available, such as Gal4-UAS, FLP-FRT, Cre-LoxP systems, and GCaMP. Development of these transgenic tools will require extensive collaboration among entomologists to unravel the genetic and epigenetic mechanisms underlying complex eusociality.

6. PERSPECTIVES

With the combination of cutting-edge technologies in genomics, genetics, and epigenetics, the study of eusocial insects has made tremendous progress in recent years. Eusocial insects provide an ideal platform to study the phenotypic effects of epigenetic changes, as different caste members, despite sharing a very similar genome, differ widely in morphology and complex caste-specific behaviors. Additionally, their relatively simple brain structure and rich repertoire of tractable social behaviors as well as the availability of tools for genetic manipulation make neuroepigenetic investigation in these organisms attractive. Important questions in the field include how epigenetic modifications are translated into functional consequences and how these changes regulate neuroplasticity.

Emerging methods in single-cell and spatial transcriptomics help visually connect a cell-type-specific gene expression profile with neural architecture (67). Use of these methods will allow an understanding of the fundamental steps of neuronal development and pinpoint specific neural circuits responsible for neuroplasticity in eusocial insects. Applying these technologies to eusocial insects could help identify caste-associated patterns of neural networks, for example, in the MBs. Identification of the functional neural circuitry in caste differentiation would be highly valuable for understanding the cellular basis of the extensive neuroplasticity in eusocial insects and, perhaps, other organisms.

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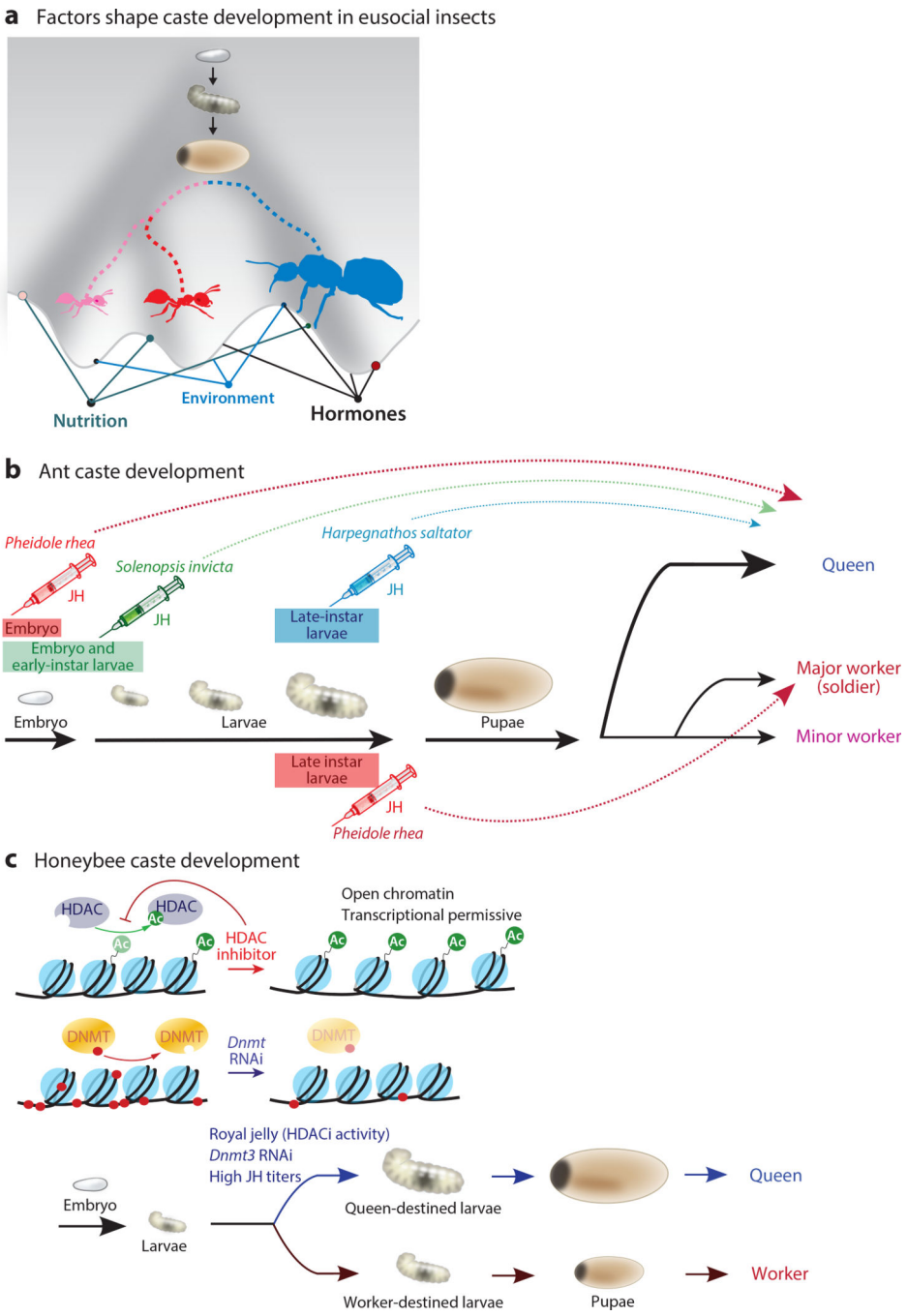

Figure 1. Major regulators during critical periods in caste determination.

(*a*) Factors including nutrition, environment, and hormones determine caste fates in eusocial insects. Embryos are pluripotent for caste determination but are highly responsive to external cues. Combinations of external cues shape development trajectories for each caste as represented by the Waddington's epigenetic landscape (134). This allows single, fertilized embryos to give rise to different female castes including queens (*blue*) and major or minor workers (*red* and *pink*, respectively). The caste is determined at early developmental stages and is maintained throughout the lifetime. (*b*) JH acts at different species-dependent

prepupal stages to trigger caste-specific development. In *Harpegnathos*, JH induces queen development at the late larval stages (94). In *Solenopsis*, JH induces queen development at the embryonic and early-instar larval stages (94, 99a, 132). In *Pheidole*, JH triggers both queen and major development at embryonic and late-instar larval stages, respectively (88, 97, 136, 137). (*c*) Queen development in the honeybee *Apis mellifera* is dependent on larval nutrition with royal jelly containing HDACi (58, 121). HDACi results in reduced removal of an acetyl group on histone tails, which may lead to an open chromatin conformation and altered transcriptional levels. Knockdown of *Dnmt3* by RNAi in young larvae mimics the developmental effect of royal jelly (63). Abbreviations: Ac, acetyl; Dnmt, DNA methyltransferase; HDACi, histone deacetylase inhibitor; JH, juvenile hormone; RNAi, RNA interference.

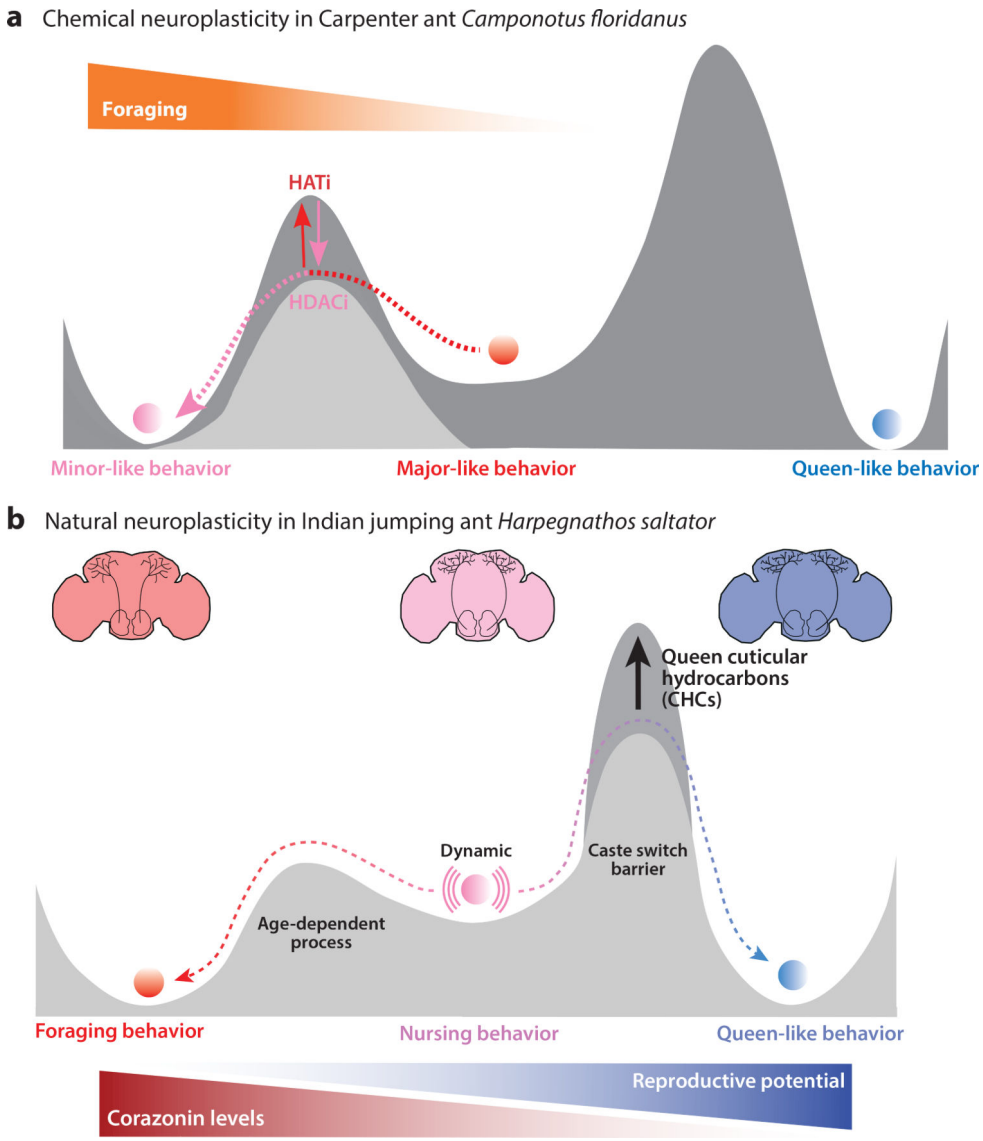

Figure 2. Neurobehavioral plasticity in eusocial insects.

(*a*) The carpenter ant *Camponotus floridanus* has two worker subcastes: minor and major. Even though *Camponotus* has a rigid caste system, caste-specific behaviors in young workers can be altered by chemical treatments: Injecting HDACi in the brain can induce minor-specific foraging behavior in majors. Coinjecting HATi antagonizes the behavioral effect (115). (*b*) In *Harpegnathos saltator*, natural environmental cues can change caste-specific behaviors: Queen CHCs repress the reproductive capabilities of *Harpegnathos* workers and prevent a caste switch. In the absence of a queen, workers develop into gamergates (*blue*), which exhibit queen-like behavior and physiology. Nursing workers (*pink*) naturally become foraging workers (*red*) as they age, in a transition related to increasing neuropeptide corazonin levels in the brain (40). Abbreviations: CHCs, cuticular hydrocarbons; HATi, histone acetyltransferase inhibitor; HDACi, histone deacetylase inhibitor.

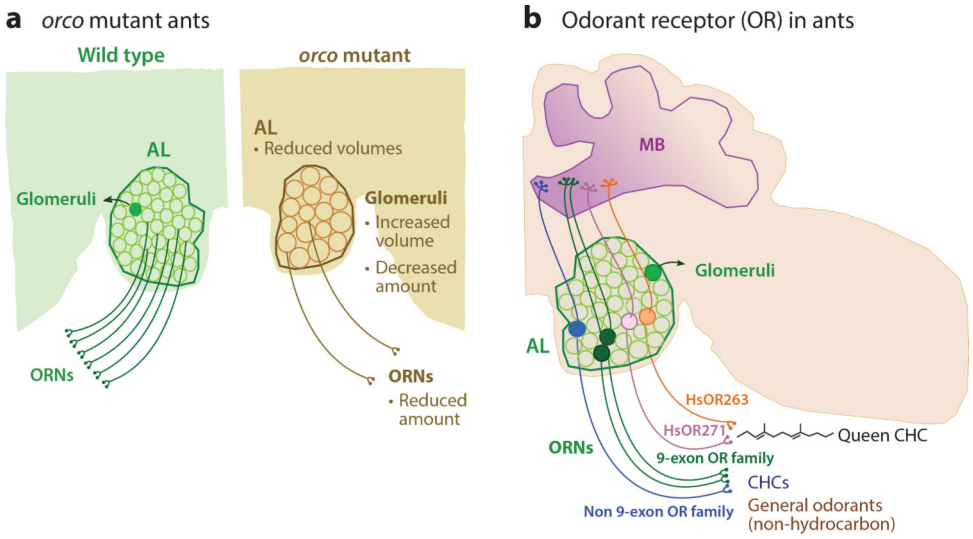

Figure 3. Anatomy and function of the olfactory system in eusocial insects.

ALs in insects consist of numerous compacted neuropil structures called glomeruli. Each glomerulus receives odorant signals from a set of ORNs that contains the same OR, while projection neurons relay the signals to the MBs and lateral horns. (*a*) *orco* mutant ants (*right*) have fewer ORNs than the wild type (*left*). The volume of mutant ALs is reduced, with fewer but enlarged glomeruli (143). (*b*) Ants have an amplified family of *Or* genes that sense a broad spectrum of odorants. In particular, the 9-exon OR family is significantly expanded in eusocial insects (147, 148). Two members of this class, HsOR263 and HsOR271, mediate response to the *Harpegnathos* queen CHC pheromone. The 9-exon and non-9-exon ORs display a broad overlap of sensitivity to general odorants and CHCs (93, 119).

Abbreviations: ALs, antennal lobes; CHC, cuticular hydrocarbon; MBs, mushroom bodies; *Or*, odorant receptor gene; *orco*, odorant receptor coreceptor gene; ORNs, odorant receptor neurons.