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Research article

Interspecific comparison of mushroom body synaptic complexes of dimorphic workers in the ant genus *Pheidole*

Darcy G. Gordon[a,*], Alejandra Zelaya[a], Katherine Ronk[a], James F.A. Traniello[a,b]

[a] Department of Biology, Boston University, 5 Cummington Mall, Boston MA, 02215, USA
[b] Graduate Program for Neuroscience, Boston University, 5 Cummington Mall, Boston, MA, 02215, USA

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ABSTRACT

Social insects may have morphologically and behaviorally specialized workers that vary in requirements for sensory information processing, making them excellent systems to examine the relationship between brain structure and behavior. The density and size of synaptic complexes (microglomeruli, MG) in the mushroom bodies (MB) have served as proxies for processing ability and synaptic plasticity, and have been shown to vary among insect species that differ in behavioral complexity. To understand the relationship between behavioral specialization and synaptic structure, we examined age-related changes in MG density and size between minor worker and soldier subcastes in two species of *Pheidole* ants, *P. dentata* and *P. morrisi,* that differ in behavior. We hypothesized that task-diverse minor workers would have more densely packed MG than soldiers, and that species-specific differences in soldier repertories would be reflected in MG structure. We also examined MG variation in young and mature minor workers and soldiers, predicting that as workers age and develop behaviorally, MG would decrease in density in both subcastes due to synaptic pruning. Results support the hypothesis that MG density in the lip (olfactory) and collar (visual) regions of the MBs decrease with age in association with increases in bouton size in the lip. However, minors had significantly lower densities of MG in the lip than soldiers, suggesting MG may not show structural variation according to subcaste-related differences in cognitive demands in either species.

1. Introduction

Flexible behavioral responses to environmental and social challenges are predicted to shape brain structure [1]. The mushroom bodies (MBs) – brain compartments that function in higher-order sensory processing – are associated with insect cognition. Kenyon cells, the principal MB neurons, receive sensory input in the mushroom body calyces (MBCs), and send axons through the peduncle to the lobes where postsynaptic efferent neurons integrate their input to control behavior [2–6]. In locusts [7], butterflies [8], fruit flies [9], and honey bees [10–12], developmental patterns of MB neuropil growth are associated with inter-individual variation in behavior. Similarly, variation in MB size correlates with broad behavioral differences across insect species; interspecific comparisons of MB circuitry can offer insight into its role in behavior [13,14].

MBCs are composed of microglomeruli (MG), synaptic complexes formed by sensory projection neuron boutons and dendritic spines of Kenyon cells, and are thought to enable neural plasticity and cognitive processing [15–17]. Information-processing challenges experienced by eusocial insect workers likely differ in association with division of labor, affecting brain structure [18–20]. In some eusocial bees and ants, MG variation is correlated with temperature during development [21–23], memory formation [24,25], and behavioral maturation [26–33]. Decreases in MG density correspond to age- and/or behavioral development-related increases in MG size [26–30]. How MG structure varies with worker morphological differentiation and task specialization, however, is not well understood.

The species-rich ant genus *Pheidole* exhibits a striking polyphenism: the worker caste is composed of small-bodied, task-generalist minors, and larger majors or "soldiers" that have disproportionately large heads and typically function in defense [34]. Strong variation in worker dimorphism and soldier specialization [34,35] make *Pheidole* an excellent system to examine how differences in task sensory processing demands impact MB synaptic structure. We selected two *Pheidole* species that differ in ecology, subcaste specialization and degree of task overlap to compare MG structure. *P. dentata* soldiers appear wholly specialized on defense [36], whereas *P. morrisi* soldiers have broad repertoires including brood care, foraging, and defense [37]. Subcaste repertoire

Abbreviations: MB, mushroom body; MBC, mushroom body calyx; MG, microglomeruli

* Corresponding author.
E-mail address: dggordon@bu.edu (D.G. Gordon).

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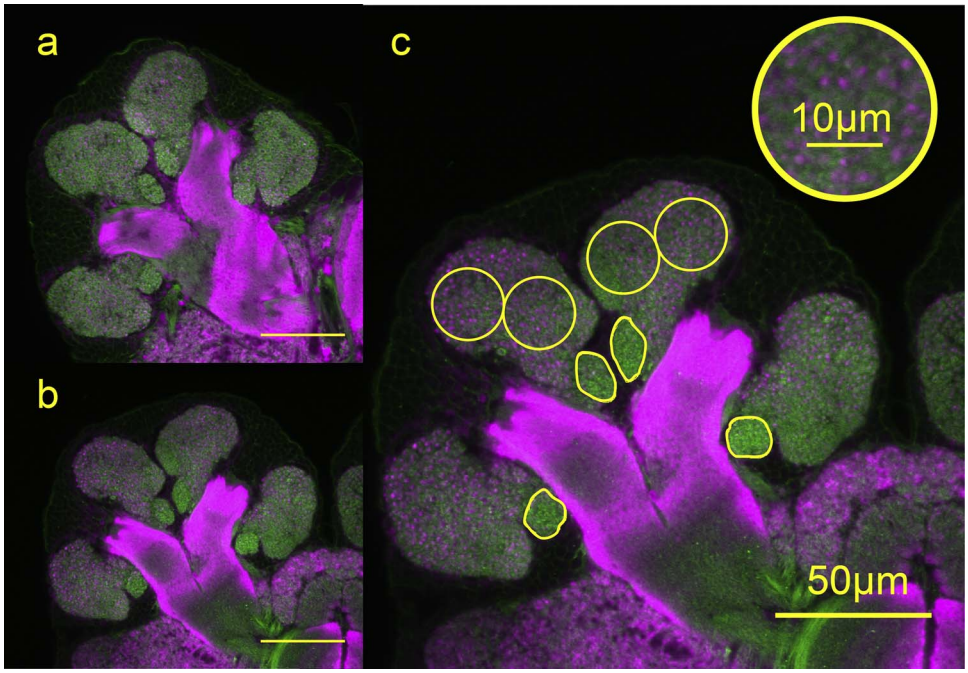

Fig. 1. Representative micrographs of mature *P. morrisi* soldier (a) and mature *P. dentata* minor (b) brains (scale bars = 50 μm). (c) Placement of counting circles in the lip region of a mature *P. dentata* minor; collar outlined in yellow. Inset: magnification of the distal medial calyx circle.

divergence between *P. dentata* and *P. morrisi* is associated with brain size and brain compartment allometries in newly eclosed (callow) and mature workers [38]. Neuropil volume differences, however, do not completely reveal behaviorally relevant neurobiological differentiation [13,39]; synaptic structure may correlate with subcaste task variation and behavioral development [33,40–42]. We used *P. dentata* and *P. morrisi* as models to test the hypothesis that species-, subcaste-, and age-related variation in behavior are associated with MG structure. We hypothesized that, in both species, MG density would be greater in minors than soldiers due to the larger task repertoires of minors, and that MG metrics would be more similar between *P. morrisi* subcastes because the more behaviorally diverse soldiers of this species have repertories more similar to minors. Furthermore, we predicted that young workers of both species would have a greater density of smaller MG than mature workers due to synaptic plasticity accompanying behavioral development.

2. Methods

2.1. Ant collection and culture

Queenright *P. dentata* (N = 9) and *P. morrisi* (N = 4) colonies were collected in Gainesville, Florida, and Long Island, New York, respectively, and maintained at 25 °C, 65% humidity with a 12 h:12 h light:dark cycle in test tubes partially filled with water plugged with cotton inside plastic boxes (20 cm × 28 cm) coated with Fluon®. Colonies were fed 1 M sucrose and live mealworms, fruit flies, or scrambled eggs alternate days.

2.2. Microglomeruli imaging and quantification

MG structure was determined in *P. dentata* and *P. morrisi* minors and soldiers of two age groups (N = 80 total, 10 per group). Age was typically estimated by cuticle pigmentation, which progressively darkens after adult eclosion. Fully pigmented workers found outside the nest were considered mature (> 3 weeks in age) and were sampled in addition to two individuals known to be 21 days old. Callows, uniformly pale in color, were conservatively considered less than three days old [43,44] and were sampled in addition to four one-day old and two six-day old individuals. Brains were dissected in ice-cold HEPES buffered saline [16,40], fixed with 4% paraformaldehyde in 0.1 M PBS overnight at 4 °C on a rotator. Brains were washed with 0.1 M PBS (3 × 20 min) before embedding in low melting point agarose (5 g/mL PBS) and cut into transverse 100 μm sections using a vibratome. Sections were incubated for 20 min each in 2% and 0.2% Triton-X-100 in 0.1 M PBS (PBST), before they were blocked in 2% normal goat serum (NGS) in 0.2% PBST for one hour at room temperature on a plate shaker. Sections were incubated in primary antibody, SYNORF1 (1:50), and AlexaFluor488-phalloidin (1:500) while covered in foil for at least three nights on a plate shaker at room temperature. Section were washed in 0.1 M PBS (5 × 20 min) and incubated in AlexaFluor568 goat anti-mouse secondary antibody (1:250). After fluorescent labeling, the brains were washed in 0.1 M PBS (5 × 20 min) before an overnight incubation in 60% glycerol at room temperature on a plate shaker while covered in foil. Finally, sections were incubated in 80% glycerol for 30 min, mounted onto glass slides in 80% glycerol, covered with glass coverslips (#1.5) and sealed with nail polish. Images were captured (1024 × 1024 pixel resolution) with an oil immersion 60X objective (NA = 1.4) without digital zoom on an Olympus Fluoview FV10i inverted laser scanning confocal microscope.

Optical sections (1.55 μm thick) that distinguished both the collar and lip, ideally in a plane in which the MBCs were bisected by the MB peduncle, were made. Each image typically contained both medial and lateral calyxes and was coded blind to the observer. MG density and size were measured using a modified protocol of Giraldo et al. [40]. Two adjacent circles (400 μm^2 each) were overlaid in the lip region and all synapsin-immunoreactive boutons not intersecting the circumference of those circles were counted using Fiji (version 1.51) at 300X digital magnification (Fig. 1). Because the collar region in *Pheidole* is small, we quantified its area in each image and counted all synapsin-immunoreactive boutons within. A grid (10 μm^2 squares) was overlaid on predefined counting areas, and a random number generator was used to select five squares in which all boutons within the square or intersecting the top or right borders were measured along the longest aspect because MG are not uniformly spherical. For each worker, average density of MG in the lip and collar were calculated by dividing the counts of boutons by the volume of the counting areas. MG size for the lip and collar was calculated from the average bouton length in each region. All analyses were conducted in R (version 3.3.2).

Analyses of variance (ANOVA) were used to determine differences