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A subpopulation of mushroom body intrinsic neurons is generated by protocerebral neuroblasts in the tobacco hornworm moth, *Manduca sexta* (Sphingidae, Lepidoptera)

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ABSTRACT

Subpopulations of Kenyon cells, the intrinsic neurons of the insect mushroom bodies, are typically sequentially generated by dedicated neuroblasts that begin proliferating during embryogenesis. When present, Class III Kenyon cells are thought to be the first born population of neurons by virtue of the location of their cell somata, farthest from the position of the mushroom body neuroblasts. In the adult tobacco hornworm moth *Manduca sexta*, the axons of Class III Kenyon cells form a separate Y tract and dorsal and ventral lobelet; surprisingly, these distinctive structures are absent from the larval *Manduca* mushroom bodies. BrdU labeling and immunohistochemical staining reveal that Class III Kenyon cells are in fact born in the mid-larval through adult stages. The peripheral position of their cell bodies is due to their genesis from two previously undescribed protocerebral neuroblasts distinct from the mushroom body neuroblasts that generate the other Kenyon cell types. These findings challenge the notion that all Kenyon cells are produced solely by the mushroom body neuroblasts, and may explain why Class III Kenyon cells are found sporadically across the insects, suggesting that when present, they may arise through *de novo* recruitment of neuroblasts outside of the mushroom bodies. In addition, lifelong neurogenesis by both the Class III neuroblasts and the mushroom body neuroblasts was observed, raising the possibility that adult neurogenesis may play a role in mushroom body function in *Manduca*.

1. Introduction

The mushroom bodies of the insect brain play a role in a multitude of higher functions including sensory integration, attention-like processes and olfactory processing and olfactory learning (Li and Strausfeld, 1999; Perez-Orive et al., 2002; Blum et al., 2009; van Swinderen and Brembs, 2010). The mushroom bodies are composed of morphologically and functionally distinct subpopulations of intrinsic neurons called Kenyon cells (Farris, 2005b). Kenyon cell dendrites form the mushroom body calyces, which receive sensory input that in most insects arises predominantly from first-order olfactory centers, the antennal lobes. Kenyon cell axons form a thick tract into the protocerebrum called the pedunculus, then bifurcate to form the medial and vertical lobes.

Kenyon cell subpopulations are produced sequentially during development, and their cell bodies and axonal processes are organized by birthdate in the adult mushroom bodies (Farris et al., 1999; Farris and Strausfeld, 2001; Kurusu et al., 2002; Farris and Sinakevitch, 2003). In all insect species studied to date, Kenyon cells appear to be generated by dedicated mushroom body neuroblasts located near the center of the calyx (Ito et al., 1997), and their somata are passively pushed outwards by continuing rounds of neurogenesis so that the earliest-born cells are located at the periphery of the Kenyon cell body cluster, farthest from the position of the neuroblasts (Farris et al., 1999; Farris and Strausfeld, 2001; Malaterre et al., 2002). Studies in the fruit fly *Drosophila melanogaster* have shown that the mushroom body neuroblasts, which begin neurogenesis in the embryo and are continually mitotically active through the mid-pupal stage, are the sole source of Kenyon cells (Ito and Hotta, 1992; Ito et al., 1997).

In some insects, the mushroom bodies contain a distinct population of mushroom body intrinsic neurons, the Class III Kenyon cells, that often receive input from gustatory centers in the deutocerebrum, tritocerebrum and subesophageal ganglion (Weiss, 1981; Frambach and Schürmann, 2004; Farris, 2008c). Class III

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Kenyon cells are further typified by a unique morphology in which their axons form separate "lobelets" outside of the medial and vertical lobes, and may also form a separate accessory calyx with their dendrites (Weiss, 1981; Farris and Strausfeld, 2003; Farris, 2008c). Despite these unique features, Class III Kenyon cells are considered true mushroom body intrinsic neurons, largely because they all possess tiny, cytoplasm-poor cell bodies and share a high affinity for the anti-DC0 polyclonal antibody (Farris and Strausfeld, 2003; Farris et al., 2004; Farris, 2005a, 2008a, 2008c; Sjöholm et al., 2006; Fukushima and Kanzaki, 2009). The anti-DC0 antibody recognizes the catalytic subunit of *Drosophila* protein kinase A, which like other components of the cAMP signaling pathway is highly expressed in Kenyon cells and is important for the learning and memory functions of the mushroom bodies (Skoulakis et al., 1993). Class III Kenyon cell bodies are located at the periphery of the Kenyon cell soma clusters in the dorso-posterior protocerebrum; they are thus presumed to be generated by the mushroom body neuroblasts at the earliest stage of mushroom body development, and their cell bodies pushed to the farthest edges of the soma clusters by adulthood (Farris and Strausfeld, 2003). Aside from this inference, however, little is known about the origin of Class III Kenyon cells and the development of the unique structures formed by their axons and dendrites.

The tobacco hornworm moth *Manduca sexta* is an important model system for studies of the development and function of the olfactory system and olfaction-driven behavior (Rössler et al., 1999; Daly et al., 2004; Ito et al., 2009; Lei et al., 2009). Most attention has focused on the antennal lobe, and very little is known about the structure and development of the mushroom bodies in this species despite their being a major target of antennal lobe output. From neuroarchitectural and developmental standpoints, *Manduca* mushroom bodies are also of interest because Class III Kenyon cells have been observed in their mushroom bodies and those of other moths belonging to the order Lepidoptera, where their axons form a morphologically unique Y tract and lobelets (Pearson, 1971; Sjöholm et al., 2005; Fukushima and Kanzaki, 2009; Huetteroth et al., 2010). The present study describes the morphology and development of the *Manduca* mushroom bodies, with particular emphasis on the developmental origin of Class III Kenyon cells and the morphogenesis of their unique neuropils.

2. Methods

2.1. Insects

All moths were reared under standard rearing conditions (Bell and Joachim, 1976). Larvae were staged according to head capsule width. At the onset of wandering (W0) in the fifth larval instar, larvae were placed in individual pupation chambers and collected as needed throughout the four day wandering stage and the 18 day pupal stage. At pupal stage 17, individuals were placed into brown paper bags and kept in an incubator (Percival Scientific I66VLC8, Perry, IA) in a reversed 16/8 light/dark cycle at 25 °C and 75% relative humidity.

2.2. Anti-DC0 and phalloidin labeling

Anti-DC0 is a polyclonal antibody derived against the catalytic subunit of protein kinase A in *D. melanogaster* (Skoulakis et al., 1993). This antibody has a high affinity for mature Kenyon cell populations across a wide range of insect species (Farris and Strausfeld, 2003; Farris et al., 2004; Farris, 2005a, 2008a, 2008c). Fluorophore-conjugated phalloidin labels filamentous actin enriched in extending axons and dendrites of newborn Kenyon cells (Farris et al., 2004).

Prior to brain dissection, insects were chilled on ice until immobile. Brains were dissected in O'Shea-Adams saline (O'Shea and Adams, 1981) and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.2) overnight at 4 °C. Fixed brains were washed 3 × 10 min in PBS, embedded in 8% agarose and vibratome sectioned at 80 μm. Sections were transferred to a 24-well culture dish and washed 3 × 10 min in PBST (PBS with 0.1% Triton X-100), then blocked in 10% normal goat serum (NGS) in PBST for at least an hour at room temperature on an orbital shaker. The blocking solution was then removed and replaced with a primary antibody solution consisting of 1% NGS in PBST and the anti-DC0 primary antibody (a generous gift of Dr. Daniel Kalderon) at a 1:1000 concentration. Tissue was incubated in the primary antibody overnight at room temperature.

The following day, tissue was washed 6 × 20 min in PBST and then placed in a secondary antibody solution of 1% NGS in PBST with Texas Red-conjugated goat anti-rabbit secondary antibody (Jackson Immunochemicals, West Grove, PA) added at a 1:200 concentration. Alexa 488-conjugated phalloidin (Invitrogen (Molecular Probes), Eugene, OR) was also added to the solution at a 1:500 concentration, and tissue was incubated overnight on the orbital shaker at room temperature. The next day, tissue was washed 6 × 20 min in PBST and cleared in 60% glycerol in PBS for 30 min and 80% glycerol in PBS for 1 h. Sections were coverslipped under 80% glycerol in PBS and viewed using an Olympus Fluoview 1000 confocal microscope. Image stacks were captured on the confocal and saved as AVI files, opened in NIH Image and broken into TIFF files of individual optical sections. Projections of portions of the stack were made in Adobe Photoshop CS4 (Adobe Systems Incorporated, USA) using the "lighten" function, and color balance, contrast and brightness adjusted as needed.

2.3. BrdU labeling

For labeling of neuroblasts and their immediate progeny in late larvae, pupae and adult moths, 25 mg/ml 5′-bromo-2-deoxyuridine (BrdU) in O′Shea-Adams saline was loaded into a 1 cc tuberculin syringe and injections made using a 31 ga needle into cold-anesthetized insects. For first and second instar larvae that were too small for injections, the insects were chilled until immobile and a droplet of BrdU solution placed on their mouthparts. The insects ingested the solution as they revived from the cold anesthesia. All BrdU-treated insects were allowed to revive at room temperature for 4–24 h, after which they were again chilled on ice and their brains dissected, fixed, embedded and sectioned as described previously.

For birthdating of Kenyon cell populations, BrdU treatment was performed as described above. Insects were then allowed to continue development until the late pupal stage and then processed for BrdU immunostaining.

Prior to BrdU immunostaining, fixed and sectioned tissue was washed 3 × 10 min in PBST, then incubated in 2 N HCl for 40 min at room temperature to denature the DNA and allow access to the anti-BrdU antibody. After acid treatment, tissue was washed 3 × 5 min in PBST and placed in 10% NGS blocking solution for 1 h. Mouse anti-BrdU (Becton–Dickinson, Franklin Lakes, NJ) was added at a 1:500 concentration to a 1% solution of NGS in PBST, and the tissue was incubated in this solution overnight at room temperature. Tissue was also double labeled with the anti-DC0 antibody as described above.

The next day, the primary antibody solution was removed and the tissue washed 6 × 20 min prior to incubation overnight in Alexa 488-conjugated goat anti-mouse secondary antibody (Invitrogen (Molecular Probes), Eugene, OR) added at a 1:200 concentration to a solution of 1% NGS in PBST. Texas Red-conjugated goat anti-rabbit secondary for visualization of the anti-DC0 antibody was also