Postembryonic development of the olfactory system in the moth *Manduca sexta*: primary-afferent control of glomerular development

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The olfactory system of the sphinx moth Manduca sexta *bears many similarities to its vertebrate counterpart in functional organization, physiology and development. In the moth, the antenna (the olfactory organ) and the antennal lobe (the primary olfactory center) of the brain arise during postembryonic metamorphic development and are accessible, independently manipulable, and structurally relatively simple. In addition, they house a conspicuous, sexually dimorphic subsystem specialized for detection of a specific pheromonal odor. These features make this system experimentally favorable for studies of development of olfactory glomeruli. Such studies have demonstrated the importance of regulatory interactions among sensory axons, glial cells and antennal-lobe neurons.*

IN HOLOMETABOLOUS INSECTS such as the moth *Manduca sexta*, a wave of 'delayed embryonic' development occurs during the metamorphic transformation of the larva into the adult moth, when the animal is large, robust and well suited to experimentation. Because of these and other technical advantages, *Manduca* has been used in extensive investigations of cellular and molecular events in neural development.[1,2] For studies of intercellular interactions during neural development, the sensory systems of such insects are especially tractable model systems. Because peripheral sensory neurons and their central postsynaptic targets generally arise independently and are physically remote from each other, the two populations of neurons can be manipulated independently with relative ease. In the case of the olfactory system of *Manduca*, these favorable characteristics are compounded by the remarkable similarity of functional organization between the olfactory systems of vertebrates and arthropods.

Common features of organization of first-order olfactory centers in vertebrates and insects

Olfactory systems of vertebrates and invertebrates share many general organizational and functional features.[3,4] In vertebrates, each olfactory receptor cell (ORC) is thought to express at least one of a large family of receptor molecules[4,5] believed to be responsible for differences in ORC response profiles. Although such receptors have not yet been identified in insects, the extremely narrow specificity of at least some insect ORCs — specifically, the sex-pheromone receptors of moth antennae — is consistent with the idea of one type of receptor per ORC. Upon binding odor molecules, the ORCs transduce chemical stimuli into patterns of action potentials. That encoded olfactory information is conveyed via ORC axons to the first synaptic stations in the olfactory pathway, the glomeruli of the primary olfactory center (the olfactory bulb, OB, in vertebrates; the antennal lobe, AL, in insects) in the brain. The similarity of glomerular organization in the first-order central olfactory neuropils of diverse invertebrates and vertebrates has been noted often.[3,4,6-8]

Each glomerulus includes arborizations of central neurons of two main classes: local interneurons (LNs) confined to the OB or AL, and projection (or principal or output) neurons (PNs) that extend axons to subsequent stations in the pathway. Each ORC axon projects to one glomerulus, and many ORC axons converge on each glomerulus, where they have synaptic connections with neurites of particular types of central neurons.[9,10] In both OBs and ALs, the convergence of ORC axons onto uniglomerular PNs (uPNs) is massive (ca 10^3:1–10^4:1). In insect ALs, virtually all synapses are in the glomeruli, whereas in vertebrate OBs there is an extensive extraglomerular synaptic plexus.[11]

The primary olfactory centers of insects, like those of vertebrates, are believed to be organized odotopically[4] — that is, attributes of odor molecules are thought to be represented spatially in the glomerular neuropil, in a manner comparable to the tonotopy of auditory systems and the visuotopy of visual systems. Although the role of olfactory sensory axons in the development of primary olfactory neuropils has been explored vigorously in many species,[12-18] relatively little is known yet about the ontogeny of this odotopy. We are addressing this question in *Manduca*, where our studies of how the glomerular array develops now serve as a backdrop for studies of how a particular cluster of glomeruli with known odor specificities arises during development of the olfactory pathway.

The olfactory system of *Manduca*

The long flagellum of the adult antenna in *Manduca* bears numerous sensilla (cuticular sensory organules) of several types, most of which are olfactory.[19,20] A male antennal flagellum has ca 3.3×10^5 ORCs associated with ca 10^5 sensilla.[19,21] Although a majority of these sensilla are similar to those present on female antennae, types that respond to volatile substances emitted by plants,[22] about 40% are long type-I trichoid (hair-like) sensilla found only on male antennae.[19,20,23,24]

The ALs of *Manduca* are organized similarly to those of most other insects.[10,25,26] ORC axons project into the AL, where they terminate in 64 ± 1 large (50–100 μm in diameter), spheroidal glomeruli arrayed around a coarse central neuropil[27,28] (Figure 1). Quantitative neuroanatomical methods have demonstrated unique identities and provided a detailed map of the glomeruli in the ALs of male *Manduca*.[29]

Each AL has approximately 1200 neurons, whose cell bodies reside in three groups at the perimeter of the AL neuropil.[27,28] These neurons send neurites through the central coarse AL neuropil to the glomeruli, where they participate in synapses. AL neurons of one major class, the LNs, have arborizations in most of all of the glomeruli, receive input directly from ORC axons, and also interact synaptically with other AL neurons. The other major class of AL neurons, the PNs, includes both uniglomerular and multiglomerular output neurons. PNs apparently receive little, if any, direct synaptic input from ORC axons but instead are involved in synaptic interactions with LNs. PN axons project to higher-order olfactory centers in the protocerebrum.

In addition to the ordinary glomeruli, a male moth's AL also possesses a sexually dimorphic macroglomerular complex (MGC; see Figure 1). As in other insects,[26,30-32] the MGC is the site of synaptic processing of afferent information about key components of the conspecific female's sex pheromone.[28,33] Two components of the female's sex-pheromone blend (here referred to as components A and B) are necessary and sufficient to elicit mate-seeking behavior in a male moth,[34] and in most of the male-specific type-I trichoid sensilla the two ORCs are tuned respectively to components A and B.[23] Axons of these pheromone-specific ORCs project exclusively to the MGC, which has two prominent glomerular divisions: the toroid and cumulus, named for their unusual shapes.[35] Axons of ORCs tuned to component A apparently project to the toroid and component-B-specific axons, to the cumulus.[35,36] A third MGC glomerulus, a smaller ring-shaped structure lying beneath the toroid,[37] has not yet been studied in detail. AL neurons that respond to antennal stimulation with sex pheromone have arborizations in the MGC.[28,38] PNs that respond selectively to stimulation of the antenna with component A have arborizations restricted to the toroid; PNs that respond selectively to component B have arborizations confined to the cumulus; and PNs that respond to both components A and B have arborizations in both cumulus and toroid.[35,39] Thus these anatomically distinct MGC glomeruli have specific roles in the processing of sex-pheromonal information and hence narrow and specific molecular receptive ranges.

Because the ordinary glomeruli in *Manduca* ALs resemble the glomeruli of vertebrate OBs both structurally and functionally, but are identifiable and more experimentally accessible, these moth glomeruli have served as useful models for studies of glomerular development. Moreover, owing to its prominence and striking sexual dimorphism, its dedication to processing of primary-afferent sex-pheromonal information, its division into discrete glomeruli representing the key components of the pheromone, and its well characterized population of LNs and PNs, the MGC is a unique model for experimental studies of the development of odotopic organization in a primary olfactory center.

Role of ORC axons in development of ordinary olfactory glomeruli

The antennae arise from imaginal disks,[19] which evert

at the onset of metamorphosis. Antennal ORCs are born in the everted disks during the first two of the 18 stages (each lasting approximately one day) of adult development[40,41] and almost immediately begin to extend axons toward the brain.

During metamorphosis, the adult AL develops from five neuroblasts that divide throughout larval life.[42] By stage 3, all AL neurons are postmitotic;[12] their somata lie outside a homogeneously textured neuropil formed by their branching neurites, and the neuropil is ensheathed by a continuous border of glial cells. As ORC axons begin to grow into the AL early in stage

4,[12,43-45] they pierce the glial border and encircle the neuropil just beneath it. During stages 5 and 6, ORC axons continue to grow into the AL, and their terminals coalesce to form spheroidal protoglomeruli,[45] in a lateral-to-medial wave of morphogenesis.[46] The protoglomeruli then serve as a template for the further development of the array of ordinary glomeruli.

uPNs with somata in the medial group of AL neurons extend processes very quickly into the newly formed protoglomeruli and even beyond, where they contact glial cells.[46] The neurites of LNs grow into the
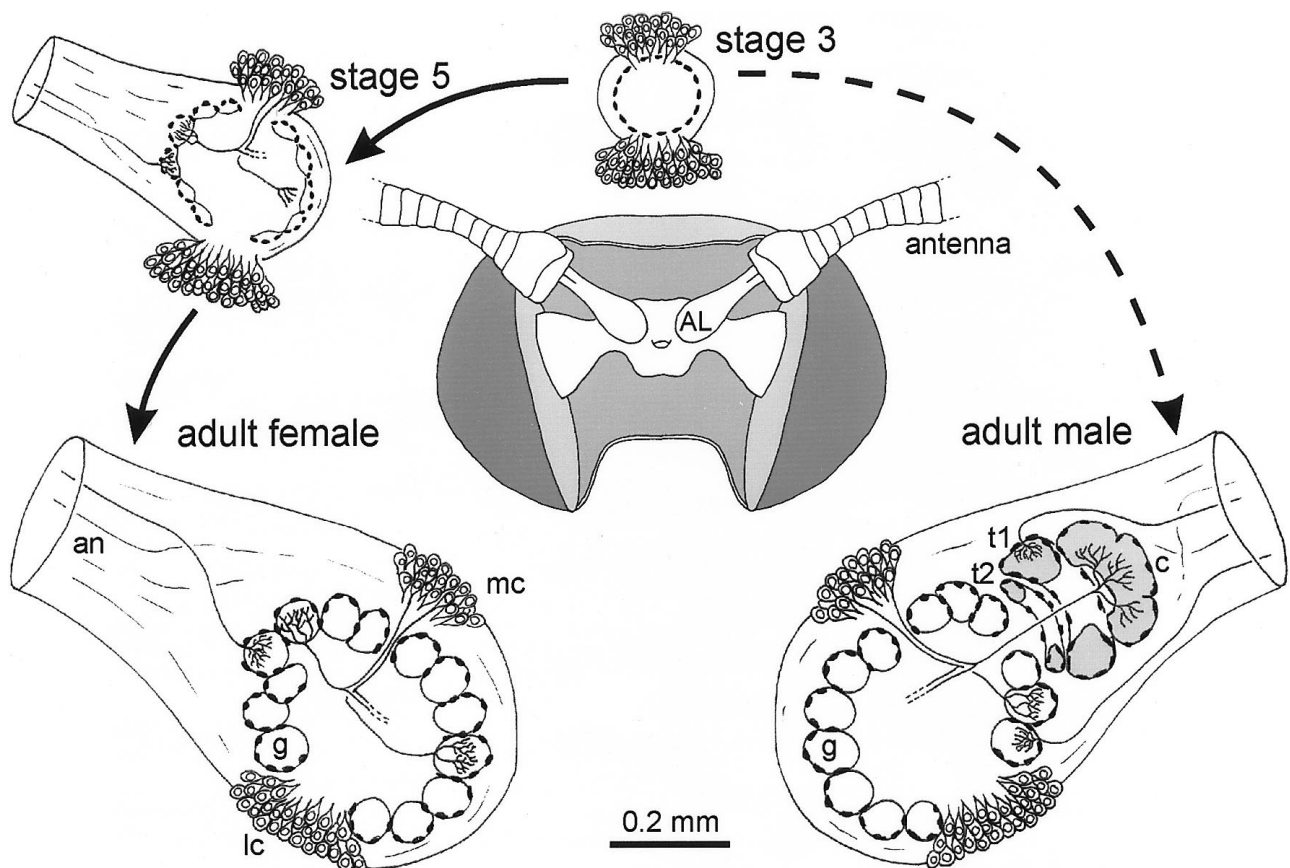

Figure 1. Schematic summary of events in development of the antennal lobes of *Manduca sexta*. At stage 3 of metamorphic adult development, antennal-lobe (AL) neurons extend neurites into a homogeneous neuropil surrounded by glial cells. Axons from olfactory receptor cells in the antenna begin to grow into the AL at stage 4, and during stage 5 their terminals coalesce to form protoglomeruli. Very soon after formation, protoglomeruli are invaded by the growing neurites of uniglomerular projection neurons (two are illustrated). Glial cells surround the protoglomeruli, and the glomeruli grow as synapses form. In the adult female moth, an array of glomeruli (g) surrounds a central, asynaptic neuropil. Adult male moths have, in addition to this array, a macroglomerular complex, or MGC, which comprises a cumulus (c) and two toroidal glomeruli (t1, t2), each of which is bounded by glial cells. an, antennal nerve; lc and mc, lateral and medial groups of AL neuronal somata.

protoglomeruli a day later, after glial cells have enveloped the protoglomeruli.[44,45] By stage 12, the AL exhibits all of the organizational features of a mature AL, although it continues to grow.

Taken together, the results of these studies suggest that uPNs are in a position to cooperate with sensory axons early, immediately after the axons have laid down the glomerular template, and that they may interact directly with glial cells at this stage; the LNs, which grow into the glomeruli later, may be constrained by glial boundaries to form their characteristic glomerular tufts.

Ingrowth of ORC axons is critical for normal development of olfactory centers. Without ORC input, the OB of the vertebrate brain does not arise at all.[18,47] In contrast, the ALs of insects develop even in the absence of ORC input,[48] but their cellular organization is abnormal.[44,45,49,50]

As in vertebrates[14,51] ORC axons play an essential instructive role in glomerulus formation in *Manduca*. If ORC axons are prevented from innervating the AL during development, the resulting AL lacks glomerular organization.[49] Development in the absence of ORC axons diverges from normal very early. Glial cells proliferate along roughly the normal timetable,[52] but they never undergo their normal shape-change and migration into the neuropil, instead remaining restricted to a rim surrounding the neuropil.[44] LNs develop diffuse rather than tufted branching patterns, and uPNs develop arborizations that are restricted in extent but larger than a normal glomerulus.[45]

Is neural activity important for the ORC axons to influence AL development? Although ORCs do not become responsive to odors until late in development,[22] spontaneous activity could play a role. Oland *et al*[53] found that the array of ordinary glomeruli develops apparently normally in female *Manduca* treated during metamorphosis with tetrodotoxin to block neural activity. Whether axons have sorted odotopically in these experimental animals, however, is not yet known.

Role of glial cells

The fact that glial-cell changes precede detectable neuronal changes in response to ingrowth of ORC axons, and that these changes do not occur in unafferented ALs, led Oland and Tolbert[44] to hypothesize that glial cells act as intermediaries in developmental interactions between ORC axons and AL neurons. By experimentally reducing the number of glial cells present during development, much more

readily accomplished in the insect than in any vertebrate, they produced ALs that were aglomerular, despite the presence of normal numbers of ORC axons.[21,54] This lends support to the argument that, despite the innate ability of ORC axons to form protoglomeruli upon entering the AL, glial cells are required for the ORC-axon-induced formation of glomeruli. Baumann *et al*[55] showed recently that the glial boundaries actually maintain the integrity of the axonal protoglomeruli; when glial numbers were reduced, protoglomeruli 'dissolved' before the dendrites of AL neurons even began to grow into them.

How might glial cells constrain axonal and/or dendritic branching to glomerular compartments? Tenascin-like molecules, which also are found on astrocytes associated with boundaries in the developing mouse brain,[56,57] may play a role. Such molecules decorate the surfaces of AL glial cells during the period of glomerulus formation,[58] and the growing neurites of many AL neurons steer away from purified mouse tenascin if confronted *in vitro*.[59] Tenascin-like molecules on glial cells might constrain neuritic growth within developing *Manduca* glomeruli, thus helping to sculpt glomerular arbors. Although the evidence is not as strong, similar sculpting roles for glial cells have been proposed for glomerulus development in vertebrate OBs.[60,61]

Special role of male-specific ORCs in development of male-specific glomeruli

A nascent MGC is recognizable in the developing male AL as early as the first protoglomeruli become discernible.[50,62] Almost immediately after the axons of the male-specific ORCs begin to terminate in the appropriate area, they form three distinct subdivisions; these become enveloped by glial borders, and MGC-specific uPNs have dendrites restricted to single subdivisions.[62] As in the ordinary glomeruli, the uPNs initially have significant overlap with glial cells in the surrounding border.

To test for a role of antennal sensory axons in the development of the MGC, Schneiderman *et al*[63] studied surgically generated antennal gynandromorphs. Taking advantage of the fact that determination of sex in insects results from the action of genes, exerted apparently in every cell, without the intermediation of hormones,[64] they produced animals in which one or both antennae were derived from antennal imaginal disks that had been transplanted from donor *Manduca* and allowed to develop in the

host animal. When a male-host AL was innervated by axons from a grafted female antenna, it lacked a recognizable MGC and resembled a normal female AL; when a female-host AL was innervated by male antennal axons, it had an 'MGC' and resembled a normal male AL.[63] Recent experiments[62] indicate that all three MGC glomeruli (see previously) develop in these female gynandromorphs. Thus, the gender of the antenna determines whether an MGC develops, and innervation by male-specific ORCs appears to be necessary and sufficient for MGC development.

At the cellular level, this influence of the male ORC axons is readily apparent. Male-like interneurons, with dendritic arborizations in the MGC in the surgically gynandromorphic AL, developed in female ALs innervated by sensory axons from grafted male antennae.[62,63] Neurons resembling normal male-specific LNs (i.e. with arborizations in the gynandromorphic MGC) received characteristic excitatory synaptic input when the grafted male antenna was stimulated with sex pheromone.[63] The cellular changes set the stage for a striking behavioral result: some female gynandromorphs (with one or two grafted male antennae) flew upwind to sources of sex pheromone, in male-like fashion.[65] Thus, male ORC axons can terminate at their characteristic target region in female ALs, form an MGC with the characteristic subdivisions, induce glial cells to form borders for these subdivisions, and even attract female AL neurons to arborize in these subdivisions and to form functional synapses there.

Outlook

Studies of the development of both vertebrate OBs and insect ALs clearly show that interactions among ORC axons, glial cells and neurons are decisive for the development of the glomerular neuropil. In OBs and ALs alike, those interactions probably involve cell-surface molecules, locally secreted substances, or both. In particular, one might predict that the fasciculated ORC axons projecting to individual glomeruli (thus determining the odotopy of the OB/AL and the molecular receptive ranges of the glomeruli) share molecular markers. Evidence supporting that idea comes from histochemical studies showing that subsets of ORC axons which express different molecular markers tend to project to distinct glomeruli.[66-68] Another attractive possibility is that ORCs projecting to an individual glomerulus and expressing one or a few members of the family of olfactory-receptor genes[5,68] might deploy those membrane proteins (or related molecules) not only in the receptor dendrites of the ORCs but also in their axons. Such a pattern of expression could enable the receptor (or receptor-related) molecules to serve both for odor transduction in the dendrites and for intercellular recognition or control in the development of glomeruli.[68,69] Analysis of the amino-acid sequences of vertebrate odor receptor molecules has suggested a possible mechanism for this result, revealing that the receptor molecules might serve as adhesion molecules governing axonal guidance.[69] These ideas clearly merit critical experimental testing.

The olfactory pathway of *Manduca* promises to be useful for such next steps in unraveling the mechanisms of glomerular development. Previous studies indicate that cell–cell interactions among ingrowing ORC axons and AL glial cells and neurons are decisive for the postembryonic development of the array of ordinary glomeruli. Because these glomeruli are individually identifiable in the AL, it should be possible eventually to characterize their molecular receptive ranges and thus the odotopy of the glomerular array, and then to dissect the developmental mechanisms responsible for generating that pattern. In the case of the MGC, however, these questions can be posed now.

The MGC is a conspicuous cluster of glomeruli, present only in males and dedicated to processing sex-pheromonal information; it possesses three discrete glomeruli, two of which represent the two key components of the pheromone; and its population of LNs and PNs have been well characterized physiologically and anatomically. Thus, we now are poised to ask the following questions aimed at elucidating mechanisms underlying the development of odotopy:

1. Do the anatomically and functionally specified glomeruli of the normal MGC form via the same interactions among neurons and glial cells that result in the formation of ordinary glomeruli? What determines the specific location at which the MGC develops?
2. In female gynandromorphs (with a female AL innervated by afferents from a grafted male antenna), do the male ORC axons sort normally, such that the toroid and cumulus of the gynandromorphic 'MGC' process information about components A and B, respectively, of the sex pheromone? Also, what mechanisms account for the developmental plasticity of neurons in the

female-host AL that become uPNs innervating the glomeruli of the gynandromorphic MGC?

3. What is the molecular basis for the sorting among pheromone-responsive axons and their projections to the cumulus and toroid? Are these MGC glomeruli good models for studies of the type of odotopy that is thought to develop throughout the array of ordinary glomeruli?

The presence of developmentally important molecules in or on the ingrowing male antennal afferent axons is inferred from the results of the antennal gynandromorph experiments. The fact that male ORC axons must grow into a target AL in order to induce development of an MGC argues that the induction results from a short-range interaction and suggests that, if molecules associated with the male afferents are involved, then these molecules must be membrane-bound or secreted but locally active. In experiments underway we seek to identify and characterize genes that might encode such molecules — i.e. genes whose products are expressed in developing male but not female ORCs and whose protein sequence is consistent with the possibility that the protein is membrane-bound or locally secreted. This is the first study, in any species, seeking the developmental rules and mechanisms underlying morphogenesis of anatomically identified glomeruli with known functional specificity.

Acknowledgements

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