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Temporally Staggered Glomerulus Development in the Moth *Manduca sexta*

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Abstract

Glomeruli, neuropilar structures composed of olfactory receptor neuron (ORN) axon terminals and central neuron dendrites, are a common feature of olfactory systems. Typically, ORN axons segregate into glomeruli based on odor specificity, making glomeruli the basic unit for initial processing of odorant information. Developmentally, glomeruli arise from protoglomeruli, loose clusters of ORN axons that gradually synapse onto dendrites. Previous work in the moth *Manduca sexta* demonstrated that protoglomeruli develop in a wave across the antennal lobe (AL) during stage 5 of the 18 stages of metamorphic adult development. However, ORN axons from the distal segments of the antenna arrive at the AL for several more days. We report that protoglomeruli present at stage 5 account for only approximately two or three of adult glomeruli with the number of structures increasing over subsequent stages. How do these later arriving axons incorporate into glomeruli? Examining the dendritic projections of a unique serotonin-containing neuron into glomeruli at later stages revealed glomeruli with immature dendritic arbors intermingled among more mature glomeruli. Labeling ORN axons that originate in proximal segments of the antenna suggested that early-arriving axons target a limited number of glomeruli. We conclude that AL glomeruli form over an extended time period, possibly as a result of ORNs expressing new odorant receptors arriving from distal antennal segments.

Key words: antennal lobe, protoglomerulus

Introduction

Glomeruli, neuropilar structures composed of olfactory receptor neuron (ORN) axon terminals and the dendrites of central neurons, are common to olfactory systems across many phyla (reviewed by Hildebrand and Shepherd, 1997; Eisthen, 2002). Axons innervating a particular glomerulus originate from ORNs responsive to a specific set of odorants (Vassar *et al.*, 1994; Mombaerts *et al.*, 1996; Gao *et al.*, 2000; Vosshall *et al.*, 2000). In many species this segregation of inputs is enhanced by glial cells which surround each glomerulus (reviewed by Boeckh and Tolbert, 1993; Hildebrand and Shepherd, 1997). Thus, glomeruli represent discrete units that contain processes encoding information about a limited subset of odorants. These discrete units develop from looser structures, the protoglomeruli. Protoglomeruli coalesce from a loose tangle of axons into tight neuropils as the dendrites of target neurons grow in and form synapses with the ORN axons, and glial cells gradually envelop the nascent structures (Oland *et al.*, 1990; Valverde *et al.*, 1992; Royal and Key, 1999; Treloar *et al.*, 1999; Potter *et al.*, 2001; Hummel and Zipursky, 2004). An understanding of the mechanisms and timing underlying the formation of protoglomeruli and their maturation into glomeruli is crucial to understanding olfactory development.

Protoglomeruli were first described in the moth *Manduca sexta*'s antennal lobe (AL), a structure analogous to the mammalian olfactory bulb (OB, Oland *et al.*, 1990). In *Manduca*, the major features of the adult olfactory system form during the first 8 of 18 stages of metamorphic adult development. Protoglomerulus formation begins at stage 5 when axons from ORNs in the antenna grow through the layer of glia surrounding the AL neuropil and begin forming discrete protoglomerular structures (Oland *et al.*, 1990). During stage 5, protoglomeruli develop in a dorsolateral to ventromedial wave across the AL (Malun *et al.*, 1994). The sequential development of protoglomeruli observed in *Manduca* ALs is also seen in mouse OBs, where the development of glomeruli in the caudal OB lags that of more rostral glomeruli by 2–3 days (Bailey *et al.*, 1999). Indeed, some evidence suggests that glomeruli are added to the OB for several weeks after birth (LaMantia and Purves, 1989; Pomeroy *et al.*, 1990).

Although protoglomeruli fill the incipient glomerular layer of the *M. sexta* AL by the end of stage 5, additional ORN

axons enter the AL for several more days, through stage 9 (Sanes and Hildebrand, 1976a,b). Experiments by Tolbert and Sirianni (1990) and Willis *et al.* (1995) indicated that these more distally originating axons are crucial to normal AL development. Truncating antennae before ORN axons grew into the brain resulted in ALs containing abnormally low numbers of glomeruli. The longer the remaining antenna, the more the glomeruli developed. Tolbert and Sirianni (1990) suggested that the later arriving axons from distal antennal segments might be needed to meet a general threshold of innervation necessary to form the full complement of glomeruli or, alternatively, that proximally arising axons might contribute to only a subset of the glomeruli with axons from more distal ORNs forming the remaining glomeruli. Here we test the possibility that protoglomeruli develop over a longer time than previously recognized and that axons from ORNs in the proximal segments of the antenna, which arrive in the AL early, contribute to only a subset of the full complement of glomeruli. We test this hypothesis by determining the number of glomerular structures in ALs through development, assessing the maturity of individual glomeruli at particular stages, and tracing axons that extend from proximal antennal segments. We conclude that new protoglomeruli form over a period of days and speculate that the antenna is heterogeneous along its extent, with the proximal part contributing input that does not represent the full spectrum of odorant inputs processed in the AL.

Materials and methods

Manduca sexta were raised on an artificial diet in a laboratory colony as previously described (Sanes and Hildebrand, 1976a,b). Animals developed under controlled temperature, humidity, and light conditions (26°C, 50–60% relative humidity, with a light:dark cycle of 17 : 7 h). *Manduca* pupae were staged according to a paradigm that divides pupal development into 18 stages, each roughly 1-day long (Tolbert *et al.*, 1983; Oland and Tolbert, 1987). The stage of an individual animal was determined through landmarks, such as eye pigmentation, that are unique to each stage.

Anterograde labeling of ORN axons from the antenna

Depending on the stage to be examined, axons terminating in the AL were labeled either with a rhodamine-labeled dextran or with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI). To label ORN axons in a stage-6 animal, the animal was first anesthetized by cooling on ice and the brain dissected in cold 0.1 M phosphate buffer (PB, pH 7.4). Care was taken to preserve as much of the antennal nerve as possible (approximately 5 mm). The cut end of the antennal nerve was then placed in a watertight petroleum jelly well filled with double distilled water. After 5 min, the water was replaced with an 8% rhodamine-labeled dextran (3000 MW, lysine fixable, Molecular Probes, Eugene, OR), 0.25% Triton-X (Sigma Chemical Co., St Louis, MO) solution made in double distilled water. The petroleum jelly well was sealed and the brain covered in PB. Tissue was allowed to incubate 24 h at 4°C and then was fixed overnight in 4% paraformaldehyde in a 0.1 M PB (pH 7.4). To allow visualization of labeling throughout the depth of the AL, tissue was cleared by dehydration in a graded series of alcohols and then placed in methyl salicylate. Because rhodamine-labeled dextran did not adequately label ORN axons in the AL of animals older than stage 6, we used DiI (Molecular Probes) to label glomerular structures in older animals. ALs were dissected as above, and the tissue was fixed overnight in 4% paraformaldehyde, 0.15% glutaraldehyde in 0.1 M PB. A DiI crystal was placed in the antennal nerve and the tissue placed in the above fixative solution for 7 days. Because DiI is soluble in alcohol, the tissue was cleared by placing it overnight in Hypaque (diatrizoate meglumine, Nycomed Inc., Princeton, NJ).

Experiments labeling ORN axons originating from a subset of segments were conducted in stage-8 males. After an animal was anaesthetized on ice, the brain and the antenna and antennal nerve were dissected in a manner that preserved the AL's connection with the antennae. Each antenna was slit open down the line separating the two olfactory epithelial sides of the antenna. The dissected tissue was pinned out in a 35 × 10–mm Falcon cell culture dish (Becton-Dickinson Labware, Franklin Lakes, NJ) coated with Sylgard (Dow Corning Corp., Midland, MI). DiI crystals were then placed on the olfactory epithelium on the inner surface of the selected antennal segment. The tissue was sealed and incubated for 6–8 weeks at 25°C in 4% paraformaldehyde, 0.15% glutaraldehyde in 0.1 M PB. Following incubation, tissue was removed from the fixative and placed overnight in 1 : 2000 SYTO 13 (Molecular Probes) in order to visualize the glial cell bodies surrounding the glomeruli. After three 5-min washes with 0.1 M PB, the tissue was cleared overnight in Hypaque.

Counting glomerular structures

Glomerular structures (protoglomeruli or glomeruli) were counted in Z-series of optical sections of ALs collected on a laser scanning confocal microscope (see below). For these experiments, ORN axons had been labeled either with rhodamine-labeled dextran or DiI as described above. All Z-series for a particular stage were collected prior to analysis. Every file to be examined for a particular stage was copied three times, and random names were assigned to these files. The nine stage-6 lobes analyzed, for example, generated 27 randomly named files. These files were analyzed at random over a period of days. Glomerular structures were counted by panning through a Z-series and circling identifiable structures. Test ALs (not included in the data) were used to train the observers to identify glomerular structures and to develop internal consistency. In order to avoid potential difficulties in identifying the toroid

glomeruli of the macroglomerular complex present in males (Christensen *et al.*, 1995), we performed this quantitative analysis in female ALs. We attempted to restrict our analysis to the sexually isomorphic glomeruli; however, due to the difficulty in unequivocally identifying the two large female glomeruli (King *et al.*, 2000) in developing ALs, they were most likely tallied in some counts and not in others, yielding a variation of ±1. Paired *t*-tests were used to compare the results between multiple observers, and an analysis of variance (ANOVA) test was used to compare results between stages of AL development.

Serotonin immunocytochemistry

Tissue used for immunocytochemical studies was recovered as described above and fixed overnight in 4% paraformaldehyde in a 0.1 M PB solution. Following fixation, the ALs were removed from the brain and mounted in 7% agarose (Type 1 Agarose, Sigma Chemical Co.). The tissue was then sliced into 100-μm sections using a Pelco 1000Plus Vibratome (Ted Pella Inc., Redding, CA). Sections were blocked in 5% normal goat serum, 5% normal donkey serum, and 1% Triton-X in a 0.1 M PB solution and then incubated overnight in rabbit antiserotonin antibody (1 : 4000, Incstar, now DiaSorin, Stillwater, MN) in the same blocking solution. Following several washes with 1% Triton in 0.1 M PB, tissue was incubated overnight in blocking solution containing Cy5-conjugated goat anti-rabbit antibody (1 : 250; Jackson ImmunoResearch, West Grove, PA) and Alexa-488 phalloidin (1 : 500; Molecular Probes). Following several washes with 1% Triton in 0.1 M PB, tissue was mounted in 60% glycerol in 0.1 M PB for confocal microscopy.

Image acquisition and processing

All images were collected on a Nikon PCM 2000 laser scanning confocal microscope equipped with a 50-mW argon laser, and 4-mW green and 10-mW red HeNe lasers. The PCM 2000 was controlled using Simple 32 software (Compix, Cranberry Township, PA). Sections to be visualized were mounted in 60% glycerol in PB. To visualize whole ALs, specimens were mounted in the compound in which they had been cleared (methyl salicylate or Hypaque). Serial optical sections were imaged at 1- to 3-μm intervals. Images for figures were stacked in two-dimensional projections, cropped, resized, and rotated for purposes of presentation. Additionally, brightness and contrast were minimally adjusted. Images were processed using CorelDRAW 12 and Corel PHOTO-PAINT 12 (Corel Inc., Ottawa, Canada). In no case was the essential content of images altered.

Three-dimensional reconstructions

Three-dimensional reconstructions of stage-8 ALs (n = 4) were produced using Amira (Mercury Computer Systems, San Diego, CA). The outer surface of the AL, the borders of the lateral and/or medial neuronal cell body packets, and all glomeruli were traced in each image in a series of confocal microscopic images to produce a three-dimensional model of the AL.

Results

Numbers of glomerular structures at different stages of development

In *M. sexta*, the first axons originating from ORN cell bodies located in the developing adult antennae reach the dorsolateral aspect of the AL at stage 3 of metamorphic development (Sanes and Hildebrand, 1976a,b; Tolbert *et al.*, 1983; Oland and Tolbert, 1987; Oland *et al.*, 1990). At stage 4, ORN axons begin to penetrate the glial border surrounding the AL neuropil and form a continuous fringe of terminals (Oland *et al.*, 1990). During stage 5, the terminals closest to where the antennal nerve enters the AL coalesce into protoglomeruli followed by a wave of protoglomerulus coalescence that sweeps ventromedially across the AL (Malun *et al.*, 1994). By stage 6, the protoglomeruli, now dense knots of axon terminals, fill the outer layer of AL neuropil. Protoglomeruli present at stage 6 develop into mature glomeruli as dendrites of AL neurons grow to overlap with axon terminals (Figure 1A; Oland *et al.*, 1990). Previous studies supposed that all adult glomeruli arise from protoglomeruli present at stage 6, but here we show that new protoglomeruli continue to form in later stages.

If all glomeruli present in the adult AL arise from protoglomeruli present at stage 6, then at stage 6 there should be 61 sexually isomorphic protoglomeruli (Rospars and Hildebrand, 1992, 2000). To determine the number of protoglomeruli in the AL at stage 6, we took Z-series of confocal microscopic images of female ALs in which ORN axons had been anterogradely labeled with rhodamine-labeled dextran. The numbers of protoglomeruli in these ALs were independently tallied by two observers in a double-blind paradigm. Only ALs in which labeling was found through the entire depth of the lobe were used in our analysis (n = 9, e.g., Figure 1E). Each observer counted protoglomeruli in each AL three times. Across all counts, stage-6 ALs contained an average of ~43 protoglomeruli (Figure 2A). The number of protoglomeruli observed in the examined lobes was between 41 and 46 for observer 1 (average = 43.3, SD = 3.24) and between 41 and 45 for observer 2 (average = 42.8, SD = 2.55). The difference in values between the two observers as measured by a paired *t*-test was not significant ($P \leq 0.05$). These results indicate that at stage-6 ALs contain just two-thirds to three-quarters of the 61 sexually isomorphic glomeruli found in the adult (Rospars and Hildebrand, 1992, 2000).

If less than a full complement of protoglomeruli is present in the stage-6 AL, when are new glomerular structures added? To address this question, we counted glomerular structures in ALs collected at later stages of development

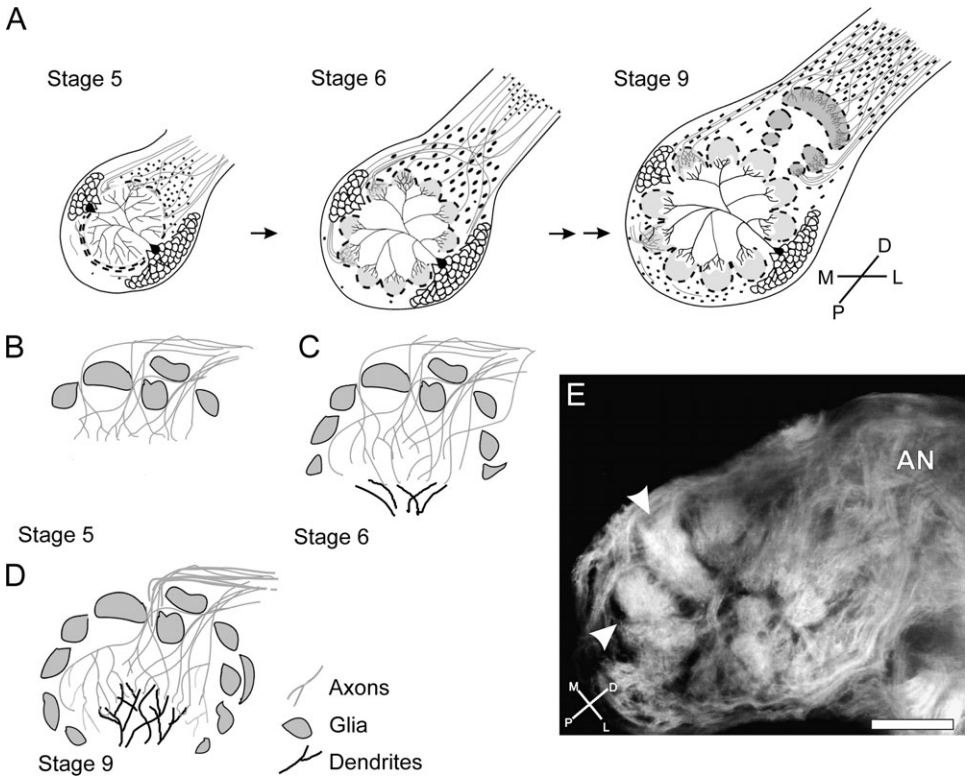

Figure 1 **(A)** Schematic representation of *Manduca sexta* AL development from stage 5 to adult. (Figure modified from Tolbert *et al.*, 2004.) **(B)** A protoglomerulus at late stage 5. ORN axons have penetrated the layer of glial cells surrounding the AL neuropil and begin to form a dense fringe. **(C)** A protoglomerulus at stage 6. Axons continue to gather in the protoglomerulus but have yet to interact with dendrites. Glia start to surround the glomerulus. **(D)** A glomerulus at stage 9. ORN axons dominate the upper portions of the glomerulus, and dendrites fill its core. Glial cells surround the glomerulus. **(E)** Rhodamine-dextran–labeled ORN axons in a stage-6 AL, a 2-μm confocal microscopy section. Many protoglomeruli are visible (arrowheads).

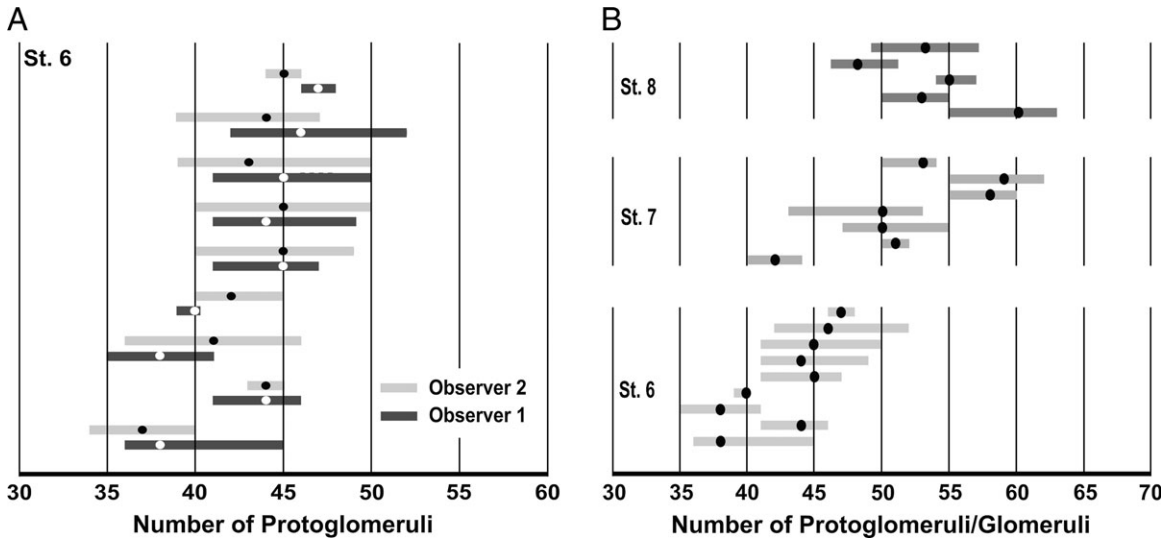

Figure 2 **(A)** Number of protoglomeruli in stage-6 ALs. Protoglomeruli revealed with rhodamine–dextran labeling were tallied independently by two observers. Each observer counted protoglomeruli in an AL three times. A single AL was counted in each of nine animals. Each bar graph represents the range of tallies observed, with the arithmetic mean denoted by a dot. **(B)** Number of protoglomeruli and glomeruli in ALs at various stages. All counts were by a single observer using the same techniques and graphing method as in (A). The stage-11 lobe was counted once. Numbers in stage-6 ALs were significantly different from those in both stage-7 and stage-8 lobes (ANOVA, $P < 0.01$). Stage-7 and stage-8 lobes were statistically indistinguishable.

(Figure 2). Previous research indicated that new axons originating in distal segments of the antennae continue to reach the AL until stage 9 (Sanes and Hildebrand, 1976a,b). We focused on determining the number of glomerular structures present at stages 7 and 8. Using counting methods described above, we found that stage-7 ALs contained 51.7 glomerular structures on average, and stage-8 ALs contained 53.6 glomerular structures on average. To accommodate the differences in sample size, we used an ANOVA test to determine the statistical significance of counts from stage-6, stage-7, and stage-8 ALs. The numbers of glomerular structures observed in stage-7 and stage-8 animals were not significantly different from each other ($P \leq 0.05$), but each stage contained significantly more glomerular structures than observed in stage-6 ALs ($P \leq 0.01$).

Differential maturation of glomeruli

The addition of glomerular structures through stages 7 and 8 indicated that ALs at stage 8 should contain glomerular structures of different developmental stages. Some glomerular structures at this stage would originate from protoglomeruli present since stage 6, roughly 2 days earlier, while other structures would have only recently begun forming. To explore the issue of mixed maturity in stage-8 ALs, we examined the maturation of the glomerular dendrites of a particular, well-studied neuron in the AL. Each AL contains the branching dendrite of a single serotonin (5-hydroxytryptamine; 5-HT)-containing neuron whose cell body is located in the lateral cell body packet of the contralateral AL (Kent *et al.*, 1987). The 5-HT–positive dendrites arborize extensively in all glomeruli in the adult (Kent *et al.*, 1987; Sun *et al.*, 1993). The pattern of growth of these dendrites into developing glomeruli has been analyzed and is similar to that observed for the dendrites of the multiglomerular local interneurons of the AL (Oland *et al.*, 1995). At stage 6, 5-HT–positive dendrites fill the central regions of the AL neuropil but have yet to interact with the protoglomeruli ringing the neuropil (Oland *et al.*, 1995; shown also in Figure 3A). At stage 7, these processes begin to enter the glomerular neuropil but remain confined to the inner portions of maturing glomeruli (Figure 3B). By stage 8, 5-HT–positive processes are found occupying the central regions of most glomeruli (arrows, Figure 3C), in a pattern similar to that observed in adults (Oland *et al.*, 1995). In the current, more detailed, study we observed that the branching pattern of 5-HT–positive processes in some glomerular structures is similar to that observed predominantly in stage 6, that is, they are just beginning to enter the glomeruli and display glomerular tufting (arrowheads, Figure 3C,D). These immature-appearing glomeruli were often adjacent to glomeruli in which 5-HT–positive dendrites displayed a more mature arborization pattern (arrows, Figure 3C,D). Developmentally delayed glomeruli were uncommon, with only two to three observed in a typical stage-8 AL. We conclude that these glomerular structures represent new glomeruli that had begun to develop after stage 6.

Segmental innervation patterns

The addition of new glomerular structures to the AL after stage 6 suggested that ORNs in the proximal segments of the antenna, whose axons arrive at the AL first, may innervate only a subset of the glomeruli found in adults. To test this possibility, we labeled ORN axons originating from small numbers of proximal antennal segments with DiI and examined their termination patterns in the AL. Ideally, these experiments would be done in adult animals to determine the termination patterns of ORN axons from particular segments in the fully mature set of glomeruli. This is prohibitively difficult to do because as the antenna matures it develops a crisp, cuticular covering and becomes too fragile for the necessary microdissection; additionally, the core of the antenna fills with tracheae and support tissue, hindering precise application of DiI to the inner surface of the olfactory epithelium. As a compromise, we chose to label the ORNs of particular segments at stage 8 when a majority of glomerular structures have formed, but the antennal cuticle is still thin. We did these experiments in male animals because the larger segments of the male antenna compared to female enabled us to apply DiI crystals to the inner surface of the olfactory epithelium without also labeling the entire antennal nerve. Additionally, the male ALs we observed at stages 6, 7, and 8 displayed a pattern of protoglomerulus addition similar to that observed in female ALs (data not shown), suggesting that the gross developmental pattern of nonsexual glomeruli is comparable in both sexes. We labeled axons from ORNs in segments 8–11 of the antenna since Tolbert and Sirianni (1990) showed that a reduced number of glomeruli develops when only the proximal 15 segments are allowed to innervate the AL and since segments 1–5, like the most distal few segments, are more sparsely populated with olfactory sensilla than the other segments (Sanes and Hildebrand, 1976a,b).

Placing DiI crystals on the inner surface of the olfactory epithelium of an antennal segment resulted in the movement of DiI label down ORN axons in the segment's nerve rootlet (arrowheads, Figure 4A). When a rootlet merged with the antennal nerve, labeled axons continued to travel together in a tight bundle (arrow, Figure 4A). Fascicles of labeled axons that originated in adjacent segments often were found in close proximity (Figure 4A). Labeled axons remained tightly fasciculated until they entered the axon-sorting zone at the base of the antennal nerve where they defasciculated (arrow, Figure 4B; Rossler *et al.*, 1999) before distributing to multiple glomeruli. While most DiI-labeled axons innervated the male-specific macroglomerular complex (arrow, Figure 4B), other axons traveled deep into the AL to reach some of the sexually isomorphic glomeruli (Figure 4B). DiI-labeled axons appeared in a small subset of those glomeruli,

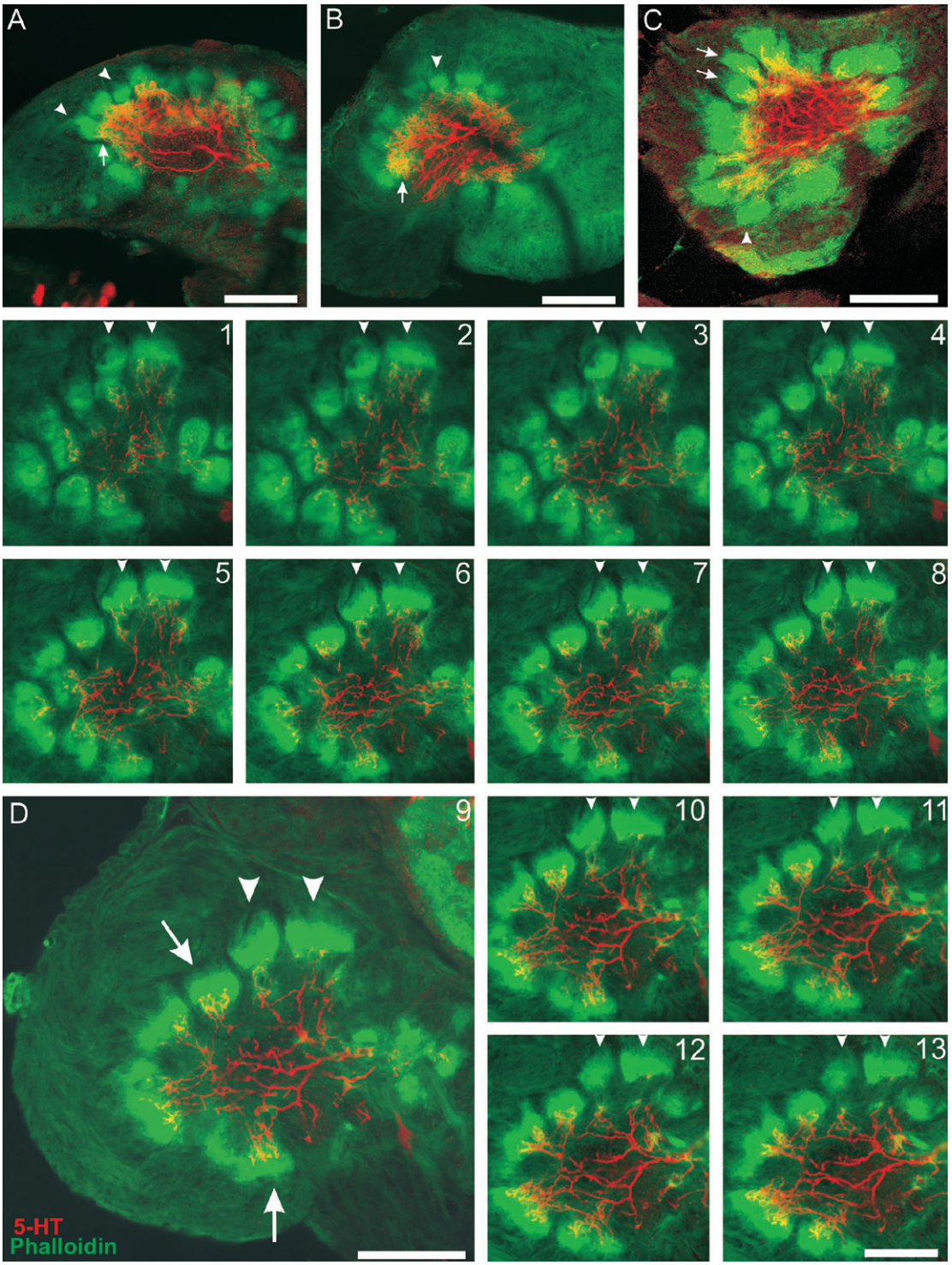

Figure 3 Arborization of the serotonergic (5-HT$^+$) interneuron (red labeling) in glomerular structures. Glomerular structures were visualized using the actin-binding toxin phalloidin conjugated to Alexa-488 (green labeling). **(A)** 5-HT$^+$ interneuron in stage-6 AL. 5-HT$^+$ dendrites have yet to enter protoglomeruli (arrowheads) but display extensive branching on the edges of protoglomeruli (arrow). **(B)** 5-HT$^+$ interneuron in a stage-7 AL. 5-HT$^+$ dendrites have begun to enter the neuropil of some glomerular structures (arrow). In other glomerular structures, 5-HT$^+$ dendrites have yet to interact with protoglomeruli (arrowhead). **(C)** 5-HT$^+$ interneuron in a stage-8 AL. 5-HT$^+$ dendrites have begun to interact strongly with the neuropil of some glomerular structures (arrows). Axons in another glomerular structure have yet to interact with 5-HT$^+$ dendrites (arrowhead). **(D)** A series of confocal images collected at 2-μm intervals from a stage-8 AL. 5-HT$^+$ dendrites have begun to interact strongly with the neuropil of some glomerular structures (arrows). Axons in two glomeruli have yet to interact with 5-HT$^+$ dendrites (arrowheads). Scale bars = 100 μm.

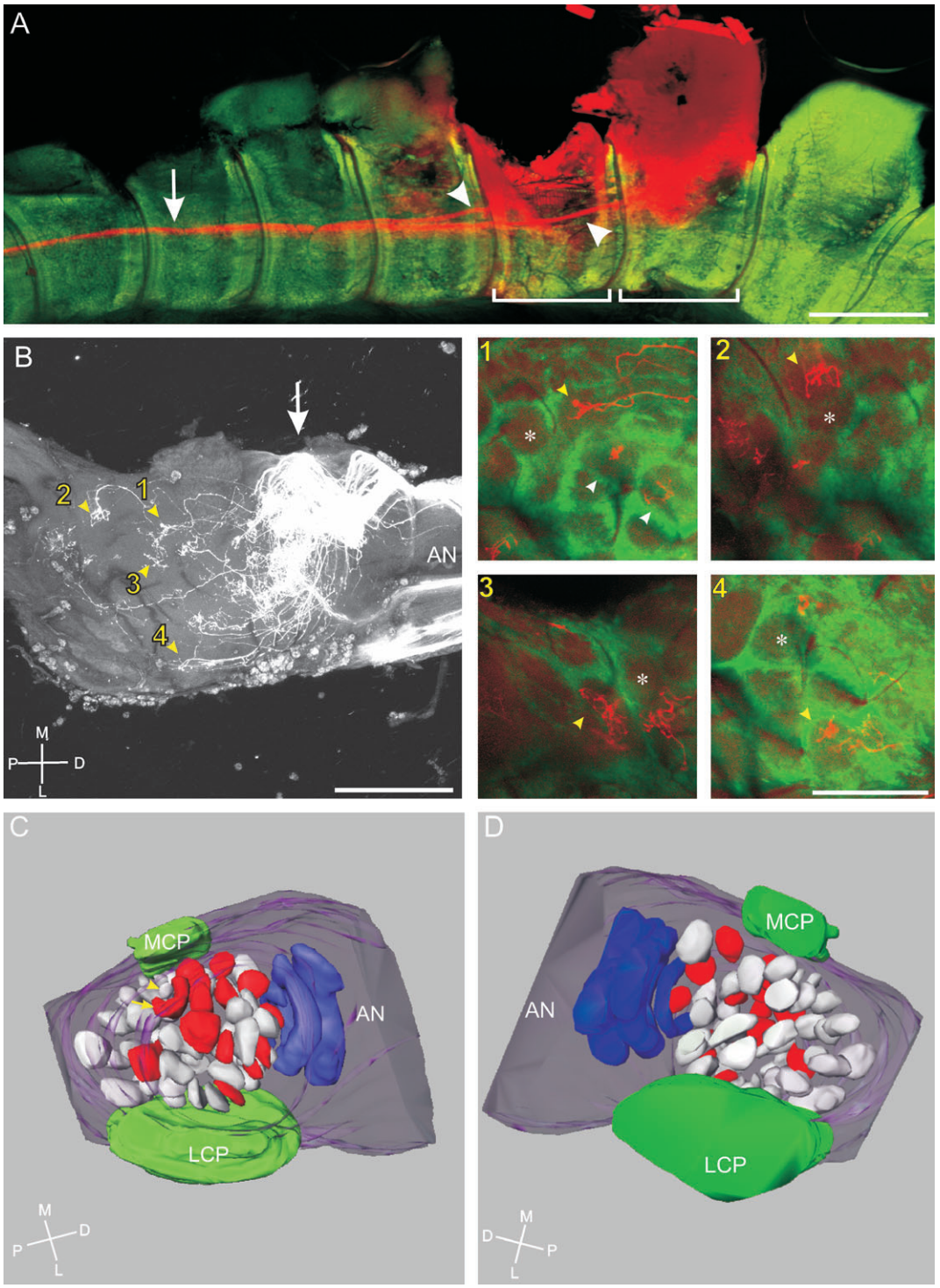

Figure 4 AL projections of axons originating in proximal antennal segments. **(A)** DiI crystals placed on two adjacent segments (brackets) label ORN axons in the nerve rootlets of each segment (arrowheads) and the antennal nerve (arrow). **(B)** Projected Z-series of a stage-8 AL with axons originating from segment 8 displaying DiI labeling. Many labeled axons are found within the macroglomerular complex (MGC, arrow), but some axons innervate ordinary glomeruli (yellow arrowheads). Numerals denote glomeruli seen at higher magnification in adjacent panels. B1–B4: DiI-labeled axons (red) in single optical sections. The glial boundaries of glomeruli are visualized with the nucleic acid dye SYTO 13 (green). While some glomeruli contain DiI-labeled ORN axons (yellow and white arrowheads), most do not (asterisks). **(C)** Three-dimensional reconstruction of an AL in which a population of axons originating in segments 8 and 9 are DiI labeled. Labeled axons innervate a subset of glomeruli (red) intermingled with glomeruli which contain no DiI-labeled axons (white). In the closely associated mortar (yellow arrow) and pestle (yellow arrowhead) glomeruli, only the mortar glomerulus contains DiI-labeled axons. The medial and lateral cell packets (MCP, LCP, green) and the MGC (blue) have also been reconstructed. **(D)** Opposite side of the AL seen in C. Scale bars A, B = 100 μm, B1–B4 = 50 μm.

and in each innervated glomerulus multiple axons commonly were labeled (Figure 4B, 1–4). Notably, these innervated glomeruli were often adjacent to glomeruli in which no DiI-labeled axons were present.

In the majority of preparations (n = 120), DiI failed to reach the AL, or a DiI crystal impinged on the antennal nerve effectively labeling axons from all axons of distal origin. We conducted a detailed analysis of DiI labeling in ALs in which DiI labeling was clearly confined to only two segments of the antenna and in which the entire AL was present in the series of sections collected. In the four ALs meeting these criteria, we observed labeled axons entering 8-, 8-, 10-, and 14-labeled glomeruli. The DiI labeling was limited to antennal segments 8–11 in this sample set, suggesting that at least the proximal antennal segments innervate just a limited number of glomeruli.

To determine the positions of the glomeruli that received DiI-labeled axons from specific antennal segments, we made three-dimensional reconstructions of the four ALs analyzed above. Although this sample size proved too small to draw conclusions about the relationship of a particular segment to a particular subset of labeled glomeruli, we could deduce that glomeruli containing axons labeled from any particular segment were scattered throughout the AL and were often found intermingled with glomeruli containing no DiI-labeled axons (Figure 4B,C). In one example, an unlabeled glomerulus (arrow, Figure 4C) was nearly totally surrounded by a DiI-labeled glomerulus (arrowhead, Figure 4C). In the reconstruction of the AL containing the most labeled glomeruli, we found that the 14 glomeruli receiving DiI-labeled axons (red, Figure 4C,D) were intermixed with 40 glomeruli containing no DiI-labeled axons (white, Figure 4C,D).

Discussion

Our results indicate that glomeruli in *M. sexta* ALs originate from protoglomerular structures that form during stage 6 to stage 9 of pupal development. While the development of each individual protoglomerulus appears to occur over a period of about a day, the development of new protoglomeruli across the AL is staggered over more stages of development than previously realized (Malun *et al.*, 1994). At stage 8, we observed glomerular structures exhibiting different stages of development as defined by their innervation by a uniquely identifiable serotonergic interneuron. Labeling of ORN axons from a subset of antennal segments suggested that segments in the proximal antenna innervate a limited subset of glomeruli, which are scattered across the AL, often intercalated among unlabeled glomeruli.

How are new glomeruli added to the AL?

Our results demonstrate that glomerular structures are added to the developing AL over a period of days. How might this process occur? From stage 6 onward the putative, and then maturing, glomerular layer of the AL appears to be completely filled with glomerular structures (Figure 1A). Given the lack of apparent aglomerular regions in which new glomeruli can form, we propose that new protoglomeruli are inserted between existing glomerular structures as the AL gradually increases in size from stage 6 through approximately stage 9 (Tolbert *et al.*, 1983; Oland *et al.*, 1990). This mechanism of glomerulus addition leads to an AL in which a consistent topography is the result of specific glomeruli forming at specific times in specific locations rather than the creation of glomeruli in a continuous spatial pattern. Such ''nonzonal,'' interspersed patterns have been observed for *Manduca* glomeruli that are positive for the putative cell adhesion molecule fasciclin II (Higgins *et al.*, 2002) and for an ephrin and an Eph-type receptor tyrosine kinase (Kaneko and Nighorn, 2003).

We propose for consideration two hypothetical mechanisms that would allow the insertion of new glomeruli into an expanding AL. One possibility is that some axons that innervate two different glomeruli in adulthood innervate the same single glomerular structure at earlier stages. As the AL matures, their terminals segregate into two (adjacent) glomeruli. Another possibility is that ORNs in the proximal antenna express a different complement of odorant receptors (ORs) than ORNs in more distal parts of the antenna. Axons originating from the proximal antennae would form protoglomeruli before axons of a more distal origin had arrived. As more distal-originating axons reached the AL, they would form new protoglomeruli with different odor specificities.

Is the antenna of *M. sexta* heterogeneous along its length?

Morphologically, the antenna of *Manduca* appears to be relatively homogenous except for the most proximal and most distal segments, which have few sensilla. With the exception of these few segments, each segment contains a variety of different types of sensilla, distributed at approximately the same density on each segment (Lee and Strausfeld, 1990; Shields and Hildebrand, 2001). Unlike in *Drosophila*, the various classes of sensilla are not associated with specific regions of the sensory organs but repeat in a pattern that is similar from segment to segment (Sanes and Hildebrand, 1976a,b; Lee and Strausfeld, 1990; Shields and Hildebrand, 2000, 2001). Comparing intersegmental responses of male antennae to female pheromones, as measured in the AL, suggests that male sensilla along the antenna are homogeneous (Baker, 1989; Heinbockel and Hildebrand, 1998). These sensilla, however, are activated by such a limited subset of odorants that their homogeneity reveals little about the homogeneity of nonsexually dimorphic sensilla along the length of the antenna. Recordings from a number of type-A trichoid sensilla located on the same segment revealed several different electrophysiological profiles, but no attempt was made to compare responses among sensilla on different antennal segments (Shields and Hildebrand, 2001). Future molecular biological and electrophysiological studies will be needed to settle the issue of antennal homogeneity or heterogeneity.

If the *Manduca* antenna is found with further study to be fairly homogenous, the addition of new glomeruli would be more likely to occur through the alternative process of glomerular splitting. Such a process might not be heretical in the context of what is known about axon targeting in the mouse olfactory system. In mice, ORNs expressing the same OR segregate soon after arriving at the surface of the presumptive OB and remain segregated as they form glomeruli over the next several days (Royal and Key, 1999; Potter *et al.*, 2001). Recent evidence from Zou *et al.* (2004), however, indicates more intermixing of axons of ORNs expressing different ORs than had been previously observed. This intermixing of ORN types was evident at birth but gradually declined, through activity-dependent mechanisms. By adulthood only a few axons expressing the same OR have failed to segregate into separate, well-defined glomeruli (Treloar *et al.*, 2002). This example of selective ORN axonal pruning late in development would be consistent with the possibility that in *Manduca* axons of different specificities initially intermingle but then are separated, at least in part by pruning. Work in *Drosophila* does not shed light on this issue. ORNs expressing a particular OR tend to cluster in the periphery (Vosshall *et al.*, 1999; Gao *et al.*, 2000), and their axons arrive at the AL as a distinct mass before forming synapses with projection neurons (Hummel and Zipursky, 2004); there appears to be little requirement or opportunity for interaction between ORN axons expressing different ORs.

We have addressed the issue of antennal heterogeneity by using anterograde labeling to identify the termination patterns of axons that originate in a particular small subset of proximal antennal segments. We found that labeled axons from this small number of segments terminated in a small subset of glomeruli (cf. Figure 4). While our results may support the hypothesis that the antenna is heterogeneous, the DiI-labeling technique used in this study has two major limitations that should be kept in mind. First, each segment of the antenna must contain thousands of ORNs since the total number of ORN axons, from all 80 segments, is estimated to be 330,000 (Sanes and Hildebrand, 1976a,b), yet the axons of only a small number of the ORNs present in the DiI-labeled segments could be traced into the AL. We suppose that these axons represent a random sampling of ORNs in labeled segments, but the possibility exists that axons of ORNs located in different sensilla are differentially labeled by DiI and therefore that the DiI-labeled glomeruli observed in our studies represent the projections of just particular sensillum types. Secondly, when we attempted to label axons from ORNs in segments 13 and higher, we found that axons did not label all the way to their terminals in the glomeruli. Thus, we were unable to compare segmental innervation patterns from the full length of the antenna. The final determination of the heterogeneity or homogeneity of the *M. sexta* antenna must await the identification of OR expression patterns.

Temporal pattering in olfactory systems

Our results indicate that the arrival of different subsets of axons at different times may be crucial to the development of the *Manduca* AL. There is experimental evidence that the timing of ORN axon arrival can influence AL development. Rössler *et al.* (2000) took advantage of the temperature sensitivity of *Manduca* development to interfere with olfactory development by exposing developing antennae to temperatures either cooler or warmer than the temperature of the brain for 48 h following the initial arrival of axons at the AL (stage 3). In experiments in which the antennae were cooled with respect to the brain, thus delaying the ingrowth of ORN axons into the AL, individual glomeruli could not be identified and glia were confined to the outer layers of the AL at stage 8. In experiments in which the antennae were heated, accelerating the ingrowth of axons, glial cells surrounded what appeared to be normal glomeruli. However, some of the axons that normally innervate the male-specific macroglomerular complex now innervated some of the sexually isomorphic glomeruli throughout the AL. These results are consistent with our hypothesis of a staggered developmental program within the AL. An initial wave of ORN axons triggers a migration of glia, which then surround nascent protoglomeruli. As new subsets of axons arrive, they also form protoglomeruli and recruit glia. Delayed ORN axons fail to initiate the maturation of glia leading to an aglomular AL. If ORN axons arrive at the AL too quickly, the normal temporal pattern of protoglomerulus addition begins to break down, and ORN axons begin to innervate the wrong glomeruli.

To what extent does the temporally staggered development of glomeruli that we have observed in *Manduca* occur in other olfactory systems? In *Drosophila*, the antenna is so short that there may be little difference in the times of arrival of various ORN axons in the AL. In mice, however, there does appear to be a general temporal pattern of maturation in the OB, with rostral glomeruli developing before caudal glomeruli, suggesting that the processes necessary for initial glomerulus development are present for an extended period. This period of new glomerulus formation is potentially extensive as indicated by the observed addition of new glomeruli to the mature OB (LaMantia and Purves, 1989; Pomeroy *et al.*, 1990). Recent microarray data suggest that OR expression patterns change throughout adulthood (Zhang *et al.*, 2004). By extension the glomeruli found in the OB may change over time, with glomeruli forming and disappearing as OR genes are up- and downregulated.

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