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The Organization of the Antennal Lobe Correlates Not Only with Phylogenetic Relationship, But Also Life History: A Basal Hymenopteran as Exemplar

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Abstract

The structure of the brain is a consequence of selective pressures and the ancestral brain structures modified by those pressures. The Hymenoptera are one of the most behaviorally complex insect orders, and the olfactory system of honeybees (one of the most derived members) has been extensively studied. To understand the context in which the olfactory system of the Hymenoptera evolved, we performed a variety of immunocytochemical and anatomical labeling techniques on the antennal lobes (ALs) of one of its most primitive members, the sawflies, to provide a comparison between the honeybee and other insect model species. The olfactory receptor neurons project from the antennae to fill the entire glomerular volume but do not form distinct tracts as in the honeybee. Labeling of projection neurons revealed 5 output tracts similar to those in moths and immunolabeling for several transmitters revealed distinct populations of local interneurons and centrifugal neurons that were also similar to moths. There were, however, no histaminergic or dopaminergic AL neurons. The similarities between sawflies and moths suggest that along with the great radiation and increased complexity of behavioral repertoire of the Hymenoptera, there were extensive modifications of AL structure.

Key words: anatomy, antennal lobes, olfaction, sawfly

Introduction

The Hymenoptera are one of the most behaviorally diverse and complex orders of insects. They encompass approximately 125 000 described species with an estimated 600 000 to 1.2 million species that have yet to be described (Grimaldi and Engel 2005). The behavioral range of the Hymenoptera includes herbivorous, parasitic, and colonial forms and with these modifications in behavior have come incredible changes in external structures that suggest that the brains of these animals must also have undergone very dramatic morphological shifts over evolutionary time.

One of the most extensively studied areas of the insect brain is the antennal lobe (AL), which is the first synaptic neuropil of the olfactory system and the equivalent of the vertebrate olfactory bulb (Hildebrand and Shepherd 1997). The AL in most insects is modular in nature, composed of many glomeruli which are synaptic neuropil compartments consisting of the processes of the olfactory receptor neurons (ORNs) that convey information from the environment to the AL, the projection neurons (PNs) which transmit information from the AL to the rest of the brain, the local interneurons (LNs) which project between glomeruli and finally the centrifugal neurons that provide feedback from the brain to the AL. The ALs of several species of insect have been the subjects of extensive study, and the ALs of each species differs from the others in terms of their anatomical structure reflecting the influences of the selective pressures that have resulted from the ecological niches occupied by each species (reviewed by Galizia and Rössler 2009). Here, we have established the basal state for the Hymenoptera that will serve as the foundation for the study of the neural structures associated with the diverse array of behaviors exhibited by this order.

The Hymenoptera can by divided up into 3 groups (Figure 1A) based on general behavioral traits (although only one is monophyletic); the herbivorous Symphyta (of which the sawflies are the most basal members), the Parasitica, and

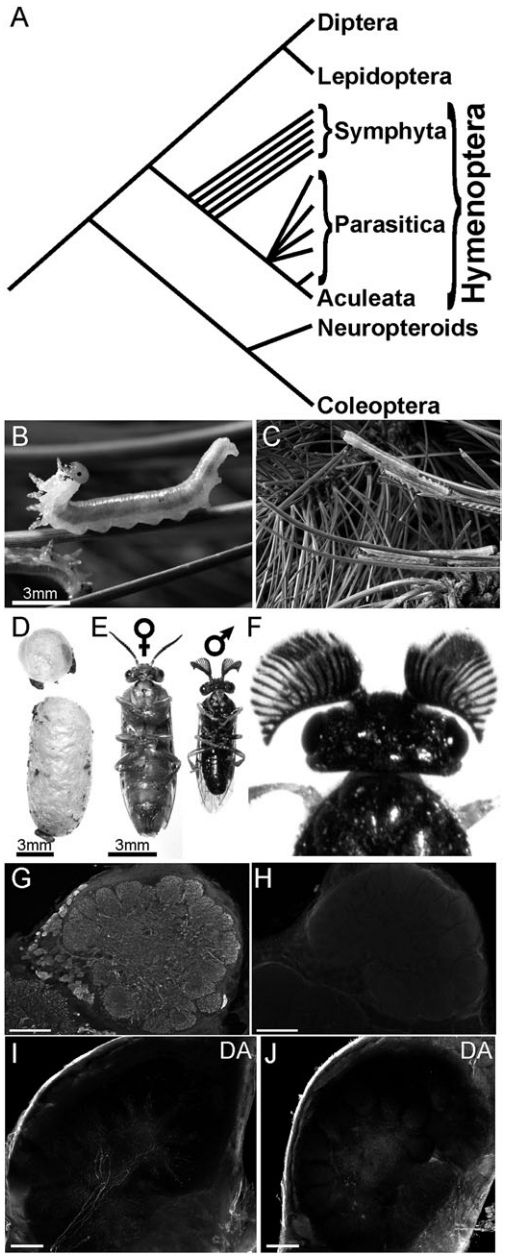

Figure 1 Phylogenetic tree depicting the phylogenetic relationships within the Hymenoptera and images of *Neodiprion* larvae and adults. **(A)** Phylogenetic tree of the Holometabola (complete metamorphosing insects) depicting the phylogenetic relationships between the Symphyta, Parasitica, and Aculeata. Tree is based on Grimaldi and Engel (2005) but is not drawn to scale and is only intended to illustrate the relationships between groups. **(B)** A late instar *Neodiprion* larvae displaying a defensive posture. **(C)** Several *Neodiprion* larvae consuming needles from clippings of a Ponderosa pine tree. **(D)** A pupal case from which an adult female emerged by cutting off a cap. **(E)** Female (left) and male (right) *Neodiprion* adults. **(F)** The head of a male *Neodiprion* highlighting the pectinate antennae. Preadsorption controls in which rabbit anti-GABA antibody has been incubated in **(G)** blocking medium or **(H)** 100 mM GABA in blocking medium before being applied to Bombus impatiens tissue. Scale bars = 100 μm. Preadsorption assays in which rabbit anti-dopamine antibody has been incubated in **(I)** blocking medium or **(J)** 100 mM dopamine in blocking medium before being applied to *Manduca sexta* tissue. Scale bars = 100 μm.

the stinging Aculeata (which include bees and ants). With regards to AL structure, these groups are relatively internally consistent, but their ALs differ markedly from each other in terms of serotonergic (Rehder et al. 1987; Dacks et al. 2006; Tsuji et al. 2007; Zube and Rössler 2008; Nakanishi et al. 2010) and histaminergic innervation (Bornhauser and Meyer 1997; Dacks et al. 2010) suggesting that the different lifestyles of these insects were accompanied by changes in the neural architecture of the ALs. The sawflies are the most basal of the Hymenoptera and the Symphyta, and their life histories are similar to moths in that the only parental care exhibited involves the female laying eggs on a host plant. This is in contrast to the Parasitica that lay their larvae in the relatively more protected internal environment of a host insect or the Aculeata many of which build nests for the raising of the young. To get a more extensive assessment of the extent to which the ALs of the more derived Hymenoptera (such as honeybees) have changed over evolutionary time compared with other insect groups, we performed a suite of anatomical and immunocytochemical assays to characterize the morphology of all 4 types of AL neurons (ORNs, PNs, LNs, and centrifugal neurons) of the sawflies *Neodiprion ventralis* and *Neodiprion autumnalis*. Our hypothesis is that the ALs of sawflies will most closely resemble those of moths due to their similar life histories and will differ significantly from honeybees which have evolved a much more complex life history compared with these basal-most members of the Hymenoptera.

Materials and methods

Animals

Several hundred final instar *N. autumnalis* (Smith and Wagner 1986) and *N. ventralis* (Ross 1955) (Figure 1B) were collected from Ponderosa pine trees on Mt Lemmon, just outside of Tucson, AZ. Because the individuals collected for this study represent these 2 very closely related species (Linnen and Farrell 2008) and were infesting the same branches, we will refer to the sawflies in this study as *Neodiprion* from this point on under the assumption that there are likely only very minor differences in the anatomy of their ALs. Furthermore, because the results of our labeling techniques were extremely consistent from animal to animal, we feel confident in the validity of grouping individuals of these 2 species together for analysis. Larvae were reared on Ponderosa pine clippings (Figure 1C) placed in 50-mL centrifuge tubes with water and held upright in a tube rack. The racks of clippings were placed in a $1 \times 1 \times 1$ m Plexiglas cage with a 1 inch layer of soil to allow the larvae to burrow and form cocoons (Figure 1D). Once all the larvae had pupated, the pupae were collected and stored in pipette tip boxes until adults emerged. Males and females (Figure 1E) were easily distinguished based on coloration (females being light brown and males being black), size (males being about 2/3 the length

of females), and antennal morphology (males having broad, pectinate antennae; Figure 1F). Adult sawflies were sacrificed for tissue processing within 1–2 days after emergence from their pupal case. There was an approximately 1:30 male:female ratio, thus female sawflies were used for all preparations except where noted, specifically for antennal nerve backfills (Figure 2B). Brains from a total of 104 adult sawflies that had emerged between September 31st and November 12th of 2009 were processed with a variety of labeling procedures. The phylogenetic tree in Figure 1A is based on Grimaldi and Engel (2005) but is not drawn to scale with respect to evolution time.

Immunohistochemistry

The details of the hosts, sources, specificity assays, and dilutions of primary antibodies are summarized in Table 1. Due to the seasonal availability of the *Neodiprion* tissue, *Bombus impatiens* and *Manduca sexta* AL tissue was used for the preadsorption assays of antibodies for which preadsorption assays had not previously been published. For the rabbit anti-γ-aminobutyric acid (GABA) antibody preadsorption, primary antibody was incubated for 24 h in either blocking medium (Figure 1G) or 100 mM GABA (Sigma) in blocking medium (Figure 1H), spun down, and then applied to *B. impatiens* AL tissue as described below. For the rabbit anti-dopamine antibody preadsorption assay, primary antibody was the incubated for 24 h in either blocking medium (Figure 1I) or 100 mM dopamine (Sigma) in blocking medium (Figure 1J), spun down, and applied to *M. sexta* AL tissue as described below. Both the GABA and dopamine preadsorptions resulted in a total loss of labeling.

Two basic protocols were used for all the antibodies described in this paper. The protocol described in Dacks et al. (2005) was used for the dopamine and octopamine labeling. Brains were dissected in 0.1 M cacodylate with 10 g/L sodium metabisulfite (SMB) and placed in a cold fixative of 0.1 M cacodylate, 2% paraformaldehyde, and 1% glutaraldehyde overnight at 4 °C. Brains were then washed in phosphate-buffered saline (PBS), embedded in 5% agarose (Sigma), and sectioned at 75 μm with a vibrating microtome (Technical Products International). Tissue was then washed in a 0.05 M Tris buffer with 8.5 g/L SMB (Tris-SMB; pH 7.4) and then incubated in Tris-SMB with glycine (10 mg/mL) for 30 min. Tissue was then washed in Tris-SMB and incubated for 2 days in primary antibody in Tris-SMB with 0.25% IgG free bovine serum albumin (BSA; Jackson Immunoresearch), 0.25% Triton X-100, 1% normal goat serum, 3% low fat milk, 50 mM sodium azide. Sections were then washed in Tris buffer with 8.5 g/L sodium chloride (Tris-NaCl; pH 7.4) and incubated overnight as above in either 1:1000 goat anti-rabbit Cy3 (Jackson Immunoresearch) for dopamine-labeled tissue or 1:1000 goat anti-mouse Cy3 (Jackson Immunoresearch) for octopamine-labeled tissue. The following day tissue was washed in the Tris-NaCl and then incubated for 10 min in 60% glycerol and 40% water and then mounted on glass slides in 80% glycerol.

For the remainder of the antibodies, a protocol based on that published in Dacks et al. (2006) was employed. Brains were dissected in insect saline and placed in 4% paraformaldehyde overnight at 4 °C with the exception of the histamine immunocytochemistry in which brains were placed in a 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma) in PBS (pH 6.9) for 1 h at room temperature before paraformaldehyde fixation. Brains were then washed in PBS, then embedded, and sectioned as described above. Sections were washed in PBS with 0.5% Triton X-100 (PBST), blocked for 1 h in PBST with 2% IgG free BSA, and incubated for 2 days in primary antibody in PBST with 1% Triton X-100, and 50 mM sodium azide (PBSAT). Sections were then washed in PBST, blocked as before, and incubated overnight in 1:1000 goat anti-rabbit Cy3 in PBSAT. Sections were then washed in PBST, cleared, and mounted as described above.

Mass fills

ORNs and PNs were backfilled as described in Dacks et al. (2006). Briefly, a broken glass electrode coated in Texas red, 3000 MW (Molecular Probes, Invitrogen) was inserted into either the antennae (for ORN labeling) or ALs (for PN labeling) of restrained adults and either antennae or head capsules (respectively) were sealed with petroleum jelly for 24 h. Brains were then dissected, fixed overnight in 4% paraformaldehyde, sectioned, cleared, and mounted as described above.

Confocal microscopy

Sections of ALs were scanned using a Zeiss 510 Meta laser scanning confocal microscope equipped with argon and green HeNe lasers and appropriate filters. The Zeiss LSM Image browser was used to create stacks of optical sections to adjust contrast and brightness of individual images and to perform cell and glomeruli counts. Images were organized in CorelDraw X4. All images are presented in a "horizontal" view with the anterior–posterior (anterior at the top of each image) and medial–lateral axes visible, unless otherwise indicated.

Results

ORNs

Retrograde dye fills of the antennal nerve resulted in labeling of hundreds of ORNs axons, which formed fascicles at the base of the antennal nerve and innervated distinct glomerular structures (44 ± 2.7 standard deviation [SD], $n = 4$; Figure 2A). The male AL was almost 2 times the size of the female AL (Figure 2B) due to an additional male-specific neuropil that sat between the base of the antennal nerve and a set of glomeruli very similar to those in the female AL. This additional

Table 1 Antisera used in this study

Antigen	Host	Source and first characterization or catalog number	Specificity assays	Dilution
Allatostatin	Rabbit	H. Agricola (Vitzthum et al. 1996)	Vitzthum et al. (1996)	1:500
Corazonin	Rabbit	J. Veenstra (Veenstra 1991)	Veenstra (1991)	1:500
Crustacean cardioactive peptide	Rabbit	H. Agricola (Agricola et al. 1995)	Agricola et al. (1995)	1:500
Dopamine (DA)	Rabbit	Immunostar (# 22939)	Figure 1G–H, this study	1:500
FMRFamide	Rabbit	E. Marder (Marder et al. 1987)	Marder et al. (1987)	1:500
GABA	Rabbit	Sigma (# A2052)	Figure 1I–J, this study	1:500
Histamine (HA)	Rabbit	Immunostar (# 22230)	Paulk et al. (2009)	1:500
Locustatachykinin II (Lom-TK II)	Rabbit	H. Agricola (Nässel 1993)	Nässel (1993)	1:500
Octopamine (OA)	Mouse	H. Agricola (Dacks et al. 2005)	Dacks et al. (2005)	1:1000
Serotonin (5HT)	Rabbit	Immunostar (# 20080)	Paulk et al. (2009)	1:5000

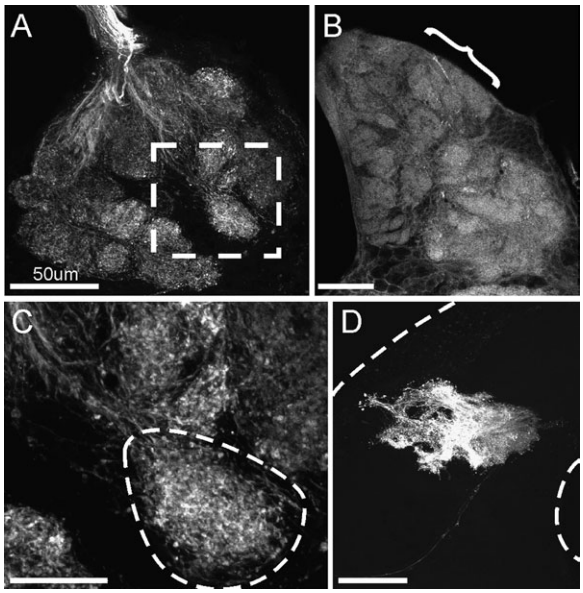

Figure 2 ORNs of *Neodiprion*. **(A)** Horizontal section of a mass fill of the ORNs in the antennae of a *Neodiprion* female highlighting the ORN innervation of the AL glomeruli. Hatched box represents the border of the high magnification view in (C). **(B)** High gain image of the AL of a *Neodiprion* male highlighting the AL glomeruli as well as an additional male-specific enlargement of the AL (bracket). **(C)** ORNs innervating a single glomerulus of *Neodiprion* delineated by the hatched border. **(D)** The antennal mechanosensory and motor center of *Neodiprion* highlighted by mass fills of the mechanosensory neurons in the antennae. Hatched lines delineate the border of the tritocerebrum on the left and the esophageal foramen on the right. All scale bars = 50 μm.

neuropil may be a macroglomerular complex similar to those found in moths (Christensen and Hildebrand 2002), but we will refrain from referring to this structure as such without further study of the physiological properties of the innervating neurons. The ORNs innervated the entire volume of the glomerulus (Figure 2C) which was quite different from the ORNs of honeybees, which innervate only the outer rind of the glomerulus (Kirschner et al. 2006) and somewhat different from the ORNs of moths which innervate the distal portion of the glomerulus (Oland et al. 1990). In addition to filling ORNs, the mass fills of the antennal nerve also resulted in the labeling of the axons of mechanosensory hairs projecting to the antennal mechanosensory and motor center, which was located just posterior and ventral to the AL (Figure 2D).

PNs

The labeling of hundreds of PNs via mass dye fills of the ALs revealed axonal output tracts produced by PNs that projected to the mushroom bodies and the lateral horn (Figure 3). *Neodiprion* possessed 5 PN axonal output tracts (Figure 3A–C,F, and G) which were very similar to those of *M. sexta* as described by Homberg et al. (1988) in terms of their relative positions, sizes, and projections to the mushroom body calyces and the lateral horn (Figure 3D,E, respectively). The majority of the volume of the calyx was occupied by the axonal terminals of the PNs, suggesting that the calyx lacked a collar region receiving visual input from the optic lobes as is found in ants and bees (Gronenberg 1999, 2001; Gronenberg and Hölldobler 1999). Studies of the PN output tracts of different species have generated several different nomenclatures. Galizia and Rössler (2009) have proposed a unifying nomenclature, which we will also use but will also make reference to the nomenclature originally established for *Manduca* (Homberg et al. 1988). The medial antennoprotocerebral tract (mAPT and the equivalent of the inner antenno-cerebral tract of *Manduca*) (Figure 3A) was the largest of the 5 tracts extending first to the calyces and then onto the lateral horn. The mAPT bifurcates to project around the peduncle of the mushroom body with both branches of the mAPT innervating the calyx (Figure 3A). The medio-lateral antenno-protocerebral tract 1 (mlAPT1

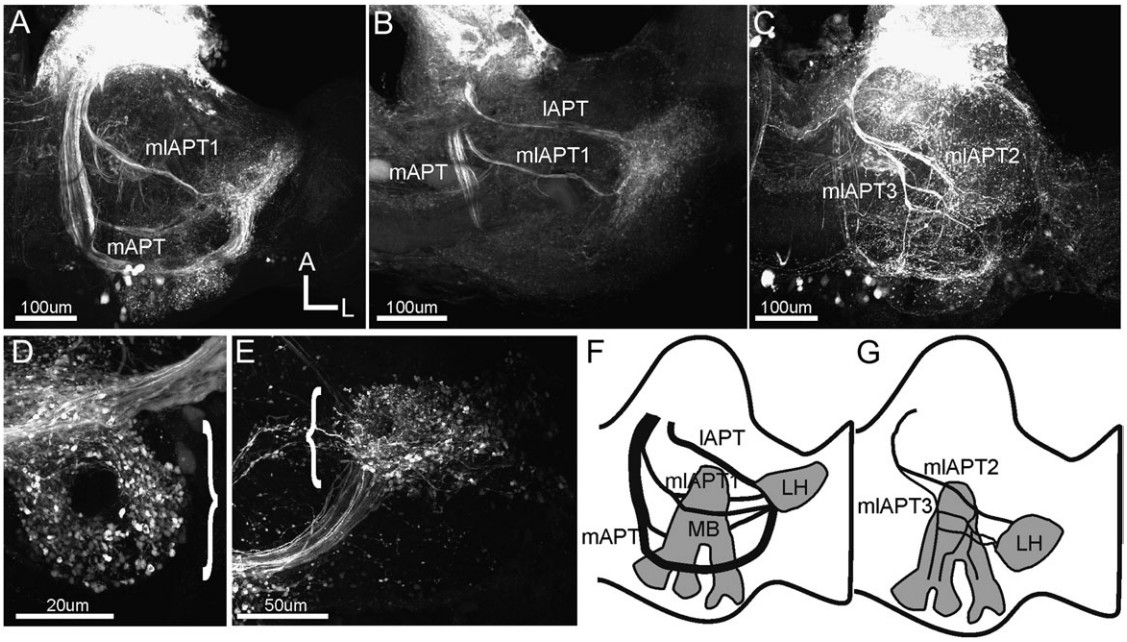

Figure 3 AL PNs of *Neodiprion*. **(A)** Horizontal section of a mass fill of AL PN axons revealing the mAPT and mlAPT1. Both the dorsal and ventral branches of the mAPT are visible projecting to the calyces of the mushroom bodies and the lateral horn. The mlAPT1 projects only to the lateral horn. **(B)** The axons of PNs forming the lAPT project first to the lateral horn and then to the calyces. **(C)** The mlAPT2 and mlAPT3 appear to bifurcate, projecting to both the calyces and the lateral horn. The synaptic boutons of AL PN axons from the various antenno-cerebral tracts highlight **(D)** the mushroom body calyces (bracket) and **(E)** the lateral horn (bracket) of *Neodiprion*. **(F)** Schematic diagram of the more ventral antenno-protocerebral tracts projecting to the mushroom bodies (MBs) and the lateral horn (LH). **(G)** Schematic diagram of the more dorsal antenno-protocerebral tracts. A, anterior; L, lateral.

and the equivalent of the medial antenno-cerebral tract) did not project to the calyces but rather extended directly to the lateral horn (Figure 3A,B). The lateral antenno-protocerebral tract (lAPT and the equivalent of the outer antenno-cerebral tract of *Manduca*) projected first to the lateral horn and then to the calyces (Figure 3B). The mAPT, mlAPT1, and the lAPT all projected from the AL to the lateral horn and mushroom bodies at a similar plane with respect to the dorsal–ventral axis of the brain. Finally, there were 2 dorsally located tracts (Figure 3C) that had the least number of contributing PN axons; the mlAPT2 (the equivalent of the dorsal antenno-cerebral tract) and the mlAPT3 (the equivalent of the dorso–medial antenno-cerebral tract) which bifurcated to project to both the calyces and the lateral horn (although only a few of the dozens of fibers in the mlAPT2 projected to the calyces). The mlAPT2 and 3 can be distinguished from each other due to the relatively small number of axons in the mlAPT3 compared with the mlAPT2 (Figure 3C). All 5 tracts are schematized in Figure 3F (the more ventral mAPT, mlAPT1, and lAPT) and Figure 3G (the more dorsal mlAPT2 and mlAPT3).

LNs

Several antibodies were used to identify different potential transmitters used by LNs in the AL of *Neodiprion*. As has been described in many different insect species, there was a large population of GABA-immunoreactive (GABA-ir) LNs localized to the cell clusters of each AL (Figure 4A). Similar to moths (Hoskins et al. 1986; Berg et al. 2009) and honeybees (Schäfer and Bicker 1986), there were a few GABA-ir neurites visible in the output tracts (Figure 4B), suggesting that there are some GABA-ir PNs in the AL of *Neodiprion*. Antibodies against the neuropeptide locustatachykinin II (Lom-TK II) (Figure 4C) revealed a population of approximately 22.4 LNs (±1.5 somata SD, $n = 4$), and against FMRFamide (Figure 4D), revealed a population of 56.1 LNs (±2.1 somata SD, $n = 4$). There were no allatostatin -ir LNs despite strong labeling of neurons in other brain regions (Figure 4E). This result was surprising as allatostatin-ir LNs have been described in the ALs of moths (Utz and Schachtner 2005; Berg et al. 2007, 2009), flies (Carlsson et al. 2010), and honeybees (Kreissl et al. 2010). Antibodies against corazonin (Figure 4F) and crustacean cardioactive peptide (Figure 4G) did not result in the labeling of any AL LNs, although there was very bright labeling of neurons in other regions of the brain.

Serotonin, octopamine, dopamine, and histamine-immunoreactive input to the AL

Labeling against 4 biogenic amines, serotonin (5HT), octopamine (OA), dopamine (DA) and histamine (HA), was performed to permit comparison of the aminergic input with the ALs of *Neodiprion* with that of other insects. Many glomeruli of *Neodiprion* received 5HT-ir innervation (Figure 5A),

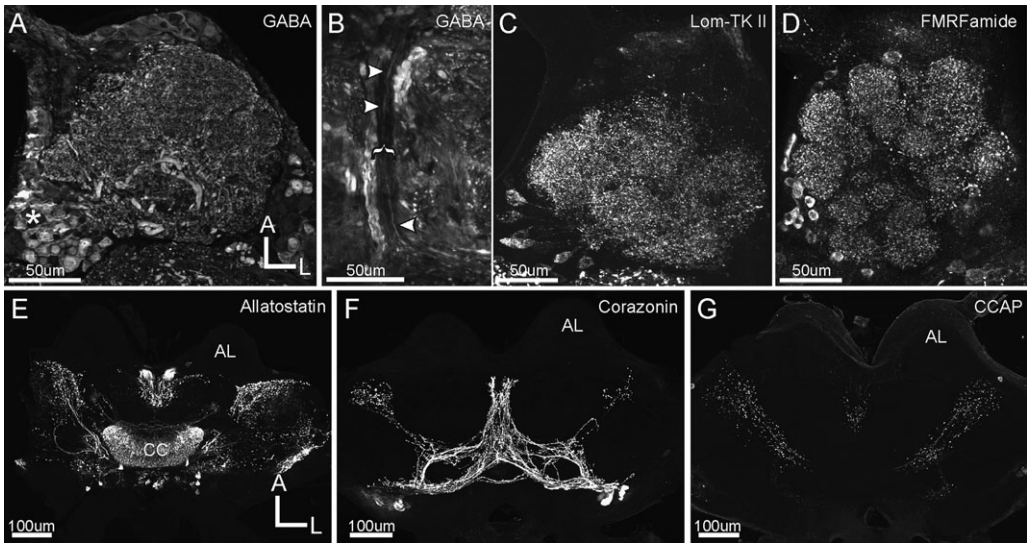

Figure 4 LNs of the ALs of *Neodiprion*. **(A)** Horizontal section through the AL showing GABA-ir LNs. The cell bodies of GABA-ir LNs in the lateral cell cluster of the AL are indicated by an asterisk. **(B)** Several GABA-ir neurites (arrowheads) in the mAPT (bracket) of *Neodiprion*. (C–D) Locustatachykinin (Lom-TK II) and FMRFamide immunolabeling of LNs, respectively. (E–G) Horizontal sections of brains immunolabeled for allatostatin, corazonin, and crustacean cardioactive peptide (CCAP), respectively. Note the absence of labeling in the ALs and strong labeling in other brain regions. Scale bars = 100 μm. A, anterior; L, lateral; CC, central complex.

although several glomeruli in the lateral anterior–ventral portion of the AL received either very little or no 5HT-ir innervation (Figure 5A,B). There was a large degree of heterogeneity in the extent to which each individual glomerulus was innervated, some receiving only 1 neurite, whereas others received several neurites that extended throughout the volume of the glomerulus (Figure 5A,B). Each AL was innervated by a centrifugal neuron that projected posterior–dorsally from the AL (Figure 5C), crossed the dorsal midline (Figure 5D), and then innervated the contralateral AL. This morphology was consistent with that of the contralaterally-projecting, serotonin-immunoreactive deutocerebral (CSD) neuron, which is widespread throughout the holometabolous (or complete metamorphosing) insects (Dacks et al. 2006). It should be noted that each CSD neuron had a small amount of branching in the ipsilateral AL (Figure 5B), which is a feature of CSD neurons that varies between species.

The AL of *Neodiprion* was sparsely innervated by OA-ir neurites (Figure 6A). Each AL was innervated by at least one centrifugal neuron that projected up from the subesophageal ganglion (SEG) (Figure 6B,C). Unfortunately, we were unable to trace the cells any further than the most dorsal aspect of the SEG as the neurites became closely associated with other OA-ir processes (Figure 6C) that form the "deep" and "superficial DUM tracts" originally described by Watson (1984). The ALs of many insects are innervated by OA-ir ventral unpaired median (VUM) neurons and we did observe in the maxillary neuromere of the SEG some OA-ir VUM neurons (Figure 6D) which projected dorsally toward the AL before bifurcating around the esophageal foramen (Figure 6D,E). However, because we were unable to trace the entirety of the OA-ir neurites innervating the AL, we can only determine that the SEG is the point of origin.

Although there are large populations of HA-ir LNs in the ALs of the aculeate and parasitic Hymenoptera (Dacks et al. 2010), there were no HA-ir LNs in the ALs of *Neodiprion* (Figure 7A). There was also no DA-ir observed in the ALs of *Neodiprion* (Figure 7B), despite obvious labeling in neighboring brain regions.

Discussion

The purpose of this study was to examine the ALs of a key taxon within the Hymenoptera to begin to understand the neural underpinnings of the diversity of olfactory behavior of this order. The most primitive Hymenoptera are essentially "moth-like" in their life history strategy. The sawflies (and other closely related taxa) lay their eggs on a host plant and leave their larvae to fend for themselves. Over evolutionary time, the parasitic Hymenoptera began to inject their eggs within the more protected environment of a host insect, thus providing somewhat more in the way of care for their offspring. Within the aculeate Hymenoptera, eusocial societies in which sterile workers care for the offspring of one or a few reproductive individuals evolved multiple times, thus producing some of the most complex societies on the planet. All of these distinct shifts in behavioral repertoire must have associated changes in brain structure, and thus, this study seeks to be a starting point for the comparison of the 2 extremes of the behavioral spectrum of the Hymenoptera. It is therefore not surprising that many of the

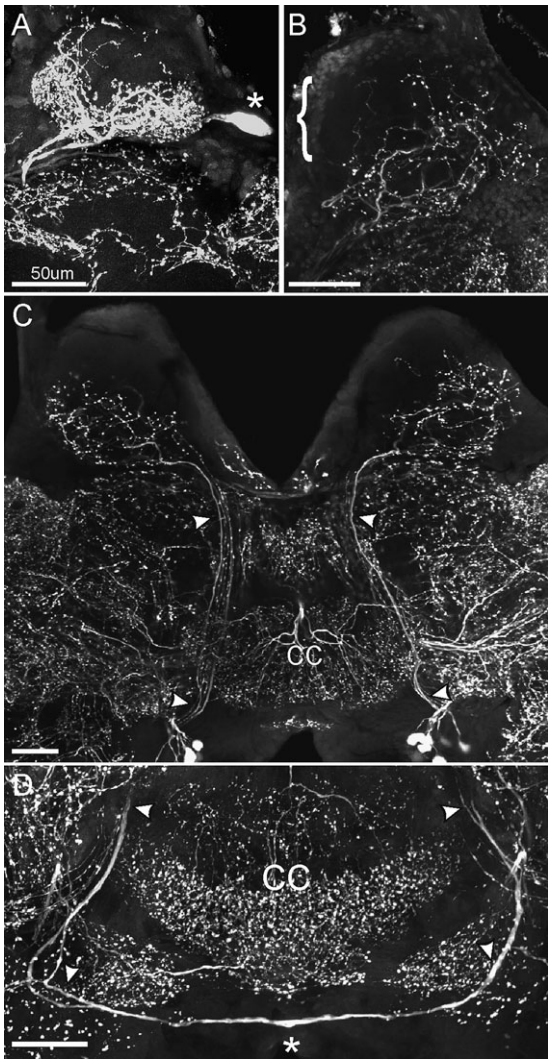

Figure 5 Serotonin-immunoreactive innervation of the ALs of *Neodiprion*. **(A)** Horizontal section AL depicting the 5HT-ir innervation of the AL and a single 5HT-ir cell body (asterisk) in the lateral cell cluster. **(B)** The more anterior glomeruli (bracket) receive less 5HT-ir compared with more posterior glomeruli, demonstrating the heterogeneity in 5HT-ir innervation of the AL. **(C)** The 5HT-ir neurons (arrowheads) in each AL project dorso–posteriorly toward the central complex (CC). **(D)** The 5HT-ir neurons (arrowheads) extend past the central complex and cross the dorsal midline before project anteriorly (arrows) toward the ALs. All scale bars = 50 μm.

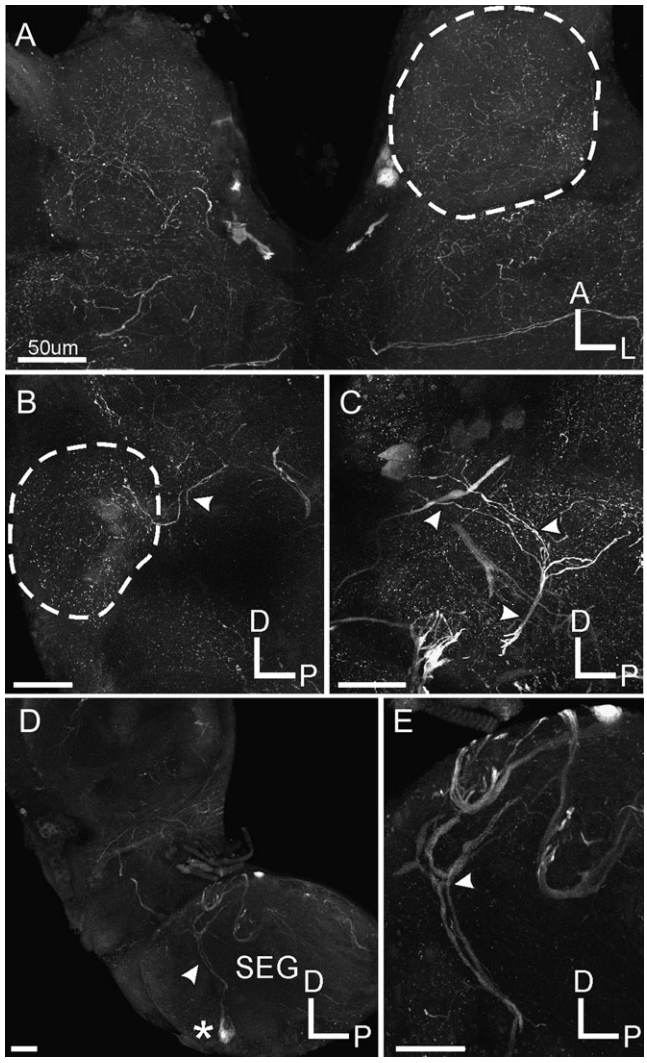

Figure 6 Octopamine-immunoreactive innervation of the ALs of *Neodiprion*. **(A)** Horizontal section revealing sparse innervation of the ALs by OA-ir neurites. **(B)** Saggital section in which OA-ir processes (arrowheads) can be seen entering the AL. Hatched border delineates the AL in (A) and (B). **(C)** Saggital section in which OA-ir processes (arrowheads) that innervate the AL project up from the SEG. **(D)** Saggital section of the SEG depicting projections of 2 VUM neurons (asterisk) toward the deep and superficial DUM neuron tracts. (E) Higher magnification view of (D) highlighting the bifurcation (arrowheads) of the VUM neurons around the esophageal foramen. All scale bars = 50 μm. A, anterior; D, dorsal; L, lateral; P, posterior.

features of the AL of *Neodiprion* were similar to those of the ALs of moths.

Comparing first the input to the glomeruli, we found that the ORN axons of *Neodiprion* did not form distinct tracts (Figure 2A) as in bees and ants and appeared to occupy the majority of the glomerular volume (Figure 2C). This was only slightly different than *Manduca* in which the ORNs occupy the distal half of each glomerulus to form a "cap" over the proximal glomerular volume (Oland et al. 1990). The ORNs of the Aculeata, on the other hand, form distinct tracts, 4 for honeybees (Suzuki 1975; Flanagan and Mercer 1989; Kirschner et al. 2006) and 6–7 for ants (Zube et al. 2008; Zube and Rössler 2008; Kelber et al. 2010) that innervate specific subsets of glomeruli and occupy the outer rind of each glomerulus (Flanagan and Mercer 1989), with the exception of the glomeruli innervated by the T4 ORN tract of the honey bee (Galizia et al. 1999; Kirschner et al. 2006). Another hallmark of the ALs of the aculeate Hymenoptera is a high number of glomeruli compared with other species. For instance, honeybees have 163 glomeruli (Galizia and Rössler 2009) and the ant *Apterostigma* cf. *mayri* has 630 glomeruli (Kelber et al. 2009), compared with 43 in Drosophila

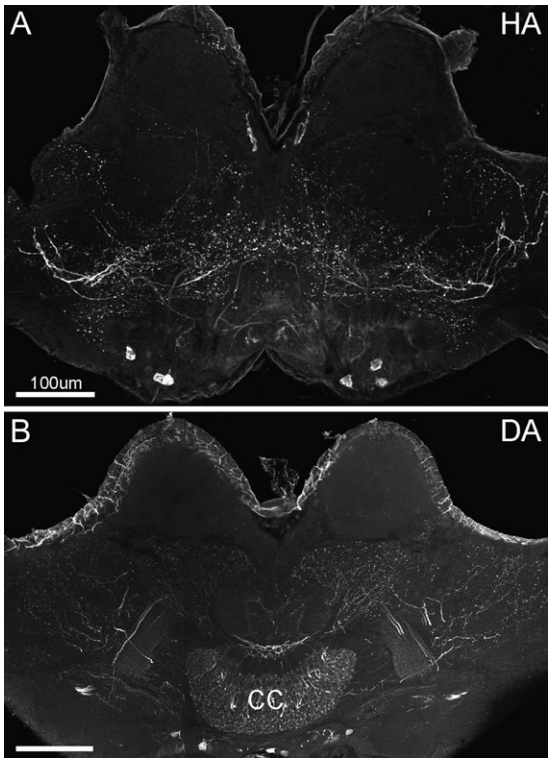

Figure 7 The ALs of *Neodiprion* receive neither histamine- nor dopamine-immunoreactive innervation. **(A)** Horizontal section through the entire brain of *Neodiprion* labeled for histamine. Despite labeling in other brain regions, the ALs do not display any histamine-immunoreactive innervation. **(B)** Horizontal section of a brain labeled for dopamine. As with histamine, there was labeling in other brain regions (notably the central complex; CC), but the ALs were devoid of any dopamine-immunoreactive innervation. All scale bars = 100 μm.

(Stocker et al. 1990; Laissue et al. 1999), 63 in *Manduca* (Rospars and Hildebrand 1992), and 44 in *Neodiprion*.

The output tracts of the PNs of *Neodiprion* also strongly resembled those of *Manduca* (Homberg et al. 1988). There were a total of 5 PN output tracts; the medial, lateral, and 3 medio–lateral antenno-protocerebral tracts (mAPT, lAPT, and mlAPT1-3). The PN output tracts of *Neodiprion* were quite similar to moths in terms of their position relative to other brain structures, their relative widths and their final destination (with the exception of the dorsal antenno-cerebral tract of *Manduca*, which only projects to the lateral horn). Notably, the mlAPT2 and mlAPT3 of *Neodiprion* projected to both the mushroom body calyces and the lateral horn, whereas all 3 medial output tracts in honeybees and ants project only to the lateral horn (Abel et al. 2001; Kirschner et al. 2006; Zube et al. 2008). However, it should be noted that only a few fibers from the mlAPT2 tract project to the dorsal base of the calyces, whereas the majority project to the lateral horn. Similar to moths, the calyx volume of *Neodiprion* was mostly occupied by PN axon terminals and lacked the obvious lip, basal ring, and collar region observed in ants and honeybees.

Similar to other insects, the AL LNs of *Neodiprion* express a variety of different transmitters. The role of GABAergic LNs in the AL has been extensively studied in many different species where they play a role in lateral inhibition, coordination of PN synchrony, refinement of the temporal response properties, and gain modulation via presynaptic inhibition (Waldrop et al. 1987; Christensen et al. 1993; MacLeod and Laurent 1996; Christensen et al. 1998a, 1998b; Lei et al. 2002; Perez-Orive et al. 2002; Sachse and Galizia 2002; Wilson and Laurent 2005; Silbering and Galizia 2007; Olsen and Wilson 2008; Root et al. 2008). It is thus hardly surprising that we observed a large number of GABA-ir LNs that innervated all the glomeruli of *Neodiprion* (Figure 4A). We also observed GABA-ir processes in the mAPT suggesting that *Neodiprion* possesses some GABA-ir PNs, which likewise have been observed in *Manduca* (Hoskins et al. 1986; Berg et al. 2009), honeybee (Schäfer and Bicker 1986), and *Drosophila* (Wilson and Laurent 2005). In addition to GABA, there have been a number of studies examining the different expression patterns of neuropeptides among the LNs of the AL (most recently examined by Hofer et al. 2005; Utz and Schachtner 2005; Berg et al. 2007, 2009; Settembrini et al. 2008; Ignell et al. 2009; Carlsson et al. 2010; Kreissl et al. 2010 and reviewed most recently by Nässel and Homberg 2006). Although little is known about the functional roles of neuropeptides in the AL, *Drosophila* tachykinin-related peptides have been shown to mediate presynaptic inhibition of ORNs in the *Drosophila* AL (Ignell et al. 2009). The distribution of neuropeptides in the AL of *Neodiprion* was very similar to those reported in other insects with the exception of allatostatin which was absent from the AL, although there was intense labeling of neurons in other brain regions. This was particularly interesting as both honeybees (Kreissl et al. 2010) and moths (Berg et al. 2009) possess allatostatin-ir LNs that coexpress GABA-ir, suggesting that this trait was lost in *Neodiprion*.

In addition to GABAergic LNs, the aculeate and parasitic Hymenoptera also possess a population of HA-ir AL LNs (Bornhauser and Meyer 1997; Dacks et al. 2010), which are thought to provide ionotropic (Roeder 2003) inhibitory input within the honeybee AL (Sachse et al. 2006). The ALs of both *Neodiprion* and the sawfly *Lophyrotoma zonalis* (Dacks et al. 2010) lack HA-ir LNs, which is similar to the ALs of moths (Homberg and Hildebrand 1991) and flies (Pollack and Hofbauer 1991; Buchner et al. 1993; Python and Stocker 2002). The ALs of other insects are innervated by one or a few HA-ir neurons (Pirvola et al. 1988; Bornhauser and Meyer 1997; Loesel and Homberg 1999; Gebhardt and Homberg 2004; Dacks et al. 2010), but these neurons do not resemble the large population of HA-ir LNs in the Hymenoptera. The absence of HA-ir LNs in the sister taxa of the Hymenoptera suggests that this population of LNs arose after the divergence of the sawflies. The presence of an additional population of inhibitory LNs in the Hymenoptera indicates the need for increased computational

complexity that may relate to the complexity of the life history or perhaps the large relative number of glomeruli per AL.

Aminergic input to the insect AL is frequently of centrifugal origin. These centrifugal neurons are often thought to signal the broad context of a behavioral state and thus extend widely throughout the AL. The ALs of many insects receive 5HT-ir input (Kent et al. 1987; Rehder et al. 1987; Salecker and Distler 1990; Wegerhoff 1999; Hill et al. 2002; Python and Stocker 2002; Settembrini and Villar 2004; Dacks et al. 2006; Tsuji et al. 2007; Zhao and Berg 2009). Although the majority of the complete metamorphosing insects possess AL 5HT-ir neurons with the CSD morphology in which the soma resides in the lateral cell cluster of one AL and the neuron projects to the contralateral AL, the aculeate and parasitic Hymenoptera do not (Rehder et al. 1987; Dacks et al. 2006; Tsuji et al. 2007). The ALs of the Parasitica are innervated by a 5HT-ir neuron that is similar to the CSD neuron but does not cross the dorsal midline (Dacks et al. 2006), and the AL of the Aculeata are innervated by a 5HT-ir neuron that originates in the ventral nerve cord (Rehder et al. 1987; Dacks et al. 2006; Tsuji et al. 2007). However, *Neodiprion* possesses 5HT-ir neurons with the CSD morphology, suggesting that the role of 5HT in the AL of *Neodiprion* may be similar to moths and flies, which also possess neurons with the CSD morphology. Serotonin enhances the responses of PNs and LNs in both moths (Kloppenburg and Hildebrand 1995; Kloppenburg et al. 1999; Hill et al. 2003; Dacks et al. 2008; Barrozo et al. 2010) and *Drosophila* (Dacks et al. 2009), and 5HT has been implicated as a circadian modulator of olfactory processing (Linn and Roelofs 1986; Linn et al. 1994; Kloppenburg et al. 1999; Gatellier et al. 2004). The remarkable change in the morphology of the 5HT-ir neurons innervating the ALs of honeybees, suggests that the role of 5HT in the hymenopteran AL also may have changed dramatically.

The role of OA in the AL has been a matter of great interest over the last 20 years. Octopamine is thought to serve as the molecular signal for the occurrence of an appetitive stimulus during olfactory conditioning in several insects (Mercer and Menzel 1982; Hammer and Menzel 1998; Farooqui et al. 2003; Schwaerzel et al. 2003; Unoki et al. 2005; Tomchik and Davis 2009; Gervasi et al. 2010), and the ALs of several insect species are innervated by OA-ir VUM neurons (Kreissl et al. 1994; Dacks et al. 2005; Sinakevitch et al. 2005; Sinakevitch and Strausfeld 2006; Schröter et al. 2007; Busch et al. 2009; Busch and Tanimoto 2010). However, we were unable to definitively determine the source of OA-ir innervation for the AL of *Neodiprion* beyond an origin in the SEG.

Although not as well studied as serotonin and octopamine, DA-ir has been reported in the ALs of bees (Kirchhof et al. 1999), flies (Chou et al. 2010), and moths (Homberg 1990). Dopamine-ir was not, however, observed in the ALs of *Neodiprion* (Figure 7B). Unfortunately because the morphology of the DA-ir AL neurons has not been characterized in moths, we could not compare these neurons with those of honeybees to determine if this trait was lost after *Neodiprion* diverged from the rest of the Hymenoptera or if this trait was lost in the most ancestral hymenopteran and regained after the sawflies diverged.

Any given morphological feature of an organism is shaped by the selective pressures of the environment and by the historical inertia of ancestral traits. Although *Neodiprion* is a member of the same order as honeybees and ants, their ALs more closely resemble those of the moths in terms of ORN innervation (Figure 2) and PN output tracts (Figure 3) as well as the serotonergic (Figure 5) and histaminergic (Figure 7A) innervation of the AL are very similar to that of moths. Furthermore, those features of the *Neodiprion* AL that differ from moths, the absence of allatostatin-ir LNs (Figure 3E), and dopaminergic innervation (Figure 7B), also differ from honeybees suggesting that these traits were lost. This supports the hypothesis that both similarity of life history and phylogenetic relatedness contribute to brain structure. Our results suggest that because the life history of sawflies is so similar to that of moths, there has been little selective pressure exerted upon the nervous system to change the gross anatomical features of the olfactory system compared with their last common ancestor. In comparison, the aculeate Hymenoptera evolved radically different life histories compared with the sawflies and thus, their olfactory systems have also changed dramatically. By examining how the brain has changed over evolutionary time, we can begin to understand what neural features are critical to information processing and which have become malleable to best suit the selective pressures exerted upon an organism.

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