Published in final edited form as:

J Comp Neurol. 2010 August 1; 518(15): 2917–2933. doi:10.1002/cne.22371.

Histamine-immunoreactive local neurons in the antennal lobes of the Hymenoptera

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Abstract

Neural networks receive input which is transformed before being sent as output to higher centers of processing. These transformations are often mediated by local interneurons (LNs) that influence output based on activity across the network. In primary olfactory centers, the LNs that mediate these lateral interactions are extremely diverse. For instance, the antennal lobes (ALs) of bumble bees possess both GABA and histamine-immunoreactive (HA-ir) LNs, and both are neurotransmitters associated with fast forms of inhibition. Although the GABAergic network of the AL has been extensively studied, we sought to examine the anatomical features of the HA-ir LNs in relation to the other cellular elements of the bumble bee AL. As a population, HA-ir LNs densely innervate the glomerular core while sparsely arborizing in the outer glomerular rind, overlapping with the terminals of olfactory receptor neurons. Individual fills of HA-ir LNs revealed heavy arborization of the outer ring of a single “principal” glomerulus and sparse arborization in the core of other glomeruli. In contrast, projection neurons, and GABA-immunoreactive LNs project throughout the glomerular volume. To provide insight as to the selective pressures that resulted in the evolution of HA-ir LNs, we determined the phylogenetic distribution of HA-ir LNs in the AL. HA-ir LNs were present in all but the most basal hymenopteran examined, although there were significant morphological differences between major groups within the Hymenoptera. The ALs of other insect taxa examined lacked HA-ir LNs, suggesting that this population of LNs arose within the Hymenoptera and underwent extensive morphological modification.

Keywords

insect brain; Hymenoptera; histamine; olfaction

Introduction

Lateral interactions mediated by local interneurons (LNs) are ubiquitous in neural networks. In the olfactory system, lateral interactions refine, modify, and transform odor information from the environment before sending the processed information as output to higher order processing centers (Christensen et al., 1998a; Laurent, 1999; Urban, 2002; Stopfer, 2005; Kay and Sherman, 2007). In insects, olfactory receptor neurons (ORNs) on the antennae detect specific suites of odors and send this information to the primary olfactory neuropil in the insect brain, the antennal lobe (AL). In the AL, ORN axonal terminals innervate spheroidal neuropil called glomeruli. Glomeruli also house the neurites of projection output

neurons (PNs), LNs and a variety of centrifugal neurons (See reviews: Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999; Ache and Young, 2005; Vosshall and Stocker, 2007). In the insect AL, most of the lateral interactions studied are mediated by a network of GABA-immunoreactive (GABA-ir) LNs (Hoskins et al., 1986; Schäfer and Bicker 1986; Homberg et al 1987, 1990; Distler 1990; Malun, 1991; Distler and Boeckh, 1997; Wegerhoff 1999; Python and Stocker, 2002; Wilson and Laurent, 2005; Seki and Kanzaki 2008). These neurons mediate lateral suppression, coordination of PN synchrony, refinement of the temporal response properties of PNs and modulation of gain control (Waldrop et al., 1987; Christensen et al., 1993; Macleod and Laurent 1996; Christensen et al., 1998b; Lei et al 2002; Perez-Orive et al., 2002; Sachse and Galizia, 2002; Wilson and Laurent, 2005; Olson and Wilson 2008; Root et al., 2008). This assortment of different lateral interactions is likely possible because LNs in the AL are morphologically diverse (Flanagan and Mercer, 1989; Christensen et al., 1993; Fonta et al., 1993; Wilson and Laurent et al., 2005; Seki and Kanzaki, 2008; Husch et al., 2009) and contain a variety of neurotransmitters (Shang et al., 2007; Seki and Kanzaki, 2008; Husch et al., 2009) and neuropeptides (most recently studied in Hofer et al., 2005; Berg et al., 2007; Settembrini et al., 2008; Utz et al., 2008 and reviewed in Nassel and Homberg, 2006). This anatomical and immunohistochemical diversity suggests that there are multiple functional sub-populations of LNs and that interglomerular interactions are neither uniform nor reciprocal (Linster et al., 2005; Reisenman et al., 2005, 2008; Olsen et al., 2007; Silbering et al., 2007).

The lateral interactions exerted within the AL differ across species. For instance, the honey bee AL contains a large number of GABA-ir (Schäfer and Bicker, 1986) and histamine-immunoreactive (HA-ir) LNs (Bornhauser and Meyer, 1997). However, the ALs of other insect species examined receive either little or no HA-ir innervation (Homberg and Hildebrand, 1991; Pollack and Hofbauer, 1991; Buchner et al., 1993; Python and Stocker, 2002), or HA-ir innervation from centrifugal neurons or from a few LNs (Pirvola et al., 1988; Bornhauser and Meyer, 1997; Loesel and Homberg, 1999; Nässel, 1999; Gebhardt and Homberg, 2004).

In the insect nervous system histamine acts as a fast inhibitory neurotransmitter. Two histamine-gated chloride channels have been described thus far in *Drosophila* (Gisselmann et al., 2001; Gengs et al., 2002; Zheng et al., 2002; Roeder, 2003; Pantazis et al., 2008), for which homologues have been described in other insect species (Iovchev et al., 2006) and histamine also activates a chloride conductance in ORNs of the spiny lobster olfactory lobe (McClintock and Ache, 1989). Indeed, in the honey bee AL, histamine completely suppresses odor-evoked responses (Sachse et al. 2006), and the application of the GABA-A receptor antagonist picrotoxin augments inhibition in some PNs (Sachse and Galizia, 2002). These data suggest that multiple forms of fast inhibition exist within the honey bee AL, one of which could be mediated by histaminergic LNs. Pharmacological blockade of HA receptors in the honey bee AL significantly impaired olfactory interference in learning assays (Dacher and Smith, 2008) further highlighting the role of HA-ir LNs in olfactory processing.

In order to shed light on the roles of these two populations of LNs (HA-ir and GABA-ir), we examined the anatomy and morphology of HA-ir LNs in relation to other cellular elements within the AL. As a population, the HA-ir LNs projected densely to the glomerular core and sparsely to the outer glomerular rind to which the ORNs project from the antennae. On an individual basis, single HA-ir LNs project sparsely to the core of many glomeruli and more extensively to the outer rind of a single glomerulus. We also examined this population of HA-ir LNs during several pupal stages and found that there is no HA-ir labeling in the AL during early pupal development, but is present in the ALs of later pupal stages. We also characterized the phylogenetic distribution of HA-ir LNs in the ALs of the Hymenoptera.

While this population of LNs was present within the stinging Hymenoptera examined, a morphologically different population of HA-ir LN was present in the parasitoid Hymenoptera and no HA-ir LNs were present in the most basal Hymenoptera or the sister taxa to the Hymenoptera.

Methods

Animals

Among the Hymenoptera, HA-ir in the AL has only been examined in honey bees. In this study we used the closely related bumble bee *Bombus impatiens* due to its greater robustness allowing a higher success rate for intracellular dye injection of single AL neurons. Many insects used in this study (see Table 1 for a complete list of species) were caught in Arizona and identified using the University of Arizona Entomology Department insect collection. *B. impatiens* specimens were obtained from Biobest Biological Systems (Belgium); *Odontotaenius disjunctus* and *Hippodamia convergens* were obtained from Carolina Biological Supply Co. (Burlington, NC). The ant species from the *Pheidole* genus were provided by Ming Huang in the laboratory of Dr. Diana Wheeler at the University of Arizona, the *Tremex columba* were provided by Dr. Boris Kondratieff of Colorado State University, the *Comperia merceti* were provided by Cara Gibson in the laboratory of Dr. Molly Hunter at the University of Arizona, the *Macrocentrus grandii* were provided by Dr. Jen White at the University of Kentucky and the *Lophyrotoma zonalis* were caught and processed in Brisbane, Australia. All phylogenies, except for a recently proposed phylogeny for the Holometabola (Savard et al. 2006) depicted in Figure 7B, are based on those provided by Grimaldi and Engel (2005). All insects collected in the field were identified at least to genus. Pupal staging was based on the staging scheme developed for the honey bee by Groh and Roesler (2008) which divides animals into pre-pupae and pupal stages 1–9 (P1–P9) based on the amount of pigmentation in the eyes, the extent of cuticular melanization and wing curling.

Immunocytochemistry

All brains were prefixed in 4% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma, St. Louis, MO) in de-ionized water (4 hours at 4°C or 2 hours at room temperature) and then fixed overnight in 4% paraformaldehyde at 4°C. Brains were embedded in 5% low melting point agarose (Sigma, St. Louis, MO), except for whole mount preparations of *Pheidole* sp. and *Comperia merceti*, and sectioned at 50–100µm with a vibrating microtome (Technical Products International, St. Louis, MO). Sections were then washed in PBS (pH 6.9) with 0.5% Triton X-100 (PBST), blocked in PBST with 2% IgG-free bovine serum albumin (BSA; Jackson ImmunoResearch, West Grove, PA) during at least one hour, and incubated overnight with primary antibody in PBST (1% Triton X-100) with BSA and 0.05% sodium azide. Both the rabbit anti-histamine antibody (Immunostar, Hudson, WI; catalog # 22939) and the mouse anti-GABA antibody (Abcam, Cambridge, MA; catalog # ab49675) were used at a concentration of 1:500.

Following incubation in primary antibody, the tissue was washed in PBST, blocked as described above and then incubated overnight at room temperature with either goat anti-rabbit Cy3 (Jackson ImmunoResearch, West Grove, PA) for single labels of histamine, or with 1:500 goat anti-rabbit FITC (Sigma) for simultaneous labeling of histamine and single/mass fills of neurons. For double labels of histamine and GABA, tissue was incubated in 1:500 goat anti-rabbit FITC and 1:500 goat anti-mouse Cy5 (Jackson ImmunoResearch, West Grove, PA). The next day tissue was washed, cleared via an ascending series of glycerol washes (40%, 60% and 80% glycerol in water) and then mounted on glass slides in 80%

glycerol. FITC incubated preparations were cleared and mounted in carbonate buffered glycerol (pH 9.0) to prevent fading.

Antibody Characterization

The histamine antiserum was raised against synthetic histamine conjugated to succinylated keyhole limpet hemocyanin (KLH) with carbodiimide and exhibited no cross-reactivity to L-histidine or L-histidine containing peptides (Immunostar Inc. histochemical histamine antiserum specification sheet). The fact that the histamine immunolabeling in the medulla of *B. impatiens* is very similar to that observed in *Drosophila* (Melzig et al., 1996; Paulk et al., 2009), and that histamine-immunoreactivity was completely eliminated in *Drosophila* null lines for the gene encoding histidine carboxylase (Melzig et al., 1996) further indicates that this antibody is highly specific. A pre-adsorption control in which the HA antiserum was incubated overnight in .2mg/ml KLH (sigma, catalog # H7017) resulted in no change in the HA-ir labeling (supplemental figures 1A, B). The specificity and activity of the mouse monoclonal anti-GABA antibody was evaluated by Abcam and was found to have no cross-reaction with L-a-aminobutyric acid, L-glutamic acid, L-aspartic acid, glycine, d-aminovaleic acid, L-threonine, L-glutamine, taurine, putrescine, L-alanine and carnosine and had weak cross-reactivity with β -alanine and ϵ -aminocaproic acid (Abcam product information sheet). Conjugated GABA-BSA was used as the immunogen for the production of the mouse anti-GABA antibody (Abcam product information sheet). Pre-adsorption controls in which the mouse anti-GABA antibody was incubated overnight at 4°C in 2% BSA (Supplemental figure 1C) did not affect labeling, while incubation under the same conditions in 100mM GABA eliminated all labeling (Supplemental figure 1D).

Mass and intracellular fills

ORNs were mass filled by opening the head capsule of adult *B. impatiens* and stabbing the antennal nerve with the tip of a broken glass electrode coated in dextran Texas Red, 3000MW (Molecular Probes, Invitrogen, Carlsbad, CA). The head capsule was then resealed with petroleum jelly and dye was allowed to diffuse overnight at 4°C. For *Ammophila bellula*, *Pogonomyrmex rugosus* and *Macrocentrus grandii* the antennae were cut after the most proximal flagellomere and dye was placed in the opening which was then sealed with vasoline. Brains were dissected and processed for histamine immunocytochemistry as described above. The same procedure was followed for mass fills of PNs, except that the glass electrodes were inserted into the medial and lateral antennocerebral tracts to inject dye.

For the labeling of single neurons, bumble bees were dissected and prepared for intracellular recording using established procedures (Paulk et al., 2008). A total of 106 bees (or 212 ALs) were used for intracellular fills and the number of neurons from the different morphological types are detailed in Table 2. Sharp microelectrodes were made from borosilicate glass capillaries with filament (1 mm outer diameter, 0.58 or 0.75 mm internal diameter, Sutter Instruments Co., Novato, CA) on a laser puller (P-2000, Sutter Instruments Co., Novato, CA). The tip of the micropipette was filled with a solution of Alexa Fluor 568 hydrazide (10 mM in 200 mM KCl, Molecular Probes, Eugene, OR), and the shaft, with 2 M LiCl. Brains were then processed for histamine immunocytochemistry as described above. We attempted to fill primarily LNs, which, in moths, can be readily distinguished from PNs in intracellular recordings prior to dye injection on the basis of one or more of the following properties: spikes with larger width at half amplitude than those of LNs, and firing regularity (unpublished observations and Christensen et al. 1993). Because this biased our sample of filled AL neurons we do not attempt to make any estimates on the relative proportions of different cell types present in the AL based on the relative proportions of different cell types that we filled. Furthermore, although the 6 HA-ir LNs intracellularly filled in this study were morphologically similar, this does not preclude the possibility that there is a greater

morphological diversity of HA-ir LN present in the hymenopteran AL, and that we simply did not manage to fill other morphological types of HA-ir LNs in *B. impatiens*.

Confocal Microscopy

Images were collected with 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 image stacks and to adjust contrast and brightness. Images were inverted (from white on black to black on white) and organized in CorelDraw 9.0. For cell body counts, brains from 13 bumblebees (5 labeled for HA, 8 labeled for HA and GABA) and 6 honey bees were processed as described above and sectioned at 50 μ m; individual cell bodies were marked using the Zeiss LSM browser. Cell body counts were performed twice for each section. The illustrations in figures 3AA and AB were produced by tracing the branching patterns of an individually filled HA-ir neuron. The branching patterns depicted show only the arborizations observed in a 100 μ m section of the AL and not all overlying branches.

Results

Anatomical characteristics of HA-ir LNs within the AL of *Bombus impatiens*

As a population, the HA-ir LNs in the ALs of *B. impatiens* innervated most if not all of the AL glomeruli and branched extensively both within the glomeruli and the coarse neuropil of the AL (Fig. 1A). HA-ir cell bodies were found only in the lateral cell cluster of neuronal cell bodies, with an average of 310 ($\pm$ 57 standard deviation, n=13) HA-ir cell bodies per AL. This was much higher than the ~35 cell bodies per AL reported by Bornhauser and Meyer (1997) in the honey bee, so we also counted the number of HA-ir cell bodies in honey bees and found an average of 263 ($\pm$ 23 standard deviation, n=6) HA-ir cell bodies per AL (Supplemental Fig. 1E; depicts a cluster of HA-ir cell bodies in one of eight 50 μ m sections that contain the lateral cell cluster of the AL). Most HA-ir neurites were restricted to the proximal core of the glomeruli and had a large number of prominent bleb-like structures within each glomerulus (Fig 1A, B). There were, however, some finer HA-ir branches in the distal glomerular rind (Fig. 1B). Each glomerulus was innervated by 1–3 thick HA-ir processes and many very thin HA-ir processes (Fig. 1B), suggesting that each glomerulus receives input from multiple HA-ir LNs. The extent of innervation by individual HA-ir LNs, however, is likely variable as the glomeruli are innervated by neurites of different diameters.

Anterograde fills of the antennal nerve revealed very little overlap between the olfactory receptor neurons (ORNs) and the HA-ir LNs (Fig. 1C–E). The ORNs only innervated the distal glomerular rind which received only sparse HA-ir innervation (Fig. 1F–H). Mass retrograde fills (Fig. 1I–K) and individual fills of PNs (Fig. 1L–N) revealed that in contrast to the HA-ir LNs, PNs arborized throughout the glomerulus, presumably overlapping with ORN axon terminals. Furthermore, GABA-ir was present throughout the AL of *B. impatiens* (Fig. 1O). However, unlike the HA-ir LNs, GABA-ir LNs arborized throughout the entire glomerular volume (Fig. 1P). There was very little overlap ($.7\% \pm .5\%$ of HA-ir neurons also had weak GABA-ir) between the GABA-ir and HA-ir cell bodies (Fig 1Q–S), indicating that these immunoreactivity profiles represent two relatively distinct populations of LNs.

To determine if HA-ir LNs belong to a distinct morphological class of LN, we intracellularly filled single AL neurons with dye and subsequently processed the brains for HA-ir (n=89 from 106 bees, of which 6 were HA-ir). The types of AL neurons filled are summarized in Table 2. Two general morphological types of AL-LNs have been described in honey bees; homogenous LNs (“homo-LNs”) that project uniformly in many glomeruli, and heterogeneous LNs (“hetero-LNs”) that branch extensively in one or a few glomeruli

(hereafter referred to as “principal glomeruli”) and sparsely in other glomeruli (Flanagan and Mercer, 1989). We filled both non-HA-ir homo-LNs (Fig. 2A–E) and hetero-LNs (Fig. 2F–O) although there was quite a bit of morphological variability within the hetero-LNs, as previously demonstrated by Flanagan and Mercer (1989). The processes of homo-LNs (Fig. 2C–E) appeared to be thinner than those of the hetero-LNs (Fig. 2H–J) and branched more extensively throughout the entire volume of any given glomerulus. In contrast, the processes of hetero-LNs not projecting into the principal glomerulus tended to be restricted to the inner core of non-principal glomeruli (Fig. 2H–J). Some hetero-LNs projected to only a small subset of non-principal glomeruli while other hetero-LNs extended into many more glomeruli. Although all hetero-LNs produced a thick neurite that innervated the principal glomerulus, there was a large amount of variability in the projection patterns of hetero-LNs within their principal glomeruli (Fig. 2K–O). Some hetero-LNs projected throughout both the core and the rind of the principal glomerulus (Fig. 2K), whereas others branched more extensively in the outer rind of the principal glomerulus (Fig. 2L–N). There were also several hetero-LNs that projected unevenly within their principal glomerulus (Fig. 2M, N) and one hetero-LN projected into two principal glomeruli (Fig. 2O). Thus, hetero-LNs are not morphologically uniform, but rather constitute a highly diverse group of neurons.

We intracellularly filled six HA-ir LNs, all of which branched most extensively in a single principal glomerulus and sparsely in many other glomeruli (Fig. 3, SFig. 2), demonstrating an arborization pattern consistent with that of hetero-LNs (Fig. 3A–C). Figures 3G–Z depict rotated views of 3-D projections of the optical stacks of all 6 HA-ir neurons. Interestingly, all six HA-ir LNs sparsely innervated the inner core of most glomeruli with fine branches (Fig. 3D–F), but innervated the principal glomerulus with a very thick neurite that produced fine branches that were largely (although not completely) restricted to the outer rind (Fig. 3D–F). Furthermore, there was variation in the extent to which the branches of the HA-ir LNs were restricted to the glomerular rind of the principal glomerulus (Fig. 3G–Z and SFig. 2).

At first consideration the morphology of the individual HA-ir LNs appears to contradict our observations about the pattern of HA-ir innervation of glomeruli. The overall immunocytochemical labeling pattern of the HA-ir LNs as a population more densely innervated the glomerular core compared to the rind, however single HA-ir LNs projected sparsely to the core of most glomeruli and primarily to the rind of their principal glomerulus. However, based on the observation that all hetero-LNs filled in this study innervate their principal glomerulus with a single branch that is thicker than for other glomeruli (Fig. 2) and each glomerulus is innervated by between 1–3 thick HA-ir branches (Fig. 1B), we can assume that each glomerulus serves as the principal glomerulus for 1–3 HA-ir LNs. Each glomerulus is also innervated by hundreds of thin HA-ir neurites from the other HA-ir LNs. Thus, when all of the HA-ir LNs are considered, only 1–3 HA-ir neurons contribute to the HA-ir innervation of the glomerular rind, whereas upwards of hundreds of HA-ir innervate each glomerular core. This produced the overall effect of a denser HA-ir innervation of the core relative to the rind, when HA immunocytochemistry was considered. The neurites of the individually filled HA-ir neurons in their principal glomerulus spatially overlap with the volume occupied by the axon terminals of the ORNs (Fig. 1C–H) suggesting that the HA-ir LNs may receive input from ORNs in their principal glomerulus. Sequential electrophysiological and optophysiological recordings in honey bees have also suggested that the principal glomerulus is the glomerulus to which hetero-LNs receive ORN input (Galizia and Kimmerle, 2004).

HA-ir in the AL of the developing bumble bee

During metamorphosis the AL undergoes dramatic reconstruction. To determine if the HA-ir LNs play a role larval olfaction we examined ALs of late larval *B. impatiens* and found no

HA-ir in the AL (Fig. 4A). Although there were HA-ir neurons within the brain of late-larval and early pupal (stage P1 of 9) *B. impatiens*, the ALs did not possess any HA-ir LNs, nor do they receive any HA-ir innervation from neurons outside the AL (Fig. 4A and B respectively). However, by stage P8 HA-ir LNs are present in the AL and innervate glomeruli (Fig 4C). This indicates that HA-ir LNs either develop during metamorphosis or that a population of LNs already present in the AL begins to express HA-ir.

The phylogeny of HA-ir LNs in the ALs of the Hymenoptera

The presence of a large population of HA-ir LNs in the AL is a trait that, among those insects investigated thus far, appears to be unique to bees. Other holometabolous (complete metamorphosing; Fig. 8) insects examined have no HA-ir in their ALs (*Manduca*; Homberg and Hildebrand, 1991 and *Drosophila*; Pollack and Hofbauer, 1991; Buchner et al., 1993; Python and Stocker, 2002), and the ALs of some insect species are innervated by either a very small number of HA-ir LNs (cockroaches: Pirvola et al., 1988; Loesel and Homberg, 1999, locust: Gebhardt and Homberg, 2004) or by centrifugal HA-ir neurons (Cricket: Bornhauser and Meyer, 1997). To determine at what point the population of HA-ir LNs in the bee AL arose in the evolutionary history of the insects, we examined various different insects from taxa both within and outside the Hymenoptera.

The bees are members of the Aculeata which are those Hymenoptera that possess an ovipositor that has been modified into a stinger. The Aculeata are divided into three superfamilies; Apoidea (bees), Vespoidea (wasps and ants) and Chrysidoidea (Fig 5A). *B. impatiens* and *A. mellifera* belong to a single family within the Apoidea, so we first examined other members of the Apoidea and then looked at increasingly distantly related groups of hymenopteran insects. Similar to *B. impatiens*, the ALs of *Cerceris convergens* (Crabronidae) also possessed HA-ir LNs that densely innervated the majority of the glomeruli (Fig. 5B). In the ALs of *C. convergens* and the thread-waisted wasp *Ammophila bellula*, many of the antero-ventral glomeruli receive little to no HA-ir innervation (Fig. 5C–E). Anterograde fills of the antennal nerve of *A. bellula* delineate the borders of the glomeruli not innervated, and for those glomeruli that do receive HA-ir innervation there is a lack of overlap between the ORN axons and the neurites of the HA-ir LNs (Fig. 5F–H) reminiscent of the pattern observed for *B. impatiens*. Similarly, while in *Prionix* sp. (Sphecidae) many AL glomeruli receive dense HA-ir innervation (Fig. 5I), some of the more antero-ventral glomeruli were only sparsely innervated (Fig. 5I inset). Thus, there were species-specific differences among the Apoidea in the uniformity of glomerular innervation by HA-ir LNs, although, there was a large amount of variability in the number and location of glomeruli lacking HA-ir across species. However, all apoid species examined possessed a large population of HA-ir LNs that, as a population densely innervated the glomerular core, while sparsely innervating the outer rind.

The AL glomeruli of all examined specimens in the Vespoidea superfamily were also densely innervated by HA-ir LNs. For instance, in ants of the genus *Pheidole*, the AL glomeruli receive dense HA-ir innervation similar to that observed in the Apoidea (Fig. 5J). It should be noted that although the glomeruli of this *Pheidole* species are much smaller than those of the other aculeate species examined, they also received dense innervation of the glomerular core and sparse innervation of the rind. This indicates that glomerular size does not determine the uniformity of HA-ir LN glomerular innervation density. The restriction of HA-ir neurites to the glomerular core was especially pronounced in the ant *Pogonomyrmex rugosus* (Fig. 5K) and the paper wasp *Polistes fuscatus* (Fig. 5O). Furthermore, anterograde fills of the antennal nerve of *P. rugosus* demonstrate a pattern of separation between the ORNs and HA-ir LNs (Fig. 5L–N) similar to that observed for *B. impatiens* and *A. bellula*. Thus, a pattern of densely branching HA-ir AL LNs with neurites mostly restricted to the core of the glomeruli appears to be characteristic of both the Apoidea and the Vespoidea.

The Apocrita is a sub-order within the order Hymenoptera that contains the Aculeata (described above) and the Parasitica (a paraphyletic group containing several superfamilies) (Fig. 6A). While the ALs of the parasitic Hymenoptera examined had HA-ir LNs, the morphology of these LNs were very distinct from those of the Aculeata. The ALs of the Ichneumonoid parasitic wasps of the genus *Zelomorpha* (Fig. 6B) and *Eiphosoma pyralidis* (Fig. 6C) as well as those of the Chalcidoid wasp (Proctotrupomorpha; Fig. 6D) possessed HA-ir LNs that innervate the glomeruli much more sparsely and with very large bleb-like structures. These HA-ir LNs also branched sparsely within the coarse neuropil of the AL (Fig. 6B–D) and appear to innervate the entire glomerular volume (Fig. 6B and D insets). To verify that the neurites of HA-ir LNs in the ALs of parasitoid wasps project throughout the glomerular volume we performed backfills from the antennal nerves of *Macrocentrus grandii* and labeled for HA-ir (Fig. 6E). As observed in the Aculeata, the ORN axons of *M. grandii* are restricted to the outer rind of the glomerulus (Fig. 6F–H). However unlike the Aculeata, the neurites of the HA-ir LNs in the AL of *M. grandii* project throughout the glomerular volume spatially overlapping with the ORN axons (Fig. 6F–H).

We then examined the ALs of two hymenopteran species within the paraphyletic group, the Symphyta, which are the sister taxa to the Apocrita (Fig. 6I). The ALs of the horntail *Tremex columba* (Siricidae) were sparsely innervated by large bleb-like structures of HA-ir LNs (Fig. 6J, K), similar to the ALs of the Parasitica (Fig. 6B–D). However, the ALs of the sawfly *Lophyrotoma zonalis* (Pergidae) did not contain HA-ir (Fig. 6L). Because the sawflies are the most primitive among the true Hymenoptera, the lack of HA-ir in their ALs suggests that either the population of HA-ir LNs evolved within the Hymenoptera after the divergence of the sawflies, or that this trait is present in the sister taxa of the Hymenoptera but was lost by the sawflies. To test this, we examined the HA-ir in the ALs of representatives from sister taxa of the Hymenoptera. It has been widely accepted that the Coleoptera (beetles) and the Neuropteroids (lacewings, antlions, etc.) were the sister taxa and most basal of the holometabolous (complete metamorphosing) insects (Fig. 7A). However, a recent study by Savard et al (2006) suggests that the Hymenoptera are the most basal order of holometabolous insects (Fig. 7B), which would make the Paraneoptera (a group of hemimetabolous insects which includes the Hemiptera) the most primitive sister taxa to the Hymenoptera. To ensure that we examined appropriate sister taxa despite the current controversies in the phylogeny of the Holometabola, we examined ALs of species from the Coleoptera, Neuroptera and Hemiptera.

The ALs of the Coleoptera were innervated by a single HA-ir centrifugal neuron (Fig. 7C–G) that projected ventrally into the AL (Fig. 7D). The innervation of the AL by this HA-ir neuron was extremely variable depending on species. For instance, in *Odontotaenius disjunctus* this neuron projected to a large portion of the AL (although not to all regions of the AL; Fig. 7C, D). In contrast, the ALs of *Diabrotica undecimpunctata* do not receive any HA-ir innervation (Fig. 7E), and the ALs of *Hippodamia convergens* and a *Calosoma* sp. received intermediate amounts of innervation (Fig. 7F and G, respectively). The ALs of *Chrysoperla rufilabris* (Neuroptera) and *Narnia inornata* (Hemiptera) were devoid of any HA-ir innervation (Fig. 7H and I, respectively) suggesting that the basal state in the Holometabola (regardless of the phylogenetic scheme used; Fig. 7A, B) was a lack of HA-ir LNs. Figure 8 is a schematic representation summarizing the phylogenetic distribution of HA-ir LNs in the insect groups examined in this study. Our data suggests that suggest that the large population of HA-ir LNs observed in the Hymenoptera arose at some point after the divergence of the sawflies (Fig. 8B, C) as this population was not present in the sawflies, nor in any of the sister taxa to the Hymenoptera (Fig. 8D). The population of HA-ir LNs then underwent significant morphological changes in the Aculeata at some point after the split of the Aculeata and the Ichneumonoid wasps (Fig. 8A).

Discussion

In this study we examined the morphological and phylogenetic characteristics of a unique group of histamine immunoreactive (HA-ir) local interneurons (LNs) in the antennal lobes (ALs) of the Hymenoptera. One goal of this study was to determine the phylogeny of this sub-population of LNs. This population of HA-ir LNs was not present in any of the sister taxa to the Hymenoptera or Holometabola examined in this study. In the more basal Hymenoptera, glomeruli received sparse HA-ir innervation from neurites with very large bleb-like structures that appeared to project uniformly throughout each glomerulus (Fig. 6), while the aculeate Hymenoptera examined had HA-ir LNs that were morphologically similar to those of *B. impatiens* with HA-ir innervation very dense within the glomerular core and sparse within the rind. This suggests that a large population of HA-ir LNs either arose or began to express histamine at some point within the Hymenoptera (after the divergence of the sawflies) and then changed in their morphology and potentially connectivity within the aculeate Hymenoptera.

It is likely that the diversity of LNs and lateral interactions that occur within the AL reflect the diversity and complexity of the olfactory world in which insects must navigate. It is therefore important to consider the ecological relevance of any observed changes in neuroanatomical structure that have occurred on an evolutionary timescale. We observed two major morphological changes in the HA-ir population of LNs in this study. The first was the development of a population of HA-ir LNs in the AL of the Hymenoptera at some point after the divergence of the Tenthredinoidea (the majority of the sawflies). The second was the dramatic change in the characteristics of HA-ir innervation within the ALs of Aculeate Hymenoptera. Although it is tempting to ascribe a specific function for these changes based on the lifestyles of individual insect groups, we can only consider the co-occurrences of changes in anatomy and lifestyle to be correlational. For instance, the Aculeata possess a stinger that has been modified from an ovipositor and the glomeruli in their AL have a distinct morphology compared to other Hymenoptera that lack stingers. This, however, does not mean that the function of HA-ir LNs must relate to the possession of a stinger or any associated behaviors. A population of HA-ir LNs in the AL is another trait along with a stinger which is shared by this insect group, but without any evidence for a role of these HA-ir LNs in stinging behavior, we must be cautious in our speculation as to the relationship between these two traits. However, the correlations between different morphological traits provide the basis from which testable hypotheses can be formed about the role of HA-ir LNs in the life history of the Aculeata. The ALs of the Aculeata appear to have undergone extensive morphological alteration after the Aculeata diverged from the rest of the Apocrita. In addition to the morphological differences in HA-ir LNs reported in this study, the ALs of the Aculeata are innervated by serotonergic neurons that are morphologically distinct from those innervating the ALs of other Hymenoptera (Rehder et al., 1987; Dacks et al., 2006). Furthermore, in both honey bees and ants there are two parallel tracts of ORNs that innervate non-overlapping populations of glomeruli in the AL (for a review see Galizia and Rössler, 2009), while backfills of the antennal nerves of *Macrocentrus grandii* (Fig., 6F) did not reveal any distinct tracts (although further study is needed). Thus, the morphological change observed for this population of HA-ir LNs is but one of several AL traits that differ between the Aculeata and parasitic Hymenoptera.

Another major goal of this study was to characterize the general anatomy of the population of HA-ir LNs in relation to the other cellular elements of the bumble bee AL. We found that the glomerular core receives dense innervation from the HA-ir LNs, and only sparse branching in the outer rind of the glomerulus. In contrast, PNs, GABA-immunoreactive (GABA-ir) LNs and individually filled non-HA-ir LNs projected uniformly throughout the glomerular volume. Individually filled HA-ir LNs projected into many different glomeruli,

but primarily innervated the outer rind of a single “principal glomerulus”, while sparsely innervating the core of the other glomeruli. The dense HA-ir labeling observed in the core of each glomerulus is likely the sparse branching of many HA-ir LNs, while the sparse HA-ir branching observed in the outer rind is likely the neurites of just a few HA-ir LNs that are innervating their principal glomerulus. The observation that only 1–3 thick HA-ir branches entered each glomerulus, and that individually labeled HA-ir LNs only produced one thick branch which innervated the principal glomerulus, indicates that each glomerulus is innervated by 1–3 HA-ir LNs. Further, since ORNs project to the outer rind of the glomeruli, it is possible that HA-ir LNs receive direct input from the ORNs terminating in the principal glomerulus. Indeed, in experiments in which the responses of honey bee hetero-LNs were individually filled with Ca^{2+} sensitive dyes, the Ca^{2+} responses (which are presumed to represent post-synaptic Ca^{2+} influx) were observed in the principal glomerulus (Galizia and Kimmerle, 2004).

There is little doubt that AL-LNs mediate lateral interactions that are important for primary olfactory processing. However, the morphological (Flanagan and Mercer, 1989; Christensen et al., 1993; Fonta et al., 1993; Seki et al., 2008; Husch et al. 2009) and immunoreactive diversity of the numerous AL-LNs indicate that lateral interactions are likely more complex than previously thought. For instance, the ALs of *Drosophila* contain both cholinergic and GABAergic LNs (Shang et al., 2007) that respectively mediate excitatory and inhibitory lateral interactions (Root et al., 2007, 2008; Olsen et al., 2007; Silbering et al., 2008; Olsen and Wilson, 2008). In the honey bee there are ca. 1090 PNs (Kirschner et al., 2006) and ca. 3600 LNs (based on the 4704 AL neurons described by Witthoft, 1967). Of these LNs, approximately 750 are GABA-ir (Schäffer and Bicker, 1986) and 260 are HA-ir (see Results). Assuming the honey bee resembles the bumble bee in that GABA-ir and HA-ir LNs are non-overlapping populations (Fig. 1O), then there are approximately 2600 LNs that are distinct from the HA-ir and GABA-ir LNs. Thus, LNs should not be considered as a uniform population, but rather a diverse group of neurons sub-serving diverse functions such as sharpening (e.g. Sachse and Galizia, 2002) and broadening (e.g. Wilson et al., 2004; Bhandawat et al., 2007) of AL glomerular output, PN-PN synchrony (Lei et al., 2002; Perez-Orive et al., 2002), tracking of relevant temporal parameters of the odor stimulus (Christensen and Hildebrand, 1997; Christensen et al., 1998a), and gain modulation (Olsen et al., 2008; Root et al., 2008).

Lateral interactions between AL glomeruli are not homogeneous. There have been several studies indicating that both inhibitory (Reisenman et al., 2005, 2008; Silbering et al. 2007) and excitatory (Olsen et al., 2007) interactions between glomeruli are not necessarily reciprocal, nor do they exert the same level of influence. In this study we found that in some species there were glomeruli that received sparse or no HA-ir innervation compared to other glomeruli (Fig. 5C–E, I, O), suggesting that the processing of different odor stimuli may be more or less insulated from histaminergic input. Modeling studies in honey bees show that interactions between glomeruli are proportional to the similarity of their odor-response profiles regardless of their anatomical proximity (Linster et al. 2005). This suggests the presence of glomerulus-specific functional rules of connectivity for primary processing of olfactory information.

Based on intracellular fills of a small number of neurons, the HA-ir LNs in the bumble bee appear to be hetero LNs that branch most extensively in the outer rind of a single principal glomerulus and sparsely in the core of the other glomeruli (Fig. 3). However, when the HA-ir LNs were examined as a whole population, we found a much denser HA-ir innervation of the glomerular core (from many sparsely projections) and very sparse total innervation of the glomerular rind (from 1–3 HA-ir LNs that branched heavily). Since ORN axons terminate in the glomerular rind (Fig. 1C–H) and odorants that evoke strong excitatory

responses in hetero LNs also evoked strong calcium influx in the principal glomerulus (Galizia and Kimmerle, 2004), it is possible that an individual HA-ir LN receives direct excitatory input from ORNs in the principal glomerulus. This does not, however, preclude the possibility that the HA-ir LNs receive input from either PNs or non-HA-ir LNs in any of the glomeruli, nor does it suggest that the HA-ir LNs do not have output synapses within the rind of their principal glomerulus.

Physiological studies comparing the effects of GABAergic and histaminergic inhibition within the AL will be needed to fully elucidate the function of these two sub-systems. Although both GABA and histamine application suppress odor-induced responses in PNs (Sachse and Galizia 2002 and Sachse et al., 2006 respectively), it is not known if both of these neurotransmitters directly target PNs. Histamine also suppresses the odor-induced responses observed for bath application of Ca^{2+} sensing dyes (Sachse et al., 2006) which are thought to be taken up by multiple AL cell types including ORNs. It is therefore possible that histamine suppresses ORN responses, and in turn, PN responses. Sachse and Galizia (2002) proposed the existence of two forms of fast inhibition within the AL; a GABA-A dependant global form of inhibition (potentially mediated by homo LNs) that exerts an overall gain control in the AL and a glomerulus-specific GABA-A receptor independent form of inhibition (potentially mediated by hetero-LNs). This second form of inhibition could be mediated by histaminergic LNs, although we found many non HA-ir hetero LNs. It is also possible that the GABAergic and histaminergic LNs represent forms of inhibition with different temporal scales. While GABA mediates its effects via both fast acting ionotropic receptors (i.e. GABA-A receptors) and slower (yet longer lasting) metabotropic receptors (i.e. GABA-B receptors), it is thought that insects do not possess metabotropic HA receptors (Roeder, 2003), and thus the influence of HA release would be rapid and potentially short-lived. By understanding the anatomical relationships between the different cellular components of the AL (especially the diverse sub-populations of LNs), we can start to disambiguate the functional roles of each cellular component and their interactions within the AL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was funded by a grant from the National Institute of Deafness and other Communication Disorders to A.J.N. (R01DC-05652). A.M.D. was funded by the Center for Insect Science through NIH Training Grant #1 K12 Gm00708. The authors would like to thank Wulfila Gronenberg for technical advice and supplying the *B. impatiens*, Patty Jansma for help with the confocal microscopy, Andre Riveros for advice on catching *Polistes* wasps and Penny Dacks and Joshua Martin for helpful comments on this manuscript. We would also like to thank Boris Kondratieff, Cara Gibson, Josh Martin, Aaron Beyerlein, Diana Wheeler, Molly Hunter and Ming Huang for generously providing insects and Carl Olson for help with identification of wild caught insects. Finally, we would like to thank Wulfila Gronenberg and Joshua Martin for translation of German text.

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Figure 1.

Histamine-ir (HA-ir) in the antennal lobe (AL) of *Bombus impatiens*. **A** Horizontal section through the AL of *B. impatiens* showing HA-ir. Scale bar = 50µm. **B** Dense, highly varicose HA-ir innervation of a single bumble bee glomerulus. Each glomerulus was innervated by a single thick HA-ir neurite (arrow) and many thin HA-ir neurites (arrowheads). **C** Horizontal section through an AL with anterogradely filled olfactory receptor neurons (ORNs; magenta) and labeled for HA (green). **D** and **E** are single magenta and green (respectively) channel views of **C**. Scale bar = 50µm. **F** Glomeruli of an AL with anterogradely filled ORNs (magenta) and labeled against HA (green). **G** and **H** are single magenta and green (respectively) channel views of **F**. **I** Backfills of PNs (magenta) projecting to glomeruli in an AL labeled for HA (green). **J** and **K** are single magenta and green (respectively) channel views of **I**. **L** A single PN (magenta) in an AL labeled for HA (green). **M** and **N** are single magenta and green (respectively) channel views of **L**. **O** Double label against GABA (magenta) and HA (green) shows GABA-ir processes throughout the whole glomerular volume of the glomerulus, in contrast to HA-ir processes. Scale bar = 50µm. **P** Three glomeruli labeled for GABA (magenta) and HA (green). **Q** Different population of LNs in the lateral cell body cluster label for GABA (magenta) and HA (green). Red asterisks indicate HA-ir cell bodies and highlight where these cell bodies are in relation to the GABA-ir cell bodies. **R** and **S** are single magenta and green (respectively) channel views of **Q**. Scale bars = 20µm, unless indicated otherwise.

Figure 2.

Non-HA-ir LNs in the AL of *B. impatiens*. **A** Horizontal section through the AL of a bumble bee labeled for histamine (green) in which a homo-LN has been intracellularly filled (magenta). **B** depicts the magenta channel of **A**. **C** The processes of the homo-LN depicted in **A**. **D** and **E** are single magenta and green (respectively) channel views of **C**. **F** Horizontal section through the AL of a bumble labeled for histamine (green) in which a hetero-LN has been intracellularly filled (magenta). **G** depicts the magenta channel of **F**. **H** The processes of the hetero-LN depicted in **C** extending throughout the non-principal glomeruli. **I** and **J** are single magenta and green (respectively) channel views of **H**. **K–O** The projections of non-HA-ir hetero-LNs within their principal glomeruli. **K** A hetero-LN that projects evenly throughout the volume of the principal glomerulus. **L** A hetero-LN that projects evenly in the rind of the principal glomerulus, but less densely in the glomerular core. **M** A hetero-LN that projects densely to one side of the rind of the principal glomerulus. **N** A hetero-LN that projects heavily to the glomerular rind and sparsely to the glomerular core. **O** A hetero-LN that projects with equal density to 2 principal glomeruli. Scale bars = 20 μ m, except for **A**, **B**, **F** and **G** in which scale bars = 50 μ m.

Figure 3.

Individually filled HA-ir neurons in the AL of *B. impatiens*. **A** Horizontal section through the AL of a specimen in which a single HA-ir LN was intracellularly filled (magenta) and labeled for histamine (green). **B** and **C** are single magenta and green (respectively) channel views of **A**. Scale bar = 100µm. **D** The processes of the neuron in **A–C** branches heavily within its principal glomerulus and sparsely in a neighboring glomerulus. Arrowheads indicate the two thick branches entering the principal glomerulus and the arrow indicates the sparse branching in a neighboring glomerulus. Insets depict the cell body of the individual cell in **A–C**. **E** and **F** are single magenta and green (respectively) channel views of **D**. Scale bars = 20µm. **G–Z** Rotated images of 3-D optical stacks of all 6 HA-ir neurons filled in this study depicted at 0, 45, 90 and 135 degrees. The final row (**W–Z**) depicts two HA-ir LNs innervating the same glomerulus. Scale bars = 20µm. **AA** Illustration of the branching of the HA-ir LN depicted in **A–C** within the AL (grey border). **AB** Illustration of the arborizations of the HA-ir LN depicted in **A–C** within its principal glomerulus and a neighboring glomerulus depicting the inner core of the glomeruli in which there is dense innervation by the population of HA-ir LNs (grey inner area) and the area in which there is sparse innervation by the population of HA-ir LNs (white outer layer).

Figure 4.

HA-ir in the developing AL of *B. impatiens*. **A** Horizontal section through the brain and ALs of a *B. impatiens* pre-pupa shows no HA-ir in the ALs (brackets), although there are HA-ir neurons in the larval protocerebrum. **B** Horizontal section through the brain of a *B. impatiens* P1 stage pupa shows HA-ir in the protocerebrum but no HA-ir in the ALs (brackets). **C** Horizontal section through a single AL of a P8 stage pupa shows HA-ir LNs. All scale bars = 100µm.

Figure 5.

HA-ir in the ALs of the Aculeata. **A** Phylogeny of the Aculeata. **B** Horizontal section through the brain of the thread-waisted wasp *Cercerus convergens* (Crabronidae). Inset is a high magnification image of HA-ir labeling in the glomeruli. **C** Horizontal section through the brain of *Ammophila bellula* (Sphecidae) demonstrating that not all of the glomeruli in this species receive dense HA-ir innervation (bracket). Glomerular borders are delineated by anterograde fills of the ORNs (magenta) and HA-ir (green) is present only in the posterior glomeruli. **D** and **E** are single magenta and green (respectively) channel views of **C**. **F** High magnification view of a glomerulus of *A. bellula* labeled with HA-ir (green) in which the ORNs have been filled (magenta). **G** and **H** are single magenta and green (respectively) channel views of **F**. **I** Horizontal section through the brain of a thread-waisted wasp of the genus *Prionix* (Sphecidae). Inset is a high magnification image of glomeruli that receive sparse HA-ir innervation (arrowheads). **J** Whole mount brain of an ant of the genus *Pheidole* (Formicidae). Inset is a high magnification image depicting dense HA-ir innervation of glomeruli. **K** Horizontal section through the brain of the ant *Pogonomyrmex rugosus* (Formicidae). Inset is a high magnification image depicting dense HA-ir innervation of glomeruli. **L** Glomerular borders of *P. rugosus* are delineated by ORNs (magenta) and HA-ir (green) is evident in the glomerular core. **M** and **N** are single magenta and green (respectively) channel views of **L**. **O** Horizontal section through the brain of the paper wasp *Polistes fuscatus* (Vespidae). The bracket indicates a set of glomeruli receiving sparse HA-ir innervation. Inset is a high magnification image depicting dense HA-ir innervation of two glomeruli. Scale bars in primary image are 50µm and scale bars in insets are 20µm.

Figure 6.

HA-ir in the ALs of the Apocrita and Symphyta. **A** Phylogenetic tree of the Apocrita. HA-ir in the ALs of a *Zelomorpha* sp. (**B**), *Eiphosoma pyralidis* (**C**) and *Comperia merceti* (**D**). **E** Glomerular borders are delineated by anterograde fills of the ORNs (magenta) in an AL of *Macrocentrus grandii* labeled for HA-ir (green). **F** High magnification view of glomeruli of *M. grandii* labeled with HA-ir (green) in which the ORNs have been filled (magenta). **G** and **H** are single magenta and green (respectively) channel views of **F**. Scale bar is 20um. **I** Phylogenetic tree of the Neohymenoptera. HA-ir in the AL of *Tremex columba* (**J**, **K**) and *Lophyrotoma zonalis* (**L**). Scale bars are 50um for primary images and 20um for insets, unless otherwise noted.

Figure 7.

HA-ir in the ALs of the Coleoptera, Neuroptera and Hemiptera. **A** Phylogeny of the Holometabola with the Coleoptera and Neuropteroids as the basal taxa. **B** Phylogeny of the Holometabola with the Hymenoptera as the basal taxon. **C** Horizontal section depicting a HA-ir centrifugal neuron innervating the AL of *Odontotaenius disjunctus* (Passalidae; Coleoptera). **D** Frontal section through the AL of *Odontotaenius disjunctus*. **E** Horizontal section through the brain and ALs (brackets) of *Diabrotica undecimpunctata* (Chrysomelidae). Horizontal section through the AL of *Hippodamia convergens* (Coccinellidae, **F**) *Calosoma* sp. (Carabidae, **G**), *Chrysoperla rufilabris* (Chrysopidae; Neuroptera, **H**) and *Narnia inornata* (Coreidae; Hemiptera, **I**).

Figure 8.

Schematic representation of the phylogenetic distribution of characteristics of HA-ir in the ALs of the Hymenoptera and the insects. Four traits are listed; (i) “HA-ir in AL” indicates that there are HA-ir neurons that arborize within the AL, (ii) “HA-ir LNs” indicates that there are HA-ir neurons that project exclusively within the AL, (iii) “Dense innervation” indicates that there is dense HA-ir innervation within each glomerulus, (iv) “Homogenous innervation” indicates that all of the glomeruli receive HA-ir innervation. **A** Phylogeny of the Aculeata. **B** Phylogeny of the Apocrita. **C** Phylogeny of the Neohymenoptera. **D** Phylogeny of the insects (based on Grimaldi and Engel, 2005) with the Coleoptera and Neuropteroids as the basal taxa of the Holometabola. The three gray boxes in **D** indicate I) the Holometabola, II) the Paraneoptera, and III) the Polyneoptera. Filled circle indicates that the trait was present in all of the species examined within a given taxa, empty circle indicates that the trait was not present and half filled circles indicates that the trait was present in some, but not all, of the species examined within a given taxa.

Table 1
List of all species for which histamine-immunoreactivity in the AL was examined in this study.

Species	Order	Sub-order	Infra-order	Super family	Family
<i>Bombus impatiens</i>	Hymenoptera	Apocrita	Aculeata	Apoidea	Apidae
<i>Apis mellifera</i>	Hymenoptera	Apocrita	Aculeata	Apoidea	Apidae
<i>Cerceris convergens</i>	Hymenoptera	Apocrita	Aculeata	Apoidea	Crabronidae
<i>Amnophila bellula</i>	Hymenoptera	Apocrita	Aculeata	Apoidea	Sphecidae
<i>Prionix</i> sp.	Hymenoptera	Apocrita	Aculeata	Apoidea	Sphecidae
<i>Pheidole</i> sp.	Hymenoptera	Apocrita	Aculeata	Vespoidea	Formicidae
<i>Pogonomyrmex rugosus</i>	Hymenoptera	Apocrita	Aculeata	Vespoidea	Formicidae
<i>Polistes fuscatus</i>	Hymenoptera	Apocrita	Aculeata	Vespoidea	Vespidae
<i>Zelomorpha</i> sp.	Hymenoptera	Apocrita	Parasitica	Ichneumonoidae	Braconidae
<i>Macrocercus grandii</i>	Hymenoptera	Apocrita	Parasitica	Ichneumonoidae	Braconidae
<i>Eiphosoma pyralidis</i>	Hymenoptera	Apocrita	Parasitica	Ichneumonoidae	Ichneumonidae
<i>Comperia merceti</i>	Hymenoptera	Apocrita	Parasitica	Chalcidoidea	Encyrtidae
<i>Tremex columba</i>	Hymenoptera	Symphyla	-----	Siricoidea	Siricidae
<i>Lophyrotoma zonalis</i>	Hymenoptera	Symphyla	-----	Tenthredinoidea	Pergidae
<i>Diabrotica undecimpunctata</i>	Coleoptera	Polyphaga	Cucujiformia	Chrysomeloidea	Chrysomelidae
<i>Odonotauenus disjunctus</i>	Coleoptera	Polyphaga	Scarabaeiformia	Scarabaeoidea	Passalidae
<i>Hippodamia convergens</i>	Coleoptera	Polyphaga	Cucujiformia	Cucujoidea	Coccinellidae
<i>Calosoma</i> sp.	Coleoptera	Adephaga	-----	Caraboidea	Carabidae
<i>Chrysoperla rufilabris</i>	Neuroptera	-----	-----	Hemerobioidea	Chrysopidae
<i>Narnia inornata</i>	Hemiptera	Heteroptera	Pentatomomorpha	Coreoidea	Coreidae

Table 2

List of all *B. impatiens* AL neurons filled.

Total cells filled	89
HetLNs	69
-HA-ir LNs	6/69
HomLNs	11
PNs	7
Centrifugal neurons	2