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REGULAR ARTICLE

γ-Aminobutyric acid immunostaining in the antennal lobe of the moth *Heliothis virescens* and its colocalization with neuropeptides

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Abstract The antennal lobe is the primary processing center for olfactory information in insects. To understand further the neural circuitry of this brain area, we have investigated the distribution of γ-aminobutyric acid (GABA) and its colocalization with neuropeptides in the antennal lobe of the noctuid moth *Heliothis virescens*. Immunocytochemical experiments with an antiserum against GABA showed a large number of labeled somata in the antennal lobe; these somata were located exclusively in the lateral cell cluster. Stained neurites innervating all antennal-lobe glomeruli, including the male-specific macroglomerular complex, suggested a prominent role of GABA in processing olfactory information, including signals from pheromones, interspecifically acting odors, and plant odors. Fibers in two antennocerebral tracts (the middle and dorsal antennocerebral tract) exhibited prominent GABA immunoreactivity. Double-labeling experiments revealed that immunostaining for three neuropeptides, viz., A-type allatostatin, *Manduca sexta* allatotropin, and FMRFamide-related peptides, was largely colocalized with GABA in cell bodies of the lateral cell cluster. The general absence of peptide immunostaining in the antennocerebral tracts strongly indicated that these peptides were colocalized with GABA in local interneurons of the antennal lobe. In contrast, tachykinin-related peptides occurred in a distinct population of local antennal-lobe neurons that did not exhibit GABA immunostaining. Thus, local interneurons that were not GABAergic were present in the moth antennal lobe.

Keywords GABA immunocytochemistry · Antennal lobe · Olfactory system · GABA/neuropeptide colocalization · *Heliothis virescens* (Insecta)

Introduction

Odor molecules are detected by a large repertoire of olfactory receptor neurons that project to the primary olfactory center of the brain, viz., the antennal lobe in insects, and the olfactory bulb in mammals (Hildebrand and Sheperd 1997). In both groups, this first-order synaptic neuropil is characterized by an array of spherical structures termed olfactory glomeruli (Shepherd 1974; Anton and Homberg 1999). The connection of plentiful detectors directly linked to a certain number of glomeruli is obviously a favorable organization for encoding olfactory information. The logic of this projection pattern is demonstrated in the noctuid moth *Heliothis virescens* in which four male-specific receptor neuron types carry olfactory information from the external world to specific addresses in the brain, i.e., the four subunits of the macroglomerular complex (MGC; Hansson et al. 1995; Berg et al. 1998). The ligands of the male-specific receptors include pheromone substances produced by conspecific females and substances produced by females of related species. The two categories of signal information (intraspecific and interspecific) are mediated along separate pathways and induce two opposite behavioral responses: attraction versus repulsion (Almaas and Mustaparta 1991;

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Berg et al. 1995; 1998; Christensen et al. 1995; Hansson et al. 1995; Vickers et al. 1998; Vickers and Christensen 2003). The ordinary glomeruli, which are targets of plant odor sensory neurons, are present in both males and females (Stranden et al. 2003; Rø et al. 2006). In *H. virescens*, the total number of antennal-lobe glomeruli, including dimorphic and isomorphic units, comprises 64 in the male and 62 in the female (Berg et al. 2002).

In general, the antennal-lobe glomeruli are innervated by three categories of central interneurons: projection neurons that link the antennal lobe to the protocerebrum, local interneurons that are multiglomerular anaxonal elements residing within the antennal lobe, and centrifugal neurons that send axonal projections into the antennal lobe (Homberg et al. 1989). Projection neurons convey olfactory information from the antennal lobe to two main areas in the protocerebrum: the calyces of the mushroom bodies and the lateral protocerebrum. Their axons form three main pathways termed the inner, the middle, and the outer antenno-cerebral tract (Homberg et al. 1988; Rø et al. 2006). The cell bodies of projection and local interneurons are gathered into three cell groups covering the glomerular neuropil: a prominent ventro-lateral, a smaller medial, and a tiny anterior cell cluster (Homberg et al. 1989). Centrifugal neurons usually have their cell bodies located in the protocerebrum or subesophageal ganglion (for a review, see Schachtner et al. 2005).

γ-Aminobutyric acid (GABA) is the major inhibitory transmitter in the insect brain, and GABAergic synaptic input in the antennal lobe is essential for olfactory processing (for reviews, see Boeckh and Tolbert 1993; Laurent 2002). In all species studied so far, GABA is abundantly present in local interneurons of the antennal lobe, as demonstrated by immunostaining (*Manduca sexta*: Hoskins et al. 1986; *Apis mellifera*: Schäfer and Bicker 1986; *Periplaneta americana*: Distler 1989; *Schistocerca gregaria*: Leitch and Laurent 1996). Physiological studies in *M. sexta* have indicated that GABA is responsible for amplifying contrasts via interglomerular interactions (Lei et al. 2002). More precisely, GABA is believed to induce odor-released synchronization of projection neuron activity in the antennal lobe in such a way that the differences between odor signals are enhanced. Synchronization of firing patterns in groups of antennal-lobe projection neurons was demonstrated for the first time in locusts (MacLeod and Laurent 1996). Studies of vertebrates (for a review, see Möhler 2006) and invertebrates (Wilson and Laurent 2005) have led to the common acceptance that GABA influences the timing of spiking activity and consequently plays a decisive role in creating network oscillations.

As in other neural networks, many other neurotransmitter candidates have been identified in the antennal lobe. These include acetylcholine, serotonin, dopamine, octopamine, histamine, and various neuropeptides (Homberg and Müller 1999; Schachtner et al. 2005; Shang et al. 2007). In general, all nervous systems show the coexistence of peptides and small molecule transmitters within the same neuron (for a review, see Salio et al. 2006). Recently, we have investigated the distribution of neuropeptides in the central olfactory pathway of *H. virescens* (Berg et al. 2007). Immunocytochemistry and MALDI-TOF mass spectrometry have provided evidence for the presence of members of four neuropeptide families, viz., allatostatins (AST-As), *Manduca sexta* allatotropin (Mas-AT), FMRFamide-related peptides (FaRPs), and tachykinin-related peptides (TKRPs), in the antennal lobe. The various antisera label mainly local interneurons and show distinct staining patterns. Particularly noticeable is the distribution of TKRP immunostaining, which differs considerably from that of the other neuropeptides. Double-label experiments have revealed the colocalization of Ast-As, Mas-ATs, and FaRPs, whereas TKRP immunoreactivity occurs in distinct neurons.

To investigate further the distribution of neuroactive substances in the central olfactory pathway, we have studied the distribution of GABA in the antennal lobe of *H. virescens* by immunostaining. We demonstrate GABA immunoreactivity in a large number of local neurons and in two antennocerebral tracts. Double-labeling experiments have shown that AST-As, Mas-AT, and FaRPs are present in subpopulations of GABA-immunoreactive local neurons but has revealed no colocalization between GABA and TKRP immunolabeling. This demonstrates the presence of local interneurons that are not GABAergic in the antennal lobe of the moth.

Materials and methods

Insects

H. virescens (Lepidoptera: Noctuidae) pupae, originating from a laboratory culture, were kindly provided by Syngenta, Basel, Switzerland. Male and female pupae were kept on a phase-shifted light/darkness photoperiod of 14 h:10 h at 25°C. The animals were allowed to feed on sugar-water. All experiments were performed on adults, 1–5 days after ecdysis. No differences in immunostaining were observed between isomorphic brain structures of males and females.

Antisera

For the immunostaining of *H. virescens* brain sections, we used a rabbit antiserum raised against conjugates of GABA covalently coupled to keyhole limpet hemocyanin (KLH; Table 1; generously provided by Dr. T.G. Kingan, US

Department of Agriculture, Beltsville, Md., USA). In specificity tests on brain sections of *H. virescens*, the GABA antiserum (1:3,000) was preincubated prior to its application to the sections with four different concentrations (10 nM, 100 nM, 1 μM, 10 μM) of GABA conjugated to KLH (12 mg/8 ml; Sigma-Aldrich) by glutaraldehyde. Preadsorption of the antiserum with GABA-glutaraldehyde-KLH at 10 nM reduced the staining intensity, whereas higher concentrations of the antigen abolished all immunostaining (data not shown).

For double-labeling experiments, antisera, also raised in rabbit, against the following neuropeptides were utilized: *Diploptera* allatostatin 7 (Dip-Ast7), *M. sexta* allatotropin (Mas-AT), the tetrapeptide FMRFamide, and locustatachykinin II (LomTK II; for further details, see Table 1 and Berg et al. 2007). The antisera against Dip-Ast7 and LomTK II were generously provided by Dr. Hans Agricola (University of Jena, Germany), whereas antisera against Mas-AT and FMRFamide were gifts from Dr. Jan Veenstra (University of Bordeaux, Talence, France; Veenstra and Hagedorn 1993) and Dr. Eve Marder (Brandeis University, USA), respectively. The specificities of the four neuropeptide antisera in *H. virescens* nervous tissue were as previously tested by liquid-phase preadsorption (Berg et al. 2007). For labeling of neuropil structures including olfactory glomeruli, a monoclonal mouse antibody against *Drosophila* synaptic-vesicle-associated protein synapsin I, kindly provided by Dr. E. Buchner (University of Würzburg, Germany) was used.

Immunocytochemistry

GABA immunostaining was performed on mounted paraffin sections and free-floating vibratome sections by the indirect peroxidase-antiperoxidase (PAP) technique (Sternberger 1979). After the animals had been anesthetized by cooling, brains were dissected from the head capsule in cold saline (Weevers 1966) and fixed in 1 part 25% glutaralaldehyde/3 parts saturated picric acid/1% acetic acid (GPA; Boer et al. 1979) overnight at 4°C or for 1-2 h at room temperature. Brains intended for paraffin sectioning were dehydrated through a series of ethanols and toluene before being embedded in Paraplast Plus (Sigma, Steinheim, Germany). A rotary microtome was used to cut 8-μm-thick sections from frontally or dorsally positioned preparations. The sections were dried on microscope slides at room temperature for at least 4 h before being deparaffinized in xylene and ethanols and finally hydrated. To minimize non-specific staining, the sections were preincubated for 1 h in TRIS-buffered saline (TBS; 0.3 M NaCl/0.1 M TRIS-HCl, pH 7.4) containing 0.5% Triton X-100 (TrX) and 5% normal goat serum (NGS). The GABA antiserum was diluted 1:2000 in TBS containing 0.5% TrX and 1% NGS. The incubation time was 20 h at room temperature. As a secondary antibody, goat-anti-rabbit IgG (Sigma) was applied at 1:40 for 1 h, and then rabbit PAP at 1:300 for 1 h. Staining was visualized by means of a solution of 3,3′-diaminobenzidine tetrahydrochloride (DAB) containing 5 mg DAB/15 ml 0.1 M phosphate buffer (pH 7.4) and 0.015% H_2O_2. Between the various steps of the protocol, the sections were rinsed in TBS containing 0.1% TrX. The sections were dehydrated, cleared in xylene, and embedded in Entellan (Merck) under glass coverslips.

Brains prepared for free-floating vibratome sections were treated as described by Berg et al. (2007). After fixation, the preparations were embedded in a frontal or dorsal orientation in gelatin/albumin, postfixed overnight in 4% buffered

Table 1 Antisera used

Antiserum	Antigen	Source and first characterization	Specificity tests in *H. virescens*	Working dilution for PAP-technique	Working dilution for immunofluorescence
Anti-GABA (9/24)	GABA-keyhole limpet hemocyanin	T.G. Kingan (Hoskins et al. 1986)	This study	Paraffin sections, 1:2,000; vibratome sections; 1:3,000	1:2,000
Anti-*Diploptera* allatostatin 7	Allatostatin 7-thyroglobulin	H. Agricola (Vitzthum et al. 1996)	Berg et al. 2007	–	1:50,000
Anti-*Manduca* (Mas-AT) allatotropin (13.3.91)	Mas-AT thyroglobulin	J. Veenstra (Veenstra and Hagedorn 1993)			1:15,000
Anti-FMRFamide (671N)	FMRFa-thyroglobulin	E. Marder (Marder et al. 1987)			1:5,000
Anti-locustatachykinin II (Lom-TK II; K1-50820091)	Lom-TK II thyroglobulin	H. Agricola (Nässel et al. 1995)			1:10,000

formaldehyde, and sectioned at 30 μm with a vibrating blade microtome (Leica VT 1000 S; Nussloch, Germany). In these experiments, the GABA antiserum was applied at 1:3,000. Finally, the sections were mounted on chrome alum/gelatin-coated glass slides, dehydrated, cleared in xylene, and embedded in Entellan under glass coverslips.

Double-labeling experiments

To investigate the colocalization of GABA and neuropeptides in the antennal lobe of *H. virescens*, the antiserum against GABA was applied in combination with each of the four neuropeptide antisera in double-labeling experiments. Experiments were performed on vibratome sections. Brains were fixed in a mixture consisting of 0.5 ml formaldehyde, 1.2 ml 25% glutaraldehyde, and 3.3 ml 0.01 M PBS. The fixed brains were embedded and sectioned with the vibratome as described above. To minimize background fluorescence, the free-floating sections were treated with 1% sodium borohydride solution (100 mg sodium borohydride, 10 ml 0.1 M phosphate buffer, pH 7.4) containing 10% TrX. After a thorough rinse in TBS containing 1% TrX, the sections were further processed according to a method modified from that of Negoescu et al. (1994) for double-immunolabeling with primary antisera from the same species. The brain sections were preincubated in TBS containing 0.5% TrX and 5% normal donkey serum (NDS) for 1 h at room temperature. The primary antibodies were diluted in TBS containing 0.5% TrX and 1% NDS at the following concentrations: anti-GABA, 1:2,000; anti-Dip-Ast7, 1:50,000; anti-Mas-AT, 1:15,000; anti-FMRFamide, 1:5,000; anti-LomTK II, 1:10,000. Since all antibodies were raised in rabbit, they had to be applied in consecutive steps. The neuropeptide antiserum was used in the first staining cycle and anti-GABA in the second. Incubation in the neuropeptide antiserum lasted 20 h at room temperature. To block binding sites for the primary antiserum in the second staining cycle, monovalent Fab fragments (goat-anti-rabbit; Jackson ImmunoResearch, Westgrove, Pa.) diluted in TBS-0.5% TrX buffer, including 1% NDS, were then applied at a concentration of 1:40 to the preparations for 2 h at room temperature. The sections were subsequently washed and incubated for 1 h with a Cy3-conjugated donkey-anti-goat antiserum (Jackson ImmunoResearch, diluted 1:300). From this stage onward, the preparations were treated in darkness. In the second staining cycle, the sections were preincubated in TBS, containing 5% NDS, for 30 min, followed by incubation with the second primary antiserum, viz., anti-GABA, for 2 days at room temperature. To reduce background staining, 2% milk powder was added to the primary antibody in the second cycle. The sections were subsequently washed and incubated with Cy2-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) in TBS containing 0.5% TrX and 1% NDS for 1 h. For the labeling of neuropil structures, the monoclonal anti-synapsin I antibody was applied simultaneously with the anti-GABA antiserum and, likewise, the corresponding secondary antiserum, Cy5-conjugated donkey-anti-mouse (Jackson ImmunoResearch) together with Cy3-conjugated donkey anti-goat. Finally, the sections were thoroughly washed and mounted on chrome alum/gelatin-coated microscope slides as described above.

Image processing and data analysis

The DAB-treated sections were analyzed and photographed via a Zeiss digital camera mounted on a Zeiss bright-field microscope (Axiovision Z1). Images including neuropil structures of the antennal lobes and antennocerebral tracts were imported into Adobe Photoshop 7.0 for adjustments and were finally edited in Adobe Illustrator CS2. The double-labeled preparations were analyzed by using a confocal laser scanning microscope (Leica TCS SP2) and were treated further as described above. Scans for Cy2-fluorescence (Ex_{max} 490 nm, Em_{max} 508 nm), Cy3-fluorescence (Ex_{max} 550 nm, Em_{max} 570 nm), and Cy5-fluorescence (Ex_{max} 650 nm, Em_{max} 674 nm) were performed with an argon laser at 488 nm, an He/Ne laser at 543 nm, and an He/Ne laser at 633 nm, respectively, in the sequential scanning mode at 1024×1024 pixel resolution by using a 20× or 40× objective (HC PL APO 20x/0.70 lmm Corr CS and HCX PL APO 40x/1.25-0.75 Oil CS; Leica, Bensheim, Germany). The pinhole size was 1 μm, and the distance between the optical sections was usually 1 μm.

The numbers of antennal-lobe neurons immunoreactive for GABA were calculated from the paraffin sections by counting the total number of stained cell bodies in three antennal lobes originating from three individuals. To compensate for cell bodies that were cut and counted twice in adjacent sections, cell counts were corrected according to the relationship derived by Abercrombie (1946). To estimate mean cell body size, the diameter of 145 immunoreactive somata, selected from two antennal lobes, was measured. Positional information is given with respect to the body axis of the insect.

Results

Immunostaining in antennal lobe

Application of antiserum against GABA resulted in distinct staining of several neuropil areas of the central olfactory pathway (Fig. 1a). These included the antennal-lobe glomeruli, the calyces of the mushroom bodies, and the lateral protocerebrum. In the antennal lobe, each glomeru-

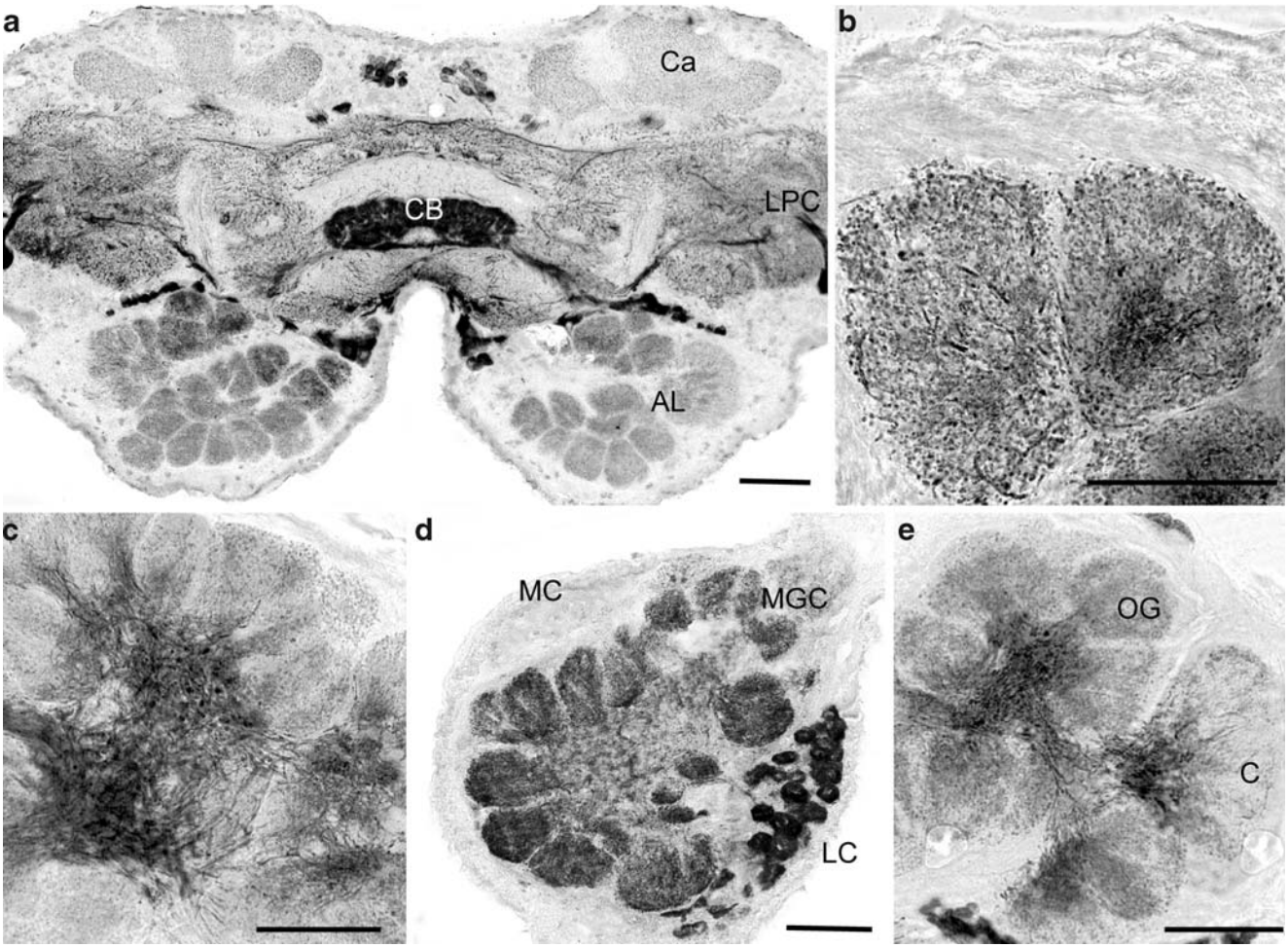

Fig. 1 GABA immunostaining in the brain of *Heliothis virescens*. **a** Horizontal section showing immunostaining in most parts of the brain including the antennal lobes (*AL*), the calyces of the mushroom body (*Ca*), and the lateral protocerebrum (*LPC*) (*CB* central body). **b** High magnification of a section through the antennal lobe. Glomerular labeling is characterized by a meshwork of fine processes throughout the glomerulus. Staining intensity is higher in the center than in the periphery of the glomeruli. **c** Part of the antennal lobe showing GABA immunostaining in the central coarse neuropil. **d** Frontal section through the antennal lobe (*MGC* macroglomerular complex). Immunoreactive somata are concentrated in the lateral cell cluster (*LC*) but are totally lacking in the medial cell cluster (*MC*). **e** Horizontal section through a male antennal lobe showing the cumulus (*C*) of the macroglomerular complex and the array of ordinary glomeruli (*OG*). The large cumulus is more sparsely invaded by immunoreactive fibers than the ordinary glomeruli. *Bars* 50 μm

lus was labeled by a fine network pervading the entire structure (Fig. 1b). Labeling was slightly more intense in the glomerular core than in the periphery. Immunostaining in the central coarse neuropil of the antennal lobe (a structure devoid of glomeruli and mainly containing primary neurites of antennal-lobe neurons) was concentrated in large-diameter fibers (Fig. 1c). In some preparations, the glomeruli located in the posterior-medial part of the antennal lobe were more extensively stained than the others. The somata of the immunoreactive neurons were exclusively located in the lateral cell cluster of the antennal lobe; none were observed in the anterior or the medial cluster (Fig. 1d). In total, 327±12 labeled cell bodies with an average diameter of 10.4±2.6 μm were counted in the antennal lobe. The male-specific glomeruli constituting the MGC could be recognized easily at the antennal nerve entrance (Fig. 1d,e). The large units of the MGC, in particular the cumulus, were more sparsely invaded by immunostained profiles than the ordinary glomeruli (Fig. 1e). No difference in immunostaining in the isomorphic glomeruli was observed between the two sexes.

Immunostaining in antennocerebral tracts

The inner antennocerebral tract projecting from the antennal lobe to the calyces of the mushroom body appeared as a thick bundle of unstained axons, except for two immunoreactive fibers (Fig. 2a). Their target projections in the protocerebrum could not be traced. In contrast to the immunonegative inner antennocerebral tract, the middle

antennocerebral tract (MACT) projecting to the lateral protocerebrum contained a considerable number of labeled axons (Fig. 2b). In the protocerebrum, the stained fiber bundle divided and targeted two different areas (Figs. 2b, 3a). The outer antennocerebral tract targeting the lateral protocerebrum via a more ventrally located pathway (Fig. 3c) also included some GABA-immunolabeled neurons, visible as they left the antennal lobe (Fig. 2c). In addition to the three main antennocerebral pathways, the dorsal antennocerebral tract running more dorsally appeared as a tight bundle of strongly labeled axons (Figs. 2d–f, 3b). In addition to the protocerebral pathways, a few stained fibers connected the antennal lobe with the antennal mechanosensory and motor center and/or the subesophageal ganglion (Fig. 2g).

Double-labeling experiments

The distribution of GABA immunostaining was compared with labeling for AST-As, Mas-AT, FaRPs, and TKRPs by

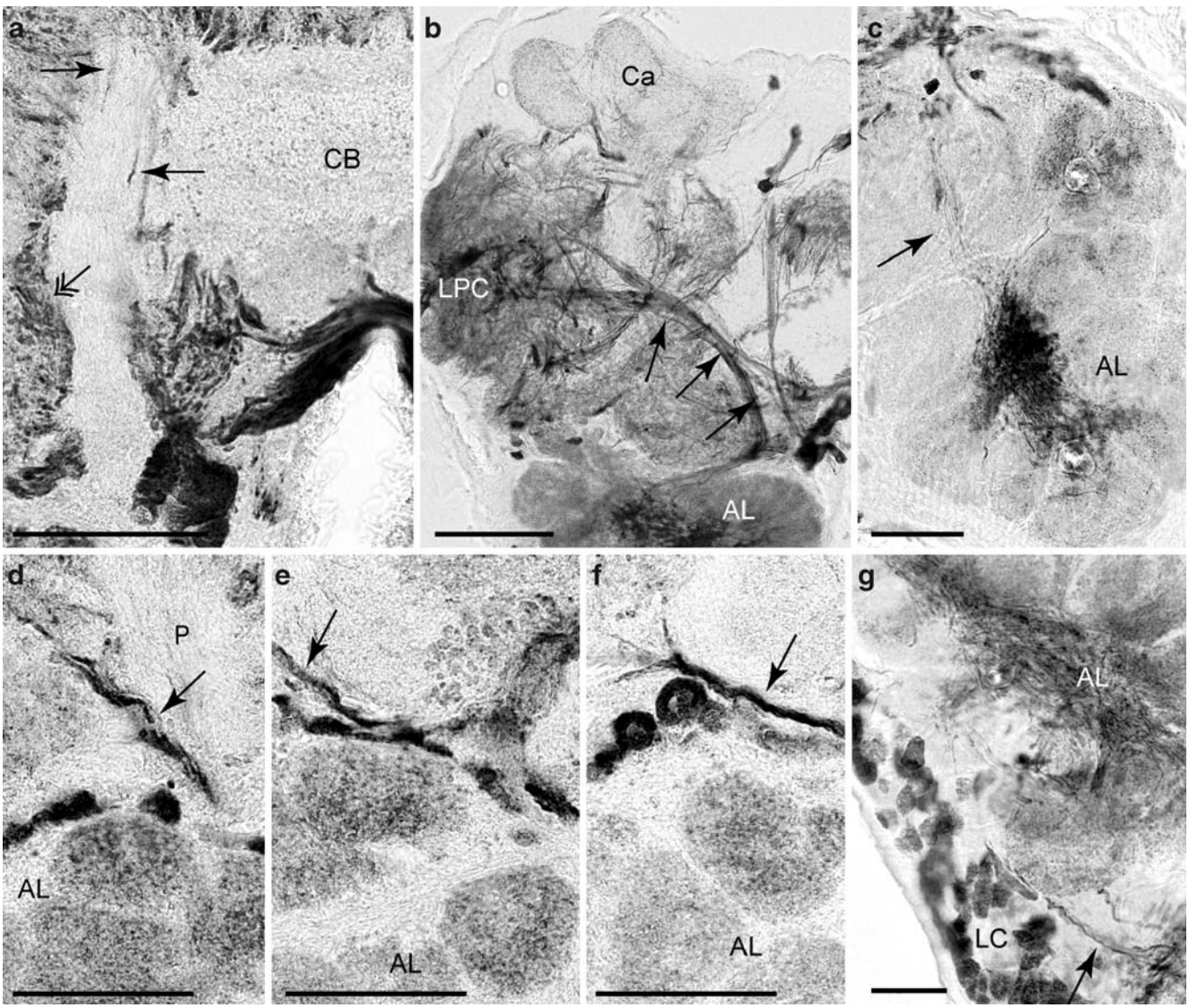

Fig. 2 GABA immunoreactivity in the antennocerebral tracts of the moth brain. **a** Horizontal section showing lack of immunostaining in the inner antennocerebral tract. Only two stained axons (*arrows*) can be seen as they pass the central body (*CB*). Note part of the middle antennocerebral tract (*double arrow*). **b** Fibers in the middle antennocerebral tract (*arrows*), which projects directly from the antennal lobe (*AL*) to the lateral protocerebrum (*LPC*), show prominent GABA immunostaining (*Ca* calyces of the mushroom body). **c** Horizontal section showing a loose bundle of stained fibers leaving the ventral part of the antennal lobe via the outer antennocerebral tract (*arrow*). **d-f** Three adjacent horizontal sections, from ventral (**d**) to dorsal (**f**), showing stained fibers in the dorsal antennocerebral tract (*arrows*). **g** Frontal section through the antennal lobe (*AL*). Several immunostained fibers (*arrow*) connect the antennal lobe with the antennal mechanosensory and motor center and/or the subesophageal ganglion (*LC* lateral cell cluster). *Bars* 50 μm

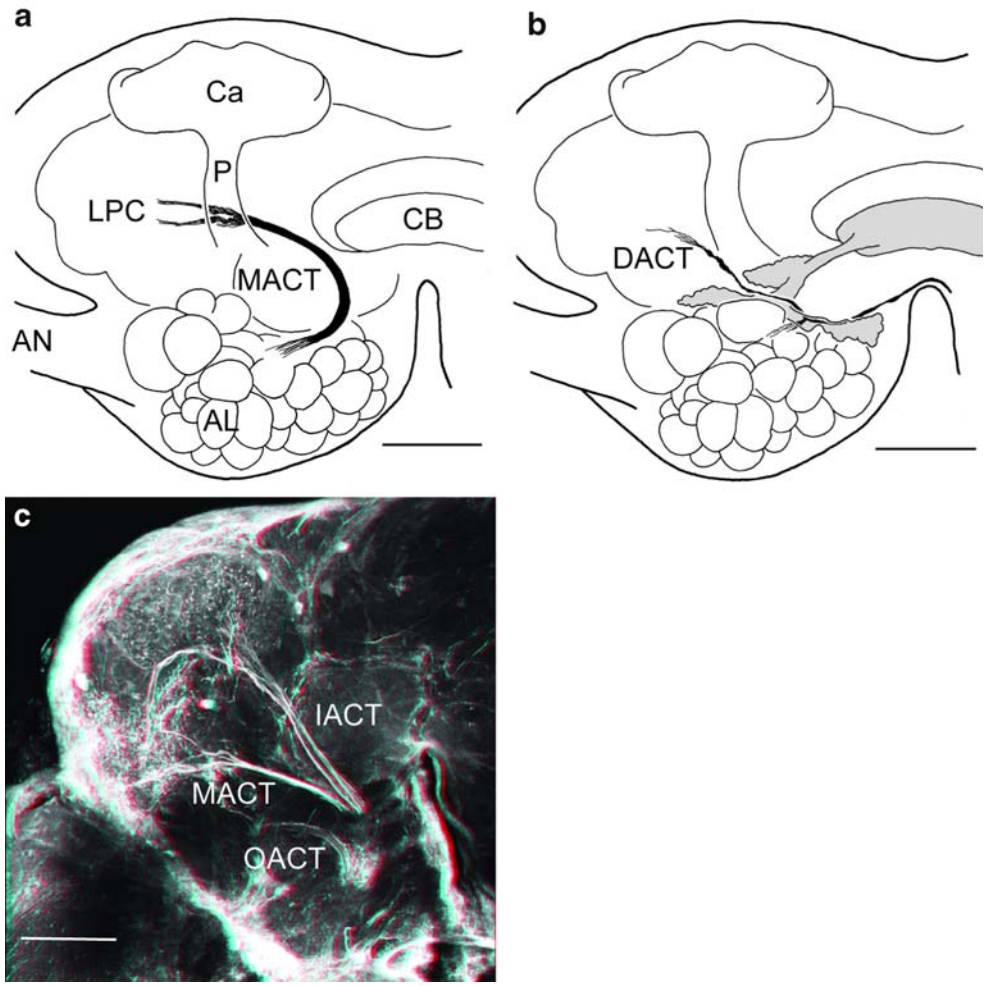

Fig. 3 GABA immunostaining in antennocerebral tracts. **a**, **b** Reconstructions originating from the preparation shown in Fig. 1a (*Ca* calyces, *P* pedunculus, *CB* central body, *LPC* lateral protocerebrum, *AL* antennal lobe, *AN* antennal nerve). **a** Camera lucida reconstruction of GABA-immunolabeled axons in the middle antennocerebral tract (*MACT*). **b** Reconstruction of GABA-immunolabeled axons in the dorsal antennocerebral tract (*DACT*). The stained axons project medially from the antennal lobe and pass around primary neurites of GABA-immunoreactive tangential neurons of the lower division of the central body (*shaded area*). One sub-bundle of fibers makes a sharp turn and projects dorso-laterally along the lower part of the pedunculus, whereas another branch joins a commissure connecting to the contralateral brain hemisphere. **c** For comparison, the three main protocerebral tracts, viz., the inner (*IACT*), middle (*MACT*), and outer (*OACT*) antennocerebral tracts are shown in a stereoscopic image (use red/green spectacles for three-dimensional viewing, with the green lens in front of the left eye). The confocal reconstruction originates from a preparation retrogradely labeled by applying fluorescent dye into the antennal lobe. The dorsal antennocerebral tract is not visible. *Bars* 50 μm

investigating the stained somata in the lateral cell cluster for double-immunofluorescence. The LomTK II antiserum revealed a mean of five immunostained cell bodies (n=6, SD=1), none of which were GABA-immunoreactive (n=6, SD=0; Fig. 4a). Likewise, LomTK II immunostaining was not colocalized with GABA immunostaining in the glomerular neuropil (Fig. 4b). In a previous study, Berg et al. (2007) estimated there being about 64 FMRFamide-, 87 Mas-AT-, and 47 Dip-Ast7-immunoreactive cells in the lateral cell cluster. The large majority of these cells showed colocalization of peptide and GABA immunoreactivities (Fig. 5). Only a few cell bodies stained by the three neuropeptide antisera were not GABA-immunolabeled. These included a single large Ast-A-labeled cell body (Fig. 6a), one large Mas-AT-labeled cell body (Fig. 6b), and one large FMRFamide-labeled cell body (Fig. 6c), all located ventrally in the lateral cell cluster.

Discussion

GABA: main inhibitory transmitter of antennal lobe

The results presented here suggest the widespread occurrence of GABA in the antennal lobe of *H. virescens*. The dense immunostaining throughout all the glomeruli supports an

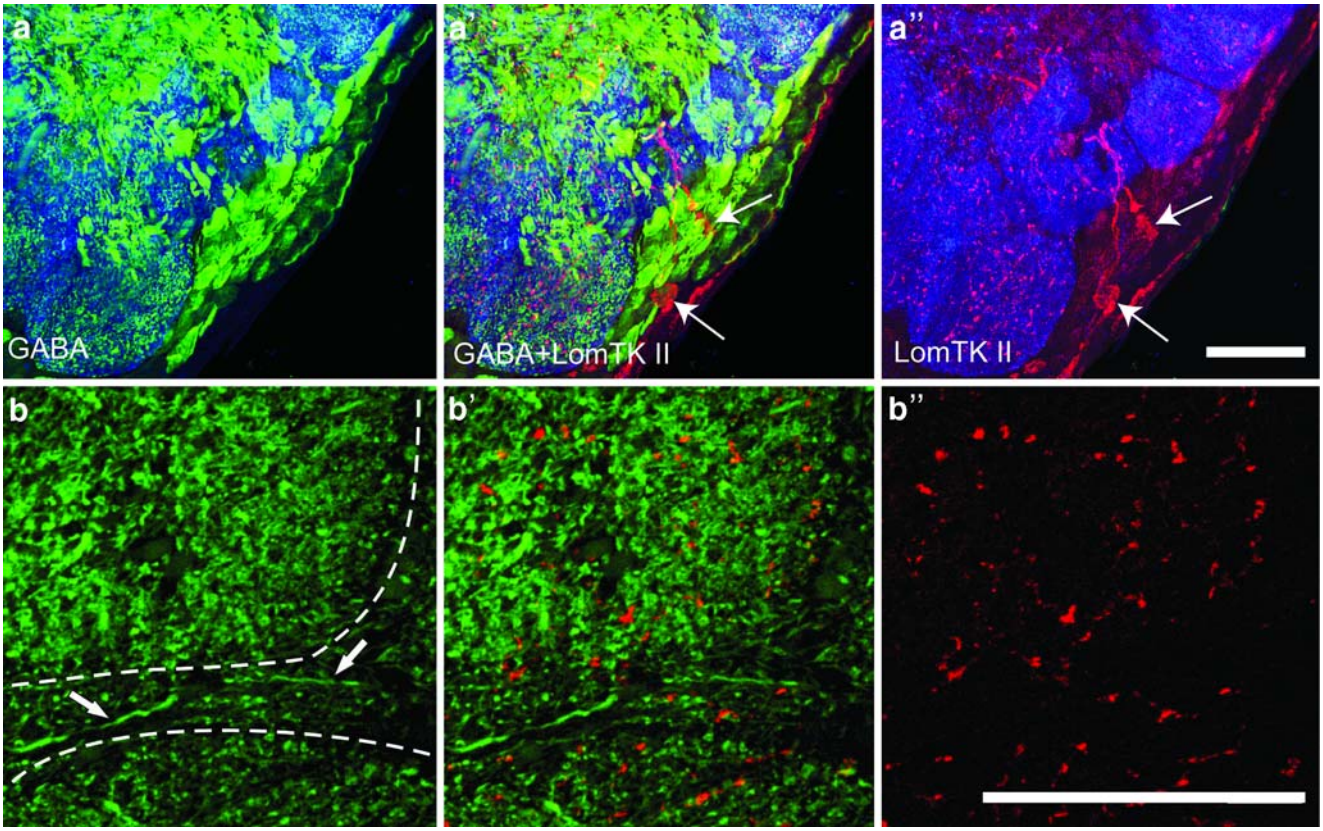

Fig. 4 Confocal images from stacks of 8–10 optical sections showing double-immunofluorescence of GABA- and LomTK II immunostaining. *Left*, *right* Images obtained at different excitation wavelengths. *Middle* Overlay. **a-a"** Immunolabeling of GABA (*green*) and LomTK II (*red*) shows somata immunoreactive for each antiserum (**a**, **a"**), but no double-labeled somata (**a'**). Note two somata and primary neurites immunoreactive for LomTK II but not for GABA (*arrows* in **a'**, **a"**). Immunolabeling of synapsin (*blue*) shows the neuropil structures. **b-b"** Images showing glomerular neuropil at high magnification. LomTK-II-immunoreactive profiles (*red*, **b"**) are, by displaying a pattern of granular staining throughout the glomerulus, considerably different from the GABA-immunoreactive profiles (*green*, **b**). Glomerular boundaries are indicated by *dashed lines* (*arrows* GABA-immunoreactive fibers running between the two glomeruli). *Bars* 50 μm

important role of GABA in processing general olfactory information, including signals from pheromones, interspecifically acting substances, and plant odors. Berg et al. (2007) have shown that neuropeptides related to Mas-AT, Dip-Ast7, and FMRFamide are largely present in local interneurons of the antennal lobe of *H. virescens*. The colocalization of these peptides and GABA in a large number of cell bodies in the lateral cell cluster therefore indicates that many GABA-immunoreactive neurons are local neurons. This is in full agreement with previous reports in the sphinx moth *M. sexta* and several other insect species (Hoskins et al. 1986; Homberg et al. 1989; for reviews, see Homberg and Müller 1999; Utz et al. 2008). The inhibitory effect of GABA was demonstrated for the moth antennal lobe more than 20 years ago (Waldrop et al. 1987). Data from several insects, including *M. sexta*, have subsequently revealed that GABA synchronizes firing patterns in groups of antennal-lobe output neurons (MacLeod and Laurent 1996; Lei et al. 2002). Two GABA-gated conductances with different kinetics have been reported in the antennal lobe of *Drosophila melanogaster*: a fast conductance induced by the activation of an ionotropic receptor ($GABA_A$ type) and a slower conductance induced by the activation of a metabotropic receptor ($GABA_B$ type; Wilson and Laurent 2005). The fast type acts at the beginning of the response and is suggested to synchronize the firing of projection neurons, whereas the slower type acts at a later phase and is proposed to influence the spatio-temporal response pattern of the neurons. A recent publication suggests that GABA mediates presynaptic inhibition by providing, via ionotropic and metabotropic receptors, a gain control that regulates the input information in proportion to the dynamic range of antennal-lobe projection neurons (Olsen and Wilson 2008). Root et al. (2008) have reported that presynaptic gain control varies between distinct input channels based on the different expression levels of $GABA_B$ receptors. In the olfactory bulb of vertebrates, a corresponding principle of gain control has been demonstrated by presynaptic inhibition via periglomerular cells releasing GABA onto axodendritic synapses (Vučinić et al. 2006; Nawroth et al. 2007).

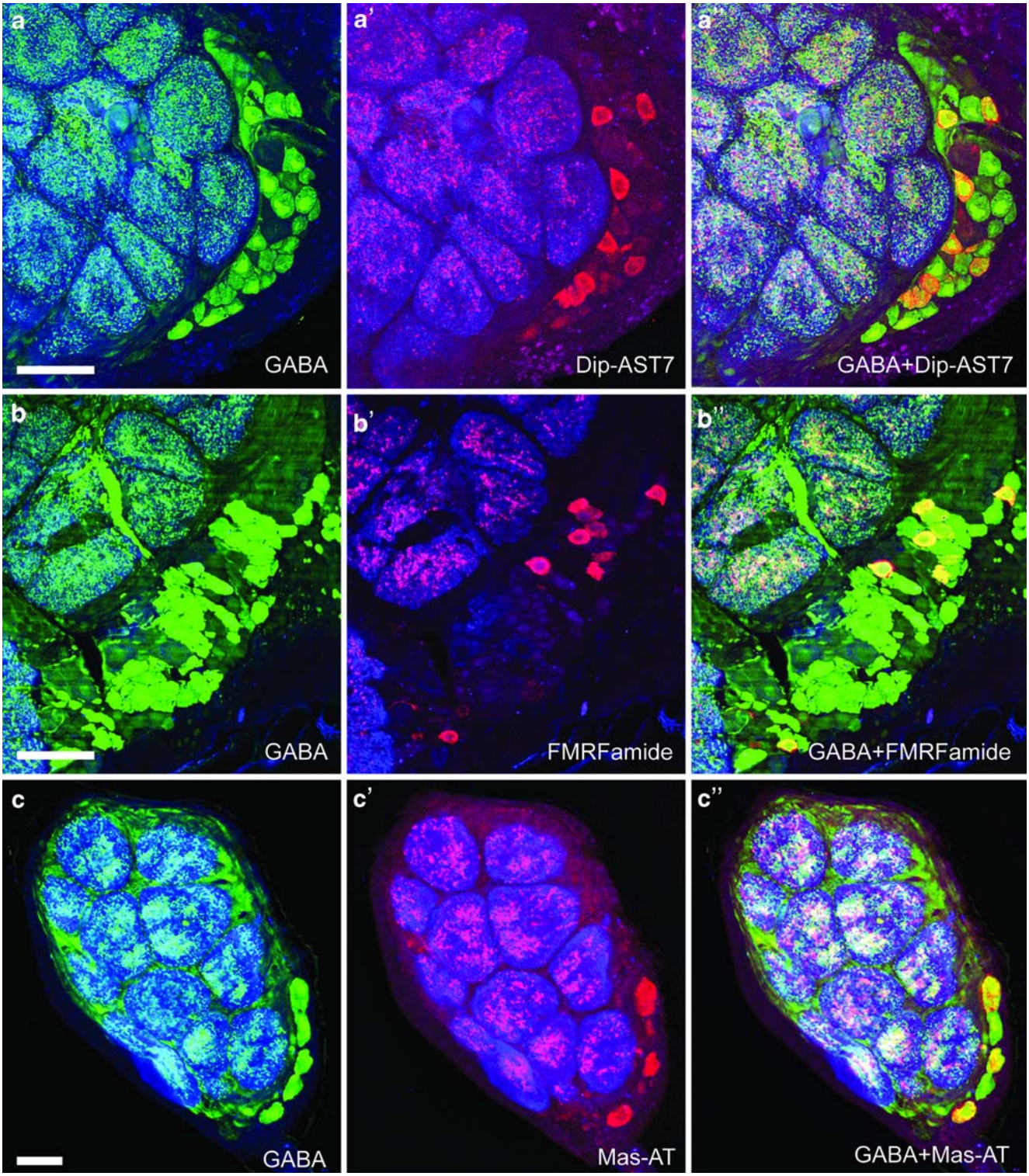

Fig. 5 Confocal images from stacks of 8–10 optical sections showing double-immunofluorescent staining for GABA in combination with one of three neuropeptides, viz., Dip-AST7, FMRFamide, and Mas-AT. *Left*, *middle* Images obtained at different excitation wavelengths. *Right* Overlay. Anti-synapsin staining is shown in *blue*. **a-a"** Immunolabeling for GABA (*green*) and Dip-AST7 (*red*) shows somata immunoreactive for each antiserum (**a**, **a'**), and the total colocalization (*orange*) of Dip-AST7 immunostaining with GABA immunolabeling (**a"**). **b-b"** Immunolabeling for GABA (*green*) and FMRFamide (*red*) shows somata immunoreactive for each antiserum (**b**, **b'**) and the colocalization (*yellow*) of GABA immunoreactivity in all FMRFamide-immunostained somata (**b"**). **c-c"** Immunolabeling for GABA (*green*) and Mas-AT (*red*) shows somata immunoreactive for each antiserum (**c**, **c'**) and colocalization (*yellow/orange*) of GABA immunostaining in all Mas-AT-immunoreactive somata (**c"**). *Bars* 50 μm

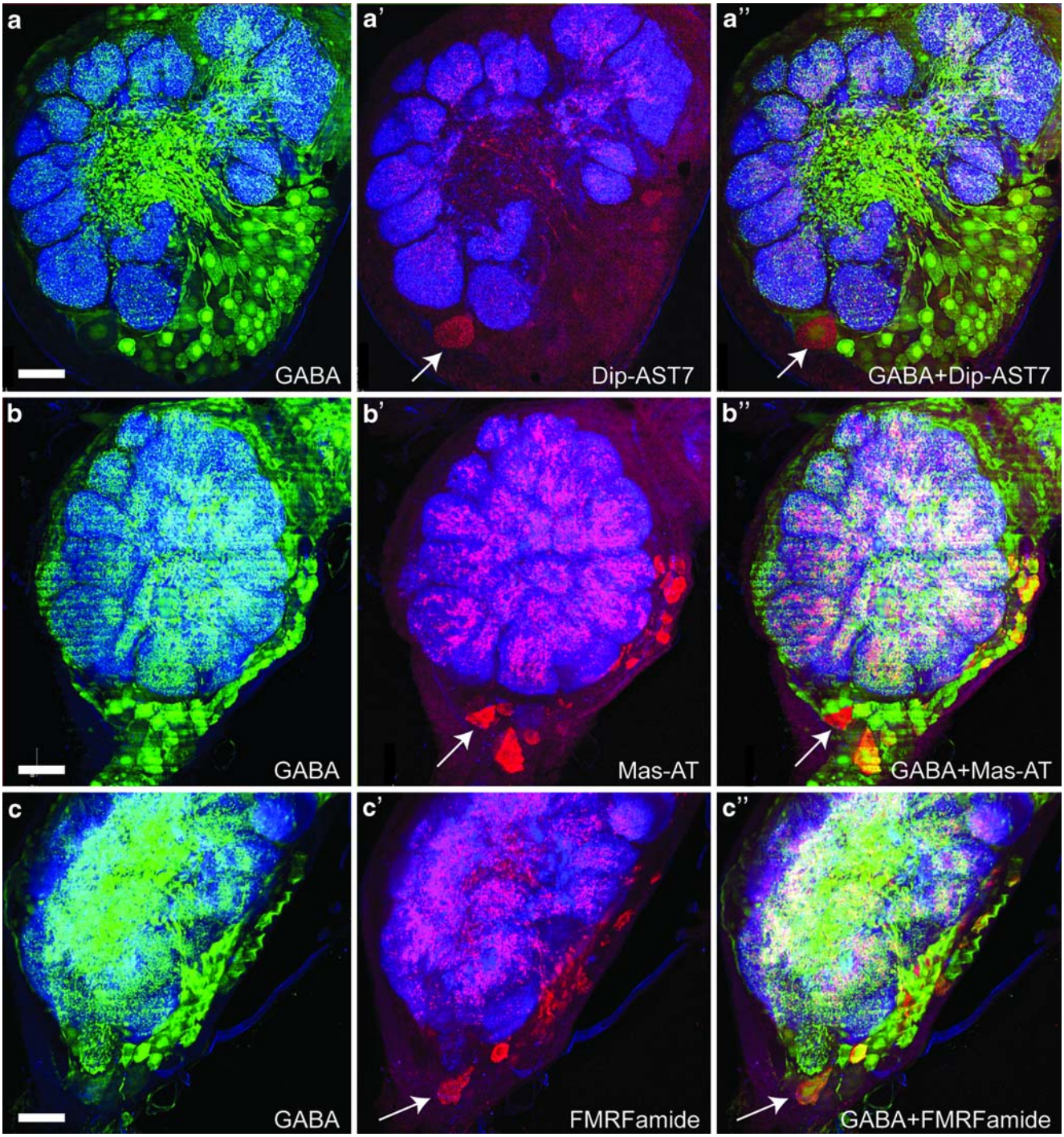

Fig. 6 Confocal images from stacks of 8–10 optical sections showing double-immunofluorescent staining for GABA in combination with various neuropeptides. *Left*, *middle* Images obtained at different excitation wavelengths. *Right* Overlay. Synapsin immunostaining (*blue*) shows neuropil structures. **a-a"** Immunolabeling for GABA (*green*) and Dip-AST7 (*red*). Note the single Dip-AST7-immunoreactive soma that lacks GABA immunostaining (*arrows* in **a'**, **a"**). **b-b"** Immunolabeling for GABA (*green*) and Mas-AT (*red*). A single large Mas-AT-immunostained soma does not show GABA immunoreactivity (*arrows* in **b'**, **b"**). **c-c"** Immunolabeling for GABA (*green*) and FMRFamide (*red*). A single large FMRFamide-immunolabeled soma (*arrows* in **c'**, **c"**) does not exhibit GABA immunoreactivity. *Bars* 50 μm

GABA in antennocerebral tracts

Prominent staining of fibers in the MACT suggests that a substantial proportion of antennal-lobe projection neurons, which target the lateral protocerebrum directly, is GABAergic. Because of dense fasciculation, the numbers of immunostained fibers have not been calculated but, as judged by the thickness of the immunostained fiber bundle,

may be in the range of 40–70 fibers. These neurons are probably multiglomerular projection neurons since intracellular recordings from the antennal lobe of *H. virescens* have shown consistently that axons passing in the MACT arborize in several glomeruli (Rø et al. 2006). A corresponding subpopulation of GABA-immunoreactive projection neurons has previously been identified in *M. sexta* (Hoskins et al. 1986; Homberg et al. 1989). In honeybees, the presence of GABA-immunoreactive fibers in a tract projecting directly to the lateral protocerebrum (the so-called mediolateral antennoglomerular tract) has also been reported (Schäfer and Bicker 1986). Labeled axons in the dorsal antennocerebral tract, estimated roughly as being 10–20 fibers, may include projection neurons targeting the contralateral hemisphere, analagous to previous findings in the sphinx moth (Homberg et al. 1989). The functional role of GABA in these projections neurons is not known, but the release of GABA probably leads to an odor-dependent inhibition of target brain areas. Some MACT neurons project, like other antennal-lobe projection neurons, to the lateral horn of the protocerebrum (Rø et al. 2006). GABAergic inhibitory input from the MACT might therefore converge in this brain area with excitatory input from the inner and outer antennocerebral tracts. This might further enhance odor discrimination and sensitivity. MACT neurons with wide projections in the protocerebrum might selectively shift attention in target areas through odor-dependent inhibition.

Co-localization of GABA and neuropeptides related to AST-A, Mas-AT, and FMRFamide

In contrast to LomTK-II-related peptides, which are present in a unique population of somata, neuropeptides related to AST-As, Mas-AT, and FMRFamide largely colocalize with GABA. As illustrated in Fig. 6, three large somata show immunoreactivity exclusively for one of the neuropeptides. This indicates that AST-As, Mas-AT, and FaRPs mainly act as co-transmitters within an inhibitory neural network of the antennal lobe. The combination of GABA and neuropeptides generates additional biochemical heterogeneity within the population of local interneurons. This heterogeneity is even more pronounced as subpopulations of the AST-A-, Mas-AT-, and FaRP-immunoreactive local interneurons contain at least a second neuropeptide (Berg et al. 2007). Mass spectrometry, furthermore, suggests that up to 50 different peptides may be present in the antennal lobe of *H. virescens* (Berg et al. 2007). Typically, neuropeptides are ligands of G-protein-coupled receptors that indirectly modulate a variety of ion channels in target cells (for a review, see Nässel 2002). Such modulation can change the intrinsic properties of a given neuron and the synaptic strength between neurons, resulting in changing properties of a neuronal network as demonstrated in the crustacean stomatogastric nervous system (Nusbaum et al. 2001; Marder and Tirumalai 2002; Marder and Bucher 2007). The variety of neuropeptides present in sets of local neurons of the antennal lobe may allow for the binding and synchronization of groups of antennal-lobe projection neurons encoding a particular odor mixture. The testing of this idea or alternative hypotheses regarding the contribution of neuropeptides together with GABA in the shaping of olfactory responses of projection neurons will be a challenge for future research.

Comparison with other moth species

The results presented here are largely in accordance with previous findings in *M. sexta*. In both species, GABA-labeled somata are located exclusively in the lateral cell cluster (Hoskins et al. 1986). A distinction between two spatially different sub-populations of somata, however, as described in *M. sexta*, has not been found in *H. virescens*. The general staining patterns in the antennocerebral tracts show striking similarities. In both species, the inner tract is largely free of immunostained fibers, whereas the middle tract displays pronounced immunoreactivity. Our findings of stained fibers in the outer and the dorsal tract and of a few labeled axons passing to the antennal mechanosensory and motor center or subesophageal ganglion are in full agreement with data obtained in *M. sexta* (Hoskins et al. 1986). Moreover, the observation of a few large cell bodies located ventrally in the lateral cell cluster, stained by neuropeptide antisera only, is in accordance with previous findings. In *M. sexta*, two large somata with positions similar to those in *H. virescens* have been labeled with antisera against Mas-AT and FMRFamide but are devoid of GABA immunoreactivity. They have been assigned as somata of projection neurons sending their axons to the tritocerebrum and probably the subesophageal ganglion (Schachtner et al. 2004; Utz et al. 2008).

Non-GABAergic local neurons

Our double-labeling experiments have shown that none of the LomTK-II-immunoreactive somata are GABA-immunoreactive. Since tachykinin-related peptides have previously been demonstrated as being present exclusively in local antennal-lobe neurons and not in projection neurons (Berg et al. 2007), the lack of colocalization with GABA demonstrates the existence of local interneurons that utilize a transmitter substance different from GABA. Behavioral tests in fruit flies have reported reduced olfactory sensitivity in animals lacking TKRPs (Winther et al. 2006).

Recently, the presence of excitatory cholinergic local neurons has been discovered in the antennal lobe of the fruit fly (Shang et al. 2007). These neurons are suggested to act as a general enhancer to guarantee the reliable transmission of olfactory information to the Kenyon cells of the mushroom bodies. Furthermore, a subpopulation of GABA-immunonegative local neurons has been identified in the antennal lobe of the silk moth, *Bombyx mori* (Seki and Kanzaki 2008). Interestingly, the LomTK-II-immunoreactive somata reported here are slightly larger than the other local neurons (Berg et al. 2007), a finding that also applies to the somata of the cholinergic local neurons in the fly (Shang et al. 2007). Whether the few non-GABAergic neurons revealed in these three species constitute corresponding populations remains to be seen.

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