Mas-Allatotropin in the Developing Antennal Lobe of the Sphinx Moth *Manduca sexta*: Distribution, Time Course, Developmental Regulation, and Colocalization with Other Neuropeptides

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ABSTRACT: The paired antennal lobes (ALs) of the sphinx moth *Manduca sexta* serve as a well-established model for studying development of the primary integration centers for odor information in the brain. To further reveal the role of neuropeptides during AL development, we have analyzed cellular distribution, developmental time course, and regulation of the neuropeptide *M. sexta* allatotropin (Mas-AT). On the basis of morphology and appearance during AL formation, seven major types of Mas-AT-immunoreactive (ir) cells could be distinguished. Mas-AT-ir cells are identified as local, projection, and centrifugal neurons, which are either persisting larval or newly added adult-specific neurons. Complementary immunostaining with antisera against two other neuropeptide families (A-type allatostatins, RFamides) revealed colocalization within three of the Mas-AT-ir cell types. On the basis of this neurochemistry, the most prominent type

of Mas-AT-ir neurons, the local AT neurons (LATn), could be divided in three subpopulations. The appearance of the Mas-AT-ir cell types occurring during metamorphosis parallels the rising titer of the developmental hormone 20-hydroxyecdysone (20E). Artificially shifting the 20E titer to an earlier developmental time point resulted in the precocious occurrence of Mas-AT immunostaining. This result supports the hypothesis that the pupal rise of 20E is causative for Mas-AT expression during AL development. Comparing localization and developmental time course of Mas-AT and other neuropeptides with the time course of AL formation suggests various functions for these neuropeptides during development, including an involvement in the formation of the olfactory glomeruli. © 2007 Wiley Periodicals, Inc. *Dev Biol* 68: 123–142, 2008

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INTRODUCTION

The antennal lobes (ALs) of the sphinx moth *Manduca sexta* serve as a well-established model for studying the neuronal development of the primary integration centers for odor information in the brain (Tolbert et al., 2004). ALs of insects compare to olfactory bulbs of vertebrates by sharing their principal morphological organization into so-called olfactory glomeruli, but also by a number of basic physiological properties with respect to information processing (Hildebrand and Shepherd, 1997; Strausfeld and

Hildebrand, 1999; Eisthen, 2002). Another characteristic of ALs and olfactory bulbs is the expression of a variety of neuropeptides (Smith et al., 1993; Caillol et al., 2003; Moody and Merali, 2004; Gutierrez-Mecinas et al., 2005; Schachtner et al., 2005). To further reveal the role of neuropeptides during ontogeny of the ALs, we focus in this study on the temporal expression pattern of the neuropeptide Mas-allatotropin (Mas-AT).

In *M. sexta* the ALs arise during metamorphosis, a hormonally-controlled postembryonic period lasting about 3 weeks. During this time, the whole brain undergoes reorganization and small larval antennal centers (LACs) develop into the adult ALs. AL development in *M. sexta* can be roughly divided into three phases (Oland and Tolbert, 1996; Dubuque et al., 2001). The preparation phase (phase I) lasts about 7–8 days beginning at pupal formation. It includes the birth of all AL neurons, the arrival of the axons of the olfactory receptor neurons (ORNs) in the ALs, and the formation of the protoglomeruli, the sites where the glomeruli form during phase II. The glomeruli formation phase (phase II) lasts about 5 days and is characterized by massive synaptogenesis between the involved neurons. It is assumed, that during phase II a basic network of synaptic contacts within and between the glomeruli is established, which ensures the principal correlation of in and output components of the AL (Dubuque et al., 2001). In contrast to phase II, in phase III, which lasts about 8 days up to adult eclosion, only little synaptogenesis occurs. During this last phase, the glomeruli grow in size between 40 and 130% (Huetteroth and Schachtner, 2005), probably because of the increasing neurite diameters, and the synaptic wiring in the glomeruli is thought to undergo further refinement and maturation (Tolbert et al., 1983; Tolbert, 1989; Dubuque et al., 2001). None of the neurons of the ALs undergo programmed cell death during phases I to III (Schachtner et al., 2004a), and, as all neurons of the ALs are born early in AL development (Hildebrand et al., 1997), the neuronal composition of the ALs does not change throughout formation of the ALs.

Immunocytochemical studies in a diversity of insects have indicated that several neuropeptides, including members of FMRFamide-related peptides (FaRPs), allatotropin, and A-type allatostatins (AST-A) are present in subpopulations of local AL neurons, which are responsible for information processing within and between glomeruli (for review see Schachtner et al., 2005). Mass spectrometric analysis of neuropeptides in the ALs of *M. sexta*, *Heliothis virescens*, the honeybee, and *Tribolium castaneum* even suggest about 40–50 different neuropeptides in the ALs of each of these species (Berg et al., 2007;

Utz et al., 2007; and unpublished). Neuropeptides might operate as cotransmitters of GABA (γ -aminobutyric acid), the principle transmitter of local AL interneurons (Homberg and Müller, 1999). Concerning AL neurochemistry, the ALs of *M. sexta* are among the best studied (for review see Schachtner et al., 2005; Utz et al., 2007). Mapping of RFamides and AST-As throughout development of the sphinx moth ALs revealed unique developmental acquisition patterns in defined sets of neurons (Schachtner et al., 2004b; Utz and Schachtner, 2005). The occurrence of these neuropeptides is developmentally regulated by the steroid hormone 20-hydroxyecdysone (20E). Furthermore, for each neuropeptide family, the temporal pattern of their occurrence correlates with defined developmental phases, suggesting defined roles during AL development, including formation of olfactory glomeruli.

Mas-allatotropin, an amidated tridecapeptide (GFKNVEMMTARGF-NH₂) was isolated from heads of pharate adults of *M. sexta* (Kataoka et al., 1989). Since then, allatotropins (-TARGFamides) have been shown to be a highly conserved insect neuropeptide family (for reviews see Elekonich and Horodyski, 2003; Homberg et al., 2004; Nässel and Homberg, 2006). In *M. sexta*, the fall armyworm *Spodoptera frugiperda*, and the Eri silkworm *Samia cynthia ricini*, the allatotropin gene is expressed as at least three mRNAs that differ by alternative splicing (*M. sexta*: Horodyski et al., 2001; Lee et al., 2002; *S. frugiperda*: Abdel-Latif et al., 2003, 2004; *S. cynthia ricini*: Sheng et al., 2007). The three different mRNAs in *M. sexta* encode Mas-AT itself and three allatotropin-like peptides (Mas-ATLs), which show bioactivities like Mas-AT, regarding stimulation of juvenile hormone (JH) biosynthesis in adults and inhibition of active ion transport on the larval midgut epithelium (Horodyski et al., 2001). Northern blot analysis revealed in the pupal brain expression of only one splice variant, which exclusively contains Mas-AT. In the pharate adult and adult brain additional low levels of a second splice variant, containing Mas-AT and Mas-ATL III, are expressed (Lee et al., 2002). A recent mass spectrometric study revealed only the ion signal of Mas-AT throughout AL development and in the adult AL, but no signal corresponding to the predicted mass of Mas-ATL III (Utz et al., 2007). This suggests that the splice form containing Mas-AT and Mas-ATL III is either not expressed in developing and adult ALs or the concentration of Mas-ATL III was too low for being detected. However, except for a single study which suggested the involvement of an allatotropin in photic entrainment of the circadian clock of the cockroach

Leucophaea maderae (Petri et al., 2002), nothing is known about possible functions of this neuropeptide family within brain circuits.

A prerequisite to understand the role of a certain neuromediator during nervous system development is the knowledge of its cellular and temporal localization. To further reveal the role of neuropeptides, we analyzed cell-type specific occurrence and hormonal regulation of allatotropin immunoreactivity during ontogeny of the ALs of *M. sexta*. Most of the Mas-AT-ir neurons are LNs which obtain their immunoreactivity with the beginning of the phase of glomeruli formation. This parallel and the developmental regulation via 20E, makes Mas-AT a candidate molecule for being actively involved during this defined phase of AL development.

With this study, we demonstrate for a third neuropeptide family distribution, time course, and regulation throughout AL development. Among the studied neuropeptides, the Mas-AT antiserum labels so far the largest amount of neurons in the ALs of *M. sexta*, including about one third of the LNs. In so far it was particularly interesting to study the developmental pattern of Mas-AT and to compare it with the earlier studies on RFamides and AST-As. This comparison revealed a third developmental pattern, namely the occurrence for Mas-AT immunoreactivity which contrasts the developmental pattern of the two other antisera.

MATERIALS AND METHODS

Animals

Manduca sexta (Lepidoptera: Sphingidae) were raised on an artificial diet under long day photoperiod (L:D = 17:7) at 26°C in walk in environmental chambers (Bell and Joachim, 1978). Under these conditions, the time required from hatching to pupal ecdysis is about 18 days, and the time from pupal to adult ecdysis is about 20 days. The start of the wandering stage (W0) occurs 3–4 days into the fifth larval instar (L5) and is characterized by the appearance of a red pigment along the heart. The following days are referred to as W1–W4. At about noon of W2, the animals go into a quiescent prepupal stage. Pupal ecdysis occurs on day W4, and the newly formed pupa is designated as day P0. Subsequent days of adult development are counted as P1–P20 and, after adult eclosion, as A0–A4. Larvae and pupae in this study have been staged according to the criteria described in Schwartz and Truman (1983) and Jindra et al. (1997). The criteria involve changes in structures that are either superficial or readily visible through the pupal cuticle under a dissecting microscope. To induce diapause, animals were reared under short day photoperiod (L:D = 12:12; Bell and Joachim, 1978).

Immunocytochemistry

For immunostaining polyclonal rabbit antisera against *M. sexta* allatotropin (Mas AT), *Diploptera* allatostatin 7 (Dip AST7), RFamide, and γ amino butyric acid (GABA), and polyclonal antisera raised in guinea pigs against GABA and the transmembrane form of *Manduca sexta* Fasciclin II (TM MFas II), and monoclonal antibodies from mouse against two ubiquitous synaptic vesicle proteins from *Drosophila* namely synaptotagmin and Synapsin I (SYNORF1) were used. Antiserum against Mas AT was used at a concentration of 1:4000 (No. 13.3.91, kindly provided by Dr. J. Veenstra, University of Bordeaux, Talence, France; Veenstra and Hagedorn, 1993). The antiserum recognizes Mas AT (Kataoka et al., 1989) and *Locusta* myotropin (Veenstra and Hagedorn, 1993), both ending with TARGFamide. Specificity of the anti Mas AT antiserum has been shown by preadsorption of the antiserum with 100 μ M synthetic Mas AT (Bachem, Bubendorf, Switzerland) for 1 h at room temperature, which abolished all immunostaining in *M. sexta* brain sections (data not shown). Preadsorption of the antiserum with 100 μ M synthetic FLRFamide, FMRFamide, or Dip AST7 (all Sigma Aldrich, St. Louis, MO) for 1 h at room temperature had no effect on immunostaining (data not shown). Antiserum against Dip AST7, which is generally thought to recognize AST A, was used at a concentration of 1:10,000 (kindly provided by Dr. H. Agricola, University of Jena, Germany; Utz and Schachtner, 2005). As shown previously by competitive ELISA the serum cross reacts with other members of the AST A peptide family, all characterized by their C terminal Y/FXFGFLamide sequence (Vitzthum et al., 1996). In a noncompetitive ELISA analysis, no cross reactivity of the antiserum was found with corazonin, crustacean cardioactive peptide, FMRFamide, leucomyosuppressin, locustatachykinin II, perisulfakinin, or proctolin (Vitzthum et al., 1996). Preadsorption of the Dip AST7 antiserum with 100 μ M synthetic Dip AST7 (Sigma Aldrich) for 1 h at room temperature abolished all immunostaining in *M. sexta* brain sections, whereas preadsorption of the antiserum with 100 μ M synthetic FLRFamide, FMRFamide (both Sigma Aldrich), or *M. sexta* allatotropin (Bachem) for 1 h at room temperature had no effect on immunostaining (Utz and Schachtner, 2005). Anti RFamide antiserum (#671, used at dilutions of 1:4000 to 1:10,000; Schachtner et al., 2004b) was kindly provided by Dr. E. Marder (Brandeis University, USA). The RFamide antiserum recognizes FMRFamide and FLRFamide peptides (Marder et al., 1987; Kingan et al., 1990), including the three FaRPs identified in *M. sexta* (Kingan et al., 1990, 1996; Miao et al., 1998).

To test whether the three used peptide antisera recognize proteins containing the short amino acid sequences, we performed Western blots. Peptide antisera were used at the same concentrations as for immunocytochemistry and preadsorptions were performed accordingly. We found for all three antisera several bands and preadsorption of the antisera resulted in the same pattern as found with the antiserum only (supplementary material Fig. S1). The results suggest that the bands recognized in the blots are due to

unspecific binding of the peptide antisera. However, as the same preadsorption protocol revealed no staining in brain sections, we conclude that the unspecific bands in the blots resulted from the sample preparation performed during the Western blot procedure.

The polyclonal anti GABA antiserum (NT 108, Protos Biotech, New York, NY) raised in guinea pig was used at a concentration of 1:1000, the polyclonal anti GABA antiserum raised in rabbits (kindly provided by Dr. T.G. Kingan, U.S. Dept. of Agriculture, Beltsville, MD) was used at a concentration of 1:2000. Both antisera were raised against conjugates of GABA covalently coupled to hemocyanin. The specificity of the rabbit GABA antiserum for *M. sexta* tissue has been shown in detail earlier (Hoskins et al., 1986). To demonstrate the specificity of the GABA antiserum raised in guinea pig, we performed double labeling experiments on 40 μ m vibrating microtome sections with both anti GABA antisera. Both antisera label the identical cells (Fig. S2).

The anti TM MFas II was used at a concentration of 1:2000 (kindly provided by Dr. P.F. Copenhaver, OHSU, USA). The specificity of the anti TM MFas II antiserum for *Manduca* nervous tissue was shown by Western blot analysis (Wright and Copenhaver, 2000). Anti synaptotagmin (1:4000, kindly provided by Dr. K. Menon, Caltech, USA) and anti synapsin antibodies (1:100, kindly provided by Dr. E. Buchner, University of Würzburg, Germany) were used to selectively label neuropil structures including olfactory glomeruli (Dubuque et al., 2001; Berg et al., 2002). *M. sexta* synaptotagmin was previously cloned and the specificity of the monoclonal anti synaptotagmin antibody in *Manduca* brain tissue was shown by Western blot analysis and by cross comparison with a polyclonal anti synaptotagmin antiserum recognizing a different region of the protein (Dubuque et al., 2001). Western blot analysis with the monoclonal anti Synapsin I antiserum revealed one double band at a molecular weight of about 70 kDa in pupal (P8) and adult (A0) *M. sexta* brain tissue which is similar to the situation in *Drosophila* larval brain tissue (supplementary material Fig. S3, procedures see below). In homogenates of *Tribolium castaneum* pupal brain tissue only one band around 66 kDa was detected (Fig. S3). The result suggests a high specificity of the antiserum for all tested brain homogenates. The MW of 70 and 74 kDa for two isoforms of Synapsin I of *Drosophila* was shown by Klagges et al. (1996) in Western blots of whole head homogenates. An additional band at 80 kDa as shown by Klagges et al. (1996) was not detected, suggesting that this isoform is not highly enough expressed in larval brain tissue of *Drosophila* to be detected in Western blot.

Goat anti rabbit antibodies conjugated to Cy2 and Cy3, goat anti guinea pig conjugated to Cy3, and goat anti mouse antibodies conjugated to Cy5 were used as secondary antisera (each 1:300; JacksonImmuno Research, West grove, PA). After dissection in cold saline (Weevers, 1966), brains of *M. sexta* were fixed in 4% formaldehyde in phosphate buffered saline for 2 h (5 h using the anti GABA antiserum) at room temperature or overnight at 4°C. For the double labeling with the two GABA antisera brains 0.1%

glutaraldehyde was added to the fixative. After fixation, brains were either embedded in gelatin/albumin, postfixed overnight in 4% buffered formaldehyde and cut at 40 μ m with a vibrating blade microtome (Leica VT 1000S) in the frontal, sagittal or horizontal plane, or processed as whole mount. Immunostaining procedure was performed exactly as described in Schachtner et al. (2004b) or, for the whole mounts, as described in Huetteroth and Schachtner (2005). For triple labeling, the anti Mas AT, anti GABA, and anti synapsin antibodies were applied simultaneously and, like wise, the corresponding secondary antisera. Double labeling with anti Mas AT and anti Dip AST7 or anti RFamide antisera was performed according to a method modified from Negoescu et al. (1994), for double immunolabeling with primary antisera from the same species as recently described in Berg et al. (2007).

Western Blot Analysis

To demonstrate the specificity of the three peptide antisera and of Synapsin I for *M. sexta* tissue, we homogenized the brains of various pupal and adult *M. sexta* stages in ice cold 10 mM Tris HCl (pH 7.4) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), 30 μ M aprotinin, 20 μ M leupeptin, 150 μ M pepstatin). For Synapsin I we additionally homogenized pupal *Tribolium castaneum* and larval (L3) *Drosophila melanogaster* brains. Total protein concentrations of the samples were determined after Bradford (1976). Then, 10 μ g of each sample was boiled for 3 min in an equal volume of reducing sample buffer (500 mM Tris HCl, pH 6.8, containing 4% sodium dodecyl sulfate (SDS), 5% 2 mercaptoethanol, 20% glycerol, and 0.2% bromophenol blue). Samples were run on a discontinuous SDS 12.5% polyacrylamide gel and blotted onto Optitran BA S 83 nitrocellulose membranes (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). The blots were blocked in 0.1M phosphate buffered saline (PBS; pH 7.4) plus 0.1% Tween 20, and 5% Slim.Fast vanilla powder (Slim.Fast, Englewood, NJ) for 2 h at room temperature (RT) and then incubated with the various antisera respectively (dilutions: Mas AT 1:4000, Dip AST7 1:10,000, RFamide 1:4000, Synapsin I 1:500) in PBS plus 0.05% Tween 20 at 4°C overnight. After washing for 45 min in PBS plus 0.05% Tween 20, 1% Triton X 100, and 1% SDS (PBT), the blots were incubated with a 1:5000 dilution of HRP conjugated anti rabbit or anti mouse antibody (JacksonImmuno Research) in PBS plus 0.05% Tween 20 for 2 h at RT. After washing again for 1 h in PBT, the blots were incubated with chemiluminescent substrate according to the SuperSignal directions (PIERCE, Rockford, IL) and exposed to Fuji Super RX film (FUJIFILM, Düsseldorf, Germany).

Hormone Manipulation Experiments

20 hydroxyecdysone (20E, Sigma Aldrich) was dissolved in saline (Ephrussi and Beadle, 1936) to a final concentration of 1 μ g/ μ L. Pupae in stage P1 were chilled on ice for 2 min and then injected once with 15 μ g 20E per gram body

Figure 1 3D reconstructions of Mas AT ir cells during AL development (P4, P7) and in the adult AL. The outlines of the AL are shown in transparent light grey and the developing neuropil (N), glomeruli, and cell groups (LC, lateral cell group; MC, median cell group; AC, anterior cell group) are shown in dark grey. Recently standardized glomeruli are color coded according to Huetteroth and Schachtner (2005; B, base (blue); C, cap (light orange); Cl, club (light green); Cu, cumulus (dark blue); D, disc (red); Db, disc base (green); g20 (light blue); Hs, horseshoe (purple); LPOG, labial pit organ glomerulus (yellow); To, toroid (orange)). Note the continuous presence of the two early Mas AT ir neurons (eATn; blue somata) in all stages shown and their primary neurite leaving the AL [compare with Fig. 5(a)]. The single large median Mas AT ir neuron (ImATn, green soma) can easily be determined from P7/8 onwards. Most Mas AT ir cells, the local Mas AT ir neurons (LATn), reside in the LC, but Mas AT ir cells were also found in the AC (aATn) and outside any cell group (dLATn). The reconstruction of the “arc” of the centrifugal Mas AT ir neuron (CATn) in A0 is shown in light green. See also supplementary material (Fig. S4 animated 3D AL). Scale: 200 μ m.

weight (Schachtner et al., 1999). Control animals were injected with 15 μ L saline per gram body weight. All injections were performed with 100 μ L Hamilton syringes dorsolaterally into the pupal thorax. Wounds were immediately

sealed with melted wax, and animals were taken back to their walk in environmental chambers. 3.5–11 days later, animals were dissected and processed according to the immunocytochemistry protocol described earlier.

Data Processing

Fluorescence was analyzed using a confocal laserscan microscope (Leica TCS SP2). Cell counts were performed at a magnification of 400×. As previously described (Utz and Schachtner, 2005) we used two strategies to obtain numbers of Mas AT labeled cell bodies in the lateral cell group (LC). (1) For up to 35 cell bodies in total, we compared section by section to ensure that each cell was only counted once. (2) For a total of more than 35 cells, we

counted every stained cell body including fragmented somata in each section and used the Abercrombie correction factor to obtain real cell numbers (Abercrombie, 1946). The mean diameter (d_m) of cell bodies (LATn) showing Mas AT immunoreactivity in LCs from stage P7 up to the adult stage was $16.3 \pm 2.7 \mu\text{m}$. Comparing cell diameters between single stages revealed a slight increase in cell diameter between stages P7 to P12 of about $3.5 \mu\text{m}$ ($p < 0.001$ assessed by unpaired *T* test) (stage P7: $d_m = 14.4 \pm 2.0 \mu\text{m}$ ($n = 14$ of 4 ALs); stage P8: $d_m = 14.6 \pm 2.5 \mu\text{m}$ ($n = 44$ of 11 ALs);

Figure 2

stage P9: $d_m = 15.7 \pm 2.3 \mu\text{m}$ ($n = 113$ of 17 ALs); stage P10: $d_m = 16.3 \pm 2.7 \mu\text{m}$ ($n = 89$ of 12 ALs); stage P11: $d_m = 16.8 \pm 2.7 \mu\text{m}$ ($n = 58$ of 10 ALs); stage P12: $d_m = 18.1 \pm 3.0 \mu\text{m}$ ($n = 72$ of 11 ALs)). Between stages P12 and adult we found a slight decrease in cell diameters of about $2.6 \mu\text{m}$ ($p < 0.001$ assessed by unpaired *T* test) (stage P14 P16: $d_m = 17.8 \pm 2.9 \mu\text{m}$ ($n = 66$ of 15 ALs); pharate: $d_m = 16.8 \pm 2.0 \mu\text{m}$ ($n = 76$ of 17 ALs); adult: $d_m = 15.5 \pm 2.6 \mu\text{m}$ ($n = 152$ of 21 ALs)). Since changes in diameter of the somata were only small, we decided to use one correction factor for whole developmental study. Thus, with a section thickness of $40 \mu\text{m}$ and a d_m of cell bodies of $16.3 \mu\text{m}$, the Abercrombie correction factor used for cell counts in stage P7 up to adult was 0.71.

3D-Reconstruction

Digitization and 3D reconstructions of wholemount ALs labeled with anti Mas AT and anti synapsin antisera, visualized by corresponding secondary antisera coupled to Cy2 and Cy5, respectively, were performed with the Leica TCS SP2 and the 3D reconstruction software AMIRA 3.1 4.1.1 (Mercury Computer Systems, Chelmsford, MA) according to the protocol described in Huetteroth and Schachtner (2005). The AL outlines and cell group borders were labeled based on background staining of either data channel.

The primary neurite of the early Mas AT ir neuron (eATn) in the P4 AL was reconstructed using the skeletonize plugin tool for AMIRA (Schmitt et al., 2004).

RESULTS

Mas-Allatotropin-ir Neurons During AL Development

On the basis of morphology and appearance during formation of the ALs, seven major types of Mas-AT-immunoreactive (ir) cell types could be distinguished (Figs. 1 and 2; Table 1). In the 5th instar larva (L5), the LAC contains numerous Mas-AT-ir fibers stemming from a cluster of about 10 smaller Mas-AT-ir larval LNs [ILATn, Fig. 2(B)]. The ILATn ceased their immunoreactivity between wandering stages W3 to W4. In contrast, two large neurons with strong Mas-AT immunoreactivity (early Mas-ATn or eATn) also present in the LAC of the 5th instar larva persisted during metamorphosis into the adult AL [Fig. 1, blue cells; Fig. 2(B,C)]. Typically, the eATn have cell bodies with diameters larger than $25 \mu\text{m}$ located in the ventral part of the lateral cell group. Without any projections in the glomeruli, the eATn send their

Figure 2 Confocal images of $40 \mu\text{m}$ sections showing frontal views of Mas AT immunoreactivity during AL development. The dashed line encircles the lateral cell group (LC), the solid line encircles the neuropil of the LAC (B) or of the developing AL (C, D). (A) Schematic of the AL presenting the orientation (frontal) of the sections B–I with respect to the position of the three cell groups (grey); LC, median cell group (MC), anterior cell group (AC). Orientation bars: D, dorsal; L, lateral. (B) The two early Mas AT ir neurons (eATn, arrowheads) and about 10 smaller LNs (ILATn, small arrows) label in the larval (L5) LC. (C) In pupal stage P5, the two eATn (arrowheads) are the only neurons labeled in the LC no staining in the neuropil could be detected. (D) P8/9: Local Mas AT ir neurons (LATn), which appear from stage P6/7 onwards and the large median Mas AT ir neuron (ImATn, arrowhead; compare with Fig. 1). Note the densely packed neurites, mainly stemming from the LATn, projecting into the central coarse neuropil (CN) and the dense Mas AT immunoreactivity in the forming glomeruli (asterisks). (E) LATn innervating all glomeruli including the male specific macroglomerular complex (MGC) in a P14 AL. (F) Adult AL of a female *M. sexta*; the ImATn can be distinguished from the LATn by its typical median position, the leaf like basal primary neurite, and the larger cell body (arrowhead, inset). (G) One Mas AT ir cell body in the anterior cell group (arrowhead) 10 days after pupal ecdysis. (H) In pupal stage P11, two dorsolateral Mas AT ir neurons (dlATn, arrowheads, inset) send their neurites (arrow) towards the AL neuropil. Typically, the neurites stained only a short distance from the cell body (arrow). (I) The “arc” neurite stemming from the centrifugal Mas AT ir neuron (CATn) gives rise to arborizations which intermingle with other Mas AT ir fibers in the AL neuropil (arrows). The arc shaped neurite is formed by a large fiber entering/leaving the AL via the inner antenno cerebral tract and entering/leaving the AL from/towards the tritocerebrum (arrowheads). Main figure shows a frontal view, inset a sagittal view. Note one of the eATn (double arrowhead) with its primary neurite leaving the AL. (J) Summary of the developmental time course for each of the Mas AT ir neuron types. The grey bar accounts for our hypothesis that the about 10 larval LNs (ILATn) might regain their Mas AT identity during pupal development. The x axis shows a simplified time scale starting during L5 larva and ending with early adult. W4, 4 days after beginning of wandering stage which is equivalent to a prepupa shortly before pupal formation. AN, antennal nerve; G, glomerulus; OL, optic lobe; SEG, subesophageal ganglion. Scale: $50 \mu\text{m}$ in B–D, G–I; $100 \mu\text{m}$ in E, F; $25 \mu\text{m}$ in insets in F, H, I.

Table 1 Mas-AT-ir Neuron Types

Mas AT ir Neuron Type	Number ^a		Origin	Identical To
Local neurons				
ILATn	~ 10		Larval	
LATn	P9/10	Adult		
Mas AT	~ 20 to 30	~ 30	Adultsp.	
Mas AT + AST A	~ 30	~ 30	Adultsp.	Type Ia AST A ^b
Mas AT + RFamide	~ 20	~ 40	Adultsp.	Type I RFamide ^c
Projection neurons				
eATn	2		Larval	Type III RFamide ^c
lmATn	1		Larval	Type II RFamide ^c
aATn ^d	2 4		Adultsp.	
dlATn ^d	1 2		Adultsp.	
Centrifugal neurons				
CATn	1		Adultsp.	

Most of the Mas AT ir neurons are LNs (LATn), which can be subdivided into three groups with respect to their different neuropeptide chemistry. Adultsp., adultspecific.

^aNumber per AL.

^bUtz and Schachtner, 2005.

^cSchachtner et al., 2004b.

^dAssignment as PN according to the location of the cell bodies.

primary neurite out of the AL towards the tritocerebrum. Owing to the numerous other Mas-AT-ir processes in the tritocerebrum, we could not further trace the neurites of the eATn.

In contrast to the ILATn and eATn, the other cell types did not obtain Mas-AT staining until after 6/7 days post-pupal eclosion (P6/7). The most prominent cell type, the local Mas-AT-ir neurons (LATn), consists exclusively of LNs located in the lateral cell group [LC; Figs. 1 and 2(D-H)]. The LATn with their smaller cell body diameter ($<20 \mu\text{m}$) could be clearly distinguished from the eATn with their large neurite leaving the AL [Figs. 1 and 6(J)]. Numbers of the LATn linearly increase between stages P6/7 and P13 from 0 to about 100 neurons [Figs. 2(J) and 3]. From P13 into adult stages, numbers of the LATn remained constant [Fig. 3]. A third cell type, the large median Mas-AT-ir neurons (lmATn), consists of a single cell with a large soma located in the median part of the LC and sends its neurite similar to the eATn into the tritocerebrum where it intermingled with other Mas-AT-ir fibers [Fig. 1, green cell; Fig. 2(D,F)].

Two other cell types (consisting of one to three cells) located outside the LC in anterior and dorsolateral positions in the AL and termed accordingly (aATn, dlATn), occurred not until after stage P7/8. Mas-AT staining in both cell types was always confined to the cell body and a neurite projecting towards the glomerular neuropil. Furthermore, aATn and dlATn were not observed in all, but a certain percentage of the preparations (of a total of 93 analyzed ALs: aATn, 45.2%; dlATn, 23.7%).

One Mas-AT-ir centrifugal neuron (CATn) could be identified from stage P7 onwards. The CATn consists of a large arc-shaped neurite in the basal median part of the AL, from where smaller fibers inter-

Figure 3 Developmental time course of numbers of Mas AT ir cell bodies in the lateral cell group (LC), mainly including the local Mas AT ir neurons (LATn). Acquisition of Mas AT immunostaining in LATn occurred in a single step from 0 up to about 100 between stage P6/7 and P13. This prominent rise of number of the Mas AT ir neurons parallels the increasing titer of the hormone 20E in the hemolymph (inset) and the main wave of synaptogenesis (phase II, shaded area). Each data point indicates mean number $\pm$ standard deviation (n numbers of ALs, dotted line adult eclosion at P20/A0). Before stage P6 only the two eATn were labeled. Inset: Hemolymph concentrations of 20E during pupal adult transition as reported by Warren and Gilbert (1986). The arrow indicates the time of 20E injection at stage P1. The small circles indicate times when injected animals were dissected and processed for immuno cytochemistry (see Fig. 5).

mingled with the other Mas-AT-ir fibers in the coarse neuropil [inset in Fig. 2(I)]. The large neurite could be followed on one side into the inner antenno-cerebral tract (IACT, Homberg et al., 1988) and on the other side it was leaving the AL towards the tritocerebrum [Figs. 1 and 2(I)]. Owing to the fact that the immunostained fiber in the IACT usually faded out after a short distance and that the neurite in the tritocerebrum intermingled with numerous Mas-AT-ir fibers we could not trace the neurite back to its cell body.

Mas-AT-ir Projections in the Developing AL Neuropil

Sparse Mas-AT-ir projections appeared in the developing AL-neuropil 6–7 days after pupal eclosion (P6/7), which is the time when formation of the protoglomeruli begins. To examine whether Mas-AT-ir fibers enter the protoglomeruli, we labeled ingrowing axons of ORNs with an antiserum against the transmembrane domain of *M. sexta* fasciclin II (TM-MFas II). A recent study by Higgins et al. (2002) demonstrated the early expression of TM-MFas II in a subset of ORNs (including the axons) before glomerulus formation; this was taken as evidence for a role of TM-MFas II during the sorting and guidance of axons. Because of its early appearance in ORN axons, TM-MFas II can be used as a reliable marker for the formation of protoglomeruli, the templates for the later-forming glomeruli. In *M. sexta*, protoglomeruli are thought to be initially formed by axon endings of ORNs and uniglomerular PNs (uPNs; Malun et al., 1994; for a review, see Tolbert et al., 2004). Triple-immunolabeling with antisera against Mas-AT, TM-MFas II, and synapsin revealed between stage P6/7 to P7/8 no overlapping areas labeled with Mas-AT and TM-MFas II antisera (Fig. 4). Mas-AT-ir fibers stayed within the confinement of the central neuropil visualized by the synapsin antiserum [Fig. 4(A,A')]. At the beginning of phase II (P7/8), Mas-AT-ir fibers start to project into the basal part of the newly forming glomeruli [Fig. 4(B,B')] to eventually display finger-like protrusions from more basal to distal parts of each glomerulus in later developmental stages and in the adult, a pattern typical for LNs [Fig. 2(D,F); Schachtner et al., 2004b; Utz and Schachtner, 2005].

Hormone Manipulation

The rising number of LATn during AL development parallels the increasing titer of 20E in the hemolymph

from P4 to P9 (inset in Fig. 3; Warren and Gilbert, 1986). To determine whether 20E is responsible for the increasing numbers of LATn, we injected 15 µg/g body mass of 20E into the hemolymph of stage P1 pupa (Schachtner et al., 1999, 2004b). Pupae were dissected 3.5–11 days after 20E injection and processed for immunocytochemistry (inset in Fig. 3). 3.5, 5.5, and 7 days after injection, the numbers of the LATn had increased to a level usually reached 2 days later during normal development. Eleven days after injection, the numbers of LATn in controls and 20E treated animals were the same as expected from normal development [Fig. 5(A,B,F)]. Across all preparations not only the soma numbers labeling with the Mas-AT antiserum increased after 20E injection but also the neurites of the cells showed strong staining [Fig. 5(B,C)].

As already shown in previous studies, formation of the olfactory glomeruli indicated by synaptotagmin immunostaining was advanced by 3–4 days compared to normal development [Fig. 5(A,B); Schachtner et al., 2004b; Utz and Schachtner, 2005]. A few of the animals which were dissected 3.5 days and all of the animals which were dissected 5.5 days after 20E injection showed an earlier occurrence of Mas-AT immunoreactivity in the ImATn and in the CATn, which normally do not appear before stage P7/8 [Fig. 5(D,E)]. Immunostainings of two pupae which had been in diapause for several weeks revealed only the two eATn, but no other Mas-AT-ir cells in the LC [Fig. 5(C)].

Colocalization of Mas-AT with GABA, RFamides, and A-Type Allatostatins

Most, if not all LNs in the LC are GABA-ir (Hoskins et al., 1986). Double immunostaining with antisera against GABA and Mas-AT demonstrated that in the LC, the eATn and the ImATn are not GABA-ir, while all of the LATn stain positively with the anti-GABA antiserum [Fig. 6(A–C)]. Inspecting the AL output tracts in horizontal, sagittal, and frontal sections, as well as in wholemounts revealed typically one Mas-AT-ir fiber in the IACT, belonging to the CATn, and, towards the SEG, the CATn fiber and two to three faintly labeled fibers very likely belonging to the eATn and the ImATn. Independently to the GABA double stainings, these results underline the identity of the LATn as LNs.

Schachtner et al. (2004b) described a large single RFamide-ir PN (type II) and two RFamide-ir somata (type III) in the LC which persisted from the larva into adulthood. Double labeling with anti-RFamide

and anti-Mas-AT antisera showed that the ImATn and the two eATn colocalize RFamide and Mas-AT staining and are thus identical with the type II and III RFamide neurons, respectively [Fig. 6(D F,J,K)]. For the LATn, double immunostaining revealed that about 17 ($\sim 20\%$ of the LATn; $n_{AL} = 6$) in P10 and

about 37 (~35% of the LATn; $n_{AL} = 5$) in adults (A0/1) additionally to their Mas-AT staining exhibited RFamide immunoreactivity [Fig. 7(A)]. Corresponding to the RFamide neurons in the LC, about 65% in P10 and about 59% in A0/1 showed colocalization with Mas-AT immunostaining.

Figure 5

Double immunolabeling with antisera against Mas-AT and AST-A revealed colocalization in many LATn [Fig. 6(G I)], but not in the other Mas-AT-ir cell types. The degree of colocalization for the LATn was examined in four developmental stages [Fig. 7(B)]. In stage P9/10 about 43% ($n_{AL} = 8$), in stage P13 about 38% ($n_{AL} = 10$), in pharate animals about 32% ($n_{AL} = 8$), and in adults (A0) about 30% ($n_{AL} = 6$) of the Mas-AT-ir neurons additionally labeled with the anti-AST-A antiserum. For about 30–40 AST-A-ir LC-neurons in the examined stages, the percentage of neurons which colocalized both immunoreactivities was about 70% in stage P9/10 ($n_{AL} = 8$), about 83% in stage P13 ($n_{AL} = 10$), about 89% in pharates ($n_{AL} = 8$), and about 81% in adults (A0; $n_{AL} = 6$). The AST-A-ir processes of a recently described centrifugal neuron are devoid of Mas-AT immunoreactivity [Fig. 6(G I); Utz and Schachtner, 2005].

To test the possibility whether all three neuropeptide antisera (Mas-AT, AST-A, RFamide) colocalize in the same neurons, we double labeled P10 and adult animals with the anti-AST-A and the anti-RFamide antisera (Fig. 6L). The result showed no overlap between these two antibody stainings in the LC, sug-

gesting that local AL neurons contain a combination of two of the three neuropeptides but not all three.

DISCUSSION

Specificity of the Mas-Allatotropin Antiserum

The polyclonal anti-Mas-AT antiserum used in this study recognizes peptides C-terminally ending with -TARGFamide (Veenstra and Hagedorn, 1993). In *M. sexta*, a single gene encodes for Mas-AT and for three allatotropin-like peptides (ATL I–III), which originate from three different mRNA splice-variants (Horodyski et al., 2001). Structurally, the three Mas-ATLs differ at their C-terminus by two to three amino acids from the conserved -TARGFamide of Mas-AT. Northern blot analysis suggested that in pupal and in adult brains predominantly the mRNA containing only Mas-AT and low amounts of the mRNA containing Mas-AT and Mas-ATL III are expressed (Lee et al., 2002). A recent mass spectrometric analysis revealed only Mas-AT, but none of the Mas-ATL

Figure 5 Hormone manipulation experiments and diapausing animals. Confocal images showing Mas AT (red, A–E) and synapsin (blue, A–C) immunoreactivity. For better visualization of the outline of the AL, Nomarski contrast images of the sections are added in A–C (grey). All frontal views if not stated otherwise. (A, B) Mas AT immunoreactivity (red) in ALs after injection of the vehicle alone (A) and after 20E injection (B) at stage P1 and dissection 3.5 days later at pupal stage P4/5. (A) After control injection, ALs were indistinguishable from ALs of untreated animals at stage P4/5. In the LC (dashed line), the two early AT-ir neurons (eATn) are labeled with their neurites leaving the AL. Typical for stage P4/5, the AL neuropil (blue, labeled with an antiserum against synapto tagmin) is undifferentiated and devoid of Mas AT immunostaining. (B) By 3.5 days after 20E injection, the AL neuropil resembles the pattern normally observed about 2 days later (P6/7) with the Mas AT immunoreactivity concentrated in an outer shell area (violet: colocalization of synapto tagmin and Mas AT). In the LC (dashed line), LATn are labeled. (C) Mas AT immunostaining in a diapausing animal revealed only the eATN in the lateral cell group. (D, E) Two examples of Mas AT immunoreactivity after 20E injection at stage P1 and dissection 5.5 days later at pupal stage P6/7. (D) 5.5 days after 20E injection, the AL neuropil resembles the pattern normally observed about 2 days later (P8/9) with the Mas AT-ir fibers projecting into the basis of the forming glomeruli [compare with Fig. 2(D)]. In the LC (dashed line), LATn and the ImATn (arrow) are labeled. (E) Oblique view, showing the fibers of the cATn entering/leaving the AL (arrowheads). In the LC, the ImATn (arrow) and LATn are labeled. (F) Numbers of Mas AT-ir neurons in the lateral cell group after hemolymph injection of 20E at P1 and dissection 3.5 days (P4/5) to 11 days (P12) later (see inset in Fig. 3). 3.5–7 days after 20E injection, hormone treated animals (open circles, means $\pm$ SD) showed a significant increase in the numbers of Mas AT-ir neurons compared with controls (filled circles, means $\pm$ SD) injected with saline ($***p < 0.001$ for each data set as assessed by unpaired *t* test). Compared with the normal developmental time course of Mas AT-ir cell numbers (Fig. 3), controls gave the expected cell numbers, whereas 20E injected animals showed a developmental advancement of about 2 days. Eleven days (P12) after 20E injection, cell numbers were not significantly different (n.s., $p > 0.05$) from control injected animals because, from stage P12 onwards, Mas AT-ir cell numbers are more or less constant (compare with Fig. 3). Scale: 40 μ m (A–E).

Figure 6 Confocal images of 40 μm sections showing triple immunostaining of Mas AT immunoreactivity (green, in (L) RFamide ir cells are shown in green) and synapsin (blue) in the lateral cell group (LC) combined with antisera against GABA, RFamide or AST A (red). In (B), an additional Nomarski contrast image is shown to better visualize the outline of the AL (grey). (A C) Adult *Manduca* male AL. Comparing Mas AT ir cell bodies (A) and GABA ir cell bodies (C) revealed that all cells containing Mas AT staining also contain GABA immunoreactivity (B, overlay). (D F) AL at pupal stage P6 showing the two early AT neurons (eATn; arrows) which are also labeled with antisera against RFamide. One large neuron, known as RFamide type II cell (Schachtner et al., 2004b), is at this stage devoid of Mas AT immunoreactivity (arrowhead in E, F). Note the forming neuropil of the AL (dashed line) which faintly stains with the antibody against synaptotagmin (E). (G I) LC of an adult labeled with antisera against Mas AT (G) and AST A (I). The overlay (H) shows a subpopulation of LATn labeling with both antisera (large arrowheads), while the ImATn only show Mas AT immunoreactivity (arrow). Note that the AST A ir fibers and varicosities stemming from centrifugal type III AST A ir neurons (small arrowheads; Utz et al., 2005) are devoid of Mas AT immunoreactivity. The AST A type III varicose meshwork is easily distinguishable from projections of the LNs, which typical for this neuron type project mainly into the basal area of each glomerulus. (J, K) In stage P10 (J) and adult (K) ALs, an antiserum against RFamide labels a subpopulation of the Mas AT ir neurons including LATn (small arrows), eATn (large arrow), and ImATn (large arrowhead). The small arrowhead points the isthmus of the OATC which labels only with the RFamide antiserum. (L) Typically, as shown in an adult LC, RFamide staining (green) never colocalizes to cells containing AST A immunoreactivity. Scale: 100 μm (A C), 50 μm (D K).

Figure 7 Colocalization of neuropeptides in local Mas AT neurons (LATn). (A) Numbers of LATn labeling with antisera against RFamide (grey), Mas AT (black) and both antisera (white) in stage P10 ($n = 6$ ALs) and in adult ALs ($n = 5$ ALs). (B) Numbers of LATn labeling with antisera against AST A (grey), Mas AT (black) and both antisera (white) in stage P10 ($n = 9$ ALs), P12 ($n = 10$), pharates ($n = 8$), and in adults ($n = 6$).

peptides in developing and adult ALs (Utz et al., 2007). This strongly suggests that the used antiserum recognizes selectively Mas-AT and no other neuropeptide.

Identity of Mas-AT-ir Neurons

Each AL of *M. sexta* contains three well-defined cell groups which house the somata of most of the LNs and PNs. All three cell groups can be easily recognized already early in AL development (Schachtner et al., 2004b). The largest of the cell groups, the lateral cell group, contains all LNs of the AL (about 360) and the largest group of PNs (about 600), while the other two cell groups contain only PNs; the median cell group about 200 and the anterior cell group about 20 PNs (Homberg et al., 1988). We describe Mas-AT immunoreactivity in seven different types of AL neurons, with four types located in the lateral cell group (lLATn, eATn, LATn, lmATn), one type located in the anterior cell group (aATn), one type located outside of any AL cell group (dlATn), and a centrifugal neuron (CATn). Owing to the projection pattern observed, the lLATn and the LATn belong to the LNs, while the eATn and the lmATn are PNs. For the neurons located outside the lateral cell group the situation is unclear. These cell types occur only in about 20–40% of the preparations, and their localization is varying. Immunostaining in the neurites of these cells typically faded out after a short distance from the soma and thus we cannot attribute them as LNs or PNs. However, as the aATn localize to the anterior cell group, they very likely are PNs because all neurons of this cell group are thought to be uniglo-

merular PNs (Homberg et al., 1988). Owing to the fact that all neurons outside the LC belong to the group of PNs, it seems very likely that also the dlATn belong to this group. The cell body of the large arc-shaped neurite of the CATn could not be traced by following the neurites in the IACT or the tritocerebrum. The “arc” formed by this neuron resembles a recently described centrifugal neuron immunoreactive to an antiserum against AST-A (Utz and Schachtner, 2005). We speculate that analogous to the AST-A-ir centrifugal neuron, the soma of the CATn could be located in the SEG.

For *M. sexta* we have a wealth of information on the developmental fates of identified neurons in the metamorphosing nervous system (for a review, see Truman, 1996a). Most neurons of the adult brain that differentiate during metamorphosis arise from neuroblasts that have been arrested during early larval stages and that start a second phase of proliferation in later larval stages. Among these cells are most of the neurons of the developing optic lobes and ALs, which are both newly formed during metamorphosis (adult specific neurons; Monsma and Booker, 1996a,b; Hildebrand et al., 1997; Champlin and Truman, 1998a, 2000). Other neurons of the CNS, which serve functions in the larva, are remodeled during metamorphosis to fit the requirements of the adult animal (larval neurons; Truman, 1996a; Weeks, 1999). A third fate of neurons during metamorphosis is programmed cell death, which has been described in many regions of the insect CNS (Weeks, 1999), but can be excluded for *M. sexta* AL development as shown by TUNEL-labeling experiments (Schachtner et al., 2004a).

Mas-AT immunoreactivity in the eATn persisted from the larva throughout pupal development and

thus these neurons can clearly be accounted for larval neurons. In contrast, in the pupa all other Mas-AT neuron types gain immunoreactivity not before stage P6/7. Judged from the acquisition of Mas-AT immunostaining these neuron types are adult specific neurons that are newly born during postembryonic development to serve functions in the adult (Truman, 1996a). In a few examples, however, it has been demonstrated that neurons can change their peptide identity (Loi and Tublitz, 1993; Tublitz and Loi, 1993; Witten and Truman, 1996). This raises the possibility, that some of the neurons could be larval neurons that change their peptide identity during metamorphosis. For a subpopulation of the LATn, the latter aspect is supported by the finding of a group of about ten Mas-AT-ir LNs (lLATn) which innervate the LAC. These larval Mas-AT-ir neurons lose their immunoreactivity during wandering stages W3 and W4. This strongly suggests that the about 100 LATn which become Mas-AT-ir from stage P6/7 to P13 consist of a mixed population of about 10 larval and about 90 adult specific LNs. A similar finding has been described for GABA-ir neurons (Homberg and Hildebrand, 1994). In the larva about 70, mostly LNs of the LAC display massive GABA-immunoreactivity. The immunostaining ceases during the wandering phase and around stage P8 over 400 mostly local AL neurons became GABA-ir. The authors could not find any typical signs of neuronal degradation in the AL of larval and wandering stages and thus suggested that the larval GABA-ir neurons regain their GABA-identity during metamorphosis. Very interestingly, the lMATn could be identified as a larval neuron by its colocalization with another neuropeptide antiserum (type II RFamide neuron (Schachtner et al., 2004b), for further discussion see later). The CATn were traced from stage P7/8. However, so far all centrifugal neurons which have been described during metamorphic development belong to the group of larval neurons (e.g. 5HT-ir and AST-A-ir centrifugal neurons; Kent et al., 1987; Utz and Schachtner, 2005). Thus it remains to be shown whether the CATn belongs to the larval or the adult specific group of neurons.

Time Course and Developmental Regulation of Mas-AT

The developmental occurrence, decrease, and increase of Mas-AT-ir neurons parallel fluctuations of the ecdysteroid titer in the hemolymph suggesting that the ecdysteroids could be causative for Mas-AT regulation. Transition from the 5th instar larva to pupa requires two surges of ecdysteroids, the commit-

ment peak and the larger prepupal peak (Riddiford, 1985).

The disappearance of the lLATn during wandering stages coincides with the prepupal peak, which is known to initiate prepupal changes, including the reorganization of neuronal networks (Levine et al., 1986; Levine, 1989; Truman and Reiss, 1995) and alterations in neuroactive substances (Loi and Tublitz, 1993; Tublitz and Loi, 1993; Witten and Truman, 1996; Žitňan et al., 1999). The occurrence of Mas-AT-ir cell types and the subsequent increase of numbers of the LATn coincide with the change in circulating ecdysteroids from primarily α -ecdysone to 20E during pupal development (inset in Fig. 3; Warren and Gilbert, 1986). Since we are particularly interested in pupal development, we have experimentally shifted the onset of the pupal 20E peak by 20E injection into an earlier developmental stage. This manipulation resulted in the precocious appearance of the LATn, the lMATn and the CATn (Fig. 5) and thus clearly suggests a regulatory role of the pupal 20E rise for Mas-AT expression in at least three different neuronal types of the AL. Besides, the cell somata also the neurites showed robust staining with the Mas-AT antiserum after 20E injection [Fig. 5(B,C)]. This finding clearly suggests that the Mas-AT-ir neurons are involved in the increased neuropil formation after 20E manipulation. The two cell types located outside the LC have not been examined in this context, because of unreliable occurrence of at least Mas-AT immunoreactivity during normal development. We account this irregular appearance to neurons which exhibit individual variability of their soma localization.

The regulatory role of 20E is further supported by our findings in diapausing pupa, which lack the pupal ecdysteroid peak and which house only the two eATn but none of the other Mas-AT cells in the LC [Fig. 5(C)].

We have previously demonstrated a similar effect of 20E injections on the expression of AST-A and RFamides during AL development (Schachtner et al., 2004b; Utz and Schachtner, 2005). Furthermore, in both studies, synaptotagmin staining has revealed that not only the presence of the neuropeptides, but also the formation of glomeruli occurred earlier following 20E treatment than during normal development. Moreover, 20E injections early in metamorphosis lead to elevated concentrations of the second messenger molecule cyclic guanosine monophosphate in LC neurons; during normal development, this does not occur before stages P7/8 (Schachtner et al., 1998, 1999). Upregulation of transmitter and receptor expression could either be directly influenced by 20E

or indirectly via mechanisms induced by 20E in the course of inducing AL development. However, the current study together with our previous findings (Schachtner et al., 2004b; Utz and Schachtner, 2005) strongly supports a major role of 20E in the orchestration of metamorphic AL development with respect to glomeruli formation and transmitter and receptor acquisition.

Effects of the pupal 20E peak on the metamorphic development of the nervous system of *M. sexta* have been shown in several other studies: the regulation of the fusion of thoracic and abdominal ganglia (Amos et al., 1996), the control of cell proliferation during the genesis of the optic lobes and the retina and also of the programmed cell death of optic lobe neuroblasts (Champlin and Truman, 1998a,b, 2000). The 20E peak also regulates the pupal expression of tyramine β -hydroxylase, an essential enzyme for octopamine biosynthesis (Lehmann et al., 2000).

Colocalization of Mas-AT with GABA, RFamides, and A-Type Allatostatins

GABA is believed to be the principal transmitter of LNs in the ALs of insects (for a review see Schachtner et al., 2005). In *M. sexta* most of the GABA-ir neurons are LNs, while a small subpopulation belongs to the group of PNs (Hoskins et al., 1986; Homberg et al., 1989). Double labeling with Mas-AT and GABA antisera revealed that in the pupa and in the adult only one Mas-AT-ir type, the LATn, displays additional GABA immunoreactivity. This result supports our morphological finding that very likely all of the LATn belong to the group of LNs.

Double labeling with anti-Mas-AT and anti-RFamide antisera revealed that the two eATn, the lmATn, and a subpopulation of the LATn colocalize RFamide and Mas-AT immunoreactivity [Figs. 6(D,F,J,K) and 7(A)]. Comparing these results with a previous study on RFamides during *M. sexta* AL development revealed that the lmATn and the eATn are identical with the type II and type III RFamide neurons (Schachtner et al., 2004b). The single type II RFamide neuron is a persisting larval neuron, which according to our finding gains additional Mas-AT immunoreactivity at stage P7/8. This result not only identifies the lmATn as a larval neuron, but also is an example for a local peptidergic neuron which gains an additional neuropeptide identity during metamorphosis. The finding that the eATn colocalize RFamide identifies the type III RFamide neurons as PNs, which could not be determined in the earlier study from Schachtner et al. (2004b).

From double labeling experiments with antisera against GABA and RFamide, it has been concluded that about 10 RFamide-ir neurons in P10 (Schachtner et al., 2004b) and about 20 RFamide-ir neurons in adults (Homberg et al., 1990) in the lateral cell group might be PNs responsible for the staining in the outer antenno-cerebral tract (OACT) and the isthmus of the AL. Our double staining experiments revealed that the fibers in the root of the OACT stained selectively with the RFamide antiserum but not with the Mas-AT antiserum [Fig. 6(J,K)]. Furthermore, about 10 RFamide-ir neurons in P10 and about 25 RFamide-ir neurons in adults are devoid of Mas-AT immunoreactivity [Fig. 7(A)]. These results strongly suggest that Mas-AT and RFamide antisera colocalize exclusively in LNs, but not in PNs.

Double labeling experiments with antisera recognizing Mas-AT and members of the AST-A family of neuropeptides revealed that throughout development about 30 neurons (about 30% of the Mas-AT-ir neurons or about 75 % of the AST-A-ir neurons) show additional Mas-AT and AST-A immunoreactivity. This result suggests that during AL development about 30 local AL neurons have in addition to their principal transmitter at least two neuropeptides at their disposal. Double labeling experiments using antisera recognizing AST-A and RFamides revealed in stage P10 and in adult animals no colocalization.

Summarizing, our double labeling experiments clearly demonstrate for the LATn that there are at least three different populations of GABA-ir and Mas-AT-ir LNs: one group which additionally expresses RFamides, a second group which additionally expresses AST-As, and a third group which expresses Mas-AT but neither one of the two other neuropeptide families (Table 1).

A recent study on the distribution of various neuropeptides in the adult AL of the noctuid moth *Heliothis virescens* revealed about the same numbers of neuropeptide immunoreactive neurons in the lateral cell group for the same three antisera used in the various *M. sexta* studies (Mas-AT: ~87; AST-A: ~47; RFamide: ~64; Berg et al., 2007). Double immunostainings with antisera against Mas-AT and AST-A or RFamide antisera using the same method as in the current study, revealed a similar number of neurons which colocalize Mas-AT and AST-A (~32) but only about half of the number which colocalize Mas-AT and RFamide (~15) immunoreactivity. Spingid and noctuid moth belong both to the group of Macrolepidoptera and have been evolutionary separated in the Paleocene about 65 Mio years ago (Grimaldi and Engel, 2005). The astonishing similarity observed in the anatomy of the neuropeptidergic systems between

these two species argues for a high conservation concerning the function of defined neuropeptides in the AL network within the Macrolepidoptera.

Possible Roles of Mas-AT and Other Neuropeptides During AL Development

Immunocytochemical studies in a diversity of insects have indicated that several neuropeptides are present primarily in subpopulations of local AL neurons (for a review see Schachtner et al., 2005). Direct profiling of ALs of several insects including *M. sexta*, *Heliothis virescens*, *Apis mellifera*, and the beetle *Tribolium castaneum* by MALDI-TOF mass spectrometry suggests about 40–50 different neuropeptides in the ALs of each of these species (Berg et al., 2007; Utz et al., 2007; and unpublished). Neuropeptides have often been considered as cotransmitters that are released in concert with a principal transmitter (Vilim et al., 2000; Nässel, 2002). In general, corelease is thought to enhance the repertoire of synaptic interactions within a neuronal network (for a review see Nusbaum et al., 2001). In the AL, neuropeptides might operate as cotransmitters of GABA (γ -aminobutyric acid), the principle transmitter of LNs (Homberg and Müller, 1999), but the function of cotransmission has so far not been studied in the AL or in general within the insect brain (Nässel and Homberg, 2006).

To date, neuropeptides are accepted as molecules responsible for shaping the activity pattern of neuronal circuits and thus as being of major importance for the functional condition and output pattern of the nervous system (Höckfelt, 1991; Marder and Bucher, 2001; Nusbaum et al., 2001; Nässel, 2002). In insects, a few studies on the role of neuropeptides revealed important functions in the brain, e.g. regulation of the insect molt (Truman, 1996b; Mesce and Fahrbach, 2002; Ewer, 2005), regulation of circadian control, and regulation of various aspects of feeding behavior (for a recent review see Nässel and Homberg, 2006).

Despite the large amount of neuropeptides present in the AL, so far only one study has addressed the question which function neuropeptides have in signal processing in the AL (Winther et al., 2006). In this study, knockdowns of the precursor gene coding for the tachykinine related peptides (TKRP), led to loss of sensitivity to three odors tested in a behavioral assay, thus suggesting an important role of the TKRPs in odor processing.

Knowledge on the functions of neuropeptides during ontogeny of the central nervous system is limited. Developmental studies attribute primarily tropic and trophic actions to certain peptides, including induc-

tion of neurogenesis and effects on neuronal migration (Strand et al., 1991; De Felipe et al., 1995; Voronezhskaya and Elekes, 1996; Croll, 2000; Höckfelt et al., 2000; Yacubova and Komuro, 2002). A few studies suggest important functions of peptides especially in the developing or regenerating olfactory system. Neuropeptide-Y and PACAP initiate proliferation of basal cells in the mouse olfactory epithelium (Hansel et al., 2001a,b), while prokineticin 2 serves as a trophic signal and induces neurogenesis in the mouse olfactory bulb (Ng et al., 2005).

The LATn start to increase their number at stage P6/7 which parallels the period when the protoglomeruli are formed. Analysis of Mas-AT-ir projections occurring at this period of AL development revealed that the protoglomeruli are devoid of Mas-AT immunostaining, suggesting that the LATn are not involved in the formation of the protoglomeruli. This fits the hypothesis that protoglomeruli are exclusively formed by ORNs and PNs, but not by LNs (Tolbert et al., 2004). With the beginning of the formation of the glomeruli (phase II) Mas-AT-ir processes enter the glomeruli, which is in accordance for a role of the LATn during formation of the synaptic network in the olfactory glomeruli. Such a role for peptidergic LNs during AL development has been postulated for interneurons displaying RFamide and AST-A immunoreactivity (Schachtner et al., 2004b; Utz and Schachtner, 2005). Comparing the developmental pattern of the three neuropeptide families, as judged by the number of immunopositive cells in LCs, suggests not only different mechanisms of developmental regulation but also distinct functions for selected developmental events (Fig. 8). Note that for the AST-

Figure 8 Summary of the developmental acquisition pattern of three antisera representing a single neuropeptide (Mas AT) or neuropeptide families (RFamide, AST A). Shown are numbers of cell bodies in the lateral cell group with label at defined developmental stages with the neuropeptide antisera. RFamide and AST A according to Schachtner et al., 2004; Utz and Schachtner, 2005.

A peptides transient increases occur, while with the other two neuropeptide antisera (Mas-AT and RFamide) only persisting increases are found. The increases around and during phase II (phase of glomeruli formation) are regulated via the increasing titer of the developmental hormone 20E, while all other fluctuations are regulated by so far unknown mechanisms (Schachtner et al., 2004b; Utz and Schachtner, 2005).

The presence of AST-A and RFamide immunoreactivity in cells of the LC and in centrifugal neurons during early AL development assigns these neurons and their neuropeptides as candidates being functionally involved during this period of AL formation. For the AST-A peptides, it has been speculated that they could provide a diffusible signal for the incoming ORN axons to find the AL and to guide the ORNs around the AL neuropil so that they can finally target their specific glomerular template sites (Utz and Schachtner, 2005).

From the beginning of phase II, processes of LNs, including those immunolabeled with the three neuropeptide antisera, enter the glomeruli to undergo massive synaptogenesis to eventually forming a glomerular network containing thousands of synapses between ORNs, PNs, and LNs. How could the neuropeptides be involved during the formation of the glomerular network during phase II and III? We hypothesize that neuropeptides could be involved (1) in generating and stabilizing neuronal activity in selected neurons establishing the glomerular network and (2) in the process of pathfinding and targeting of neurons towards their partners. The effects of the neuropeptides could be achieved by (1) a spatial and temporal release pattern which could result in a combination of transient gradients of the various peptides, and (2) by the availability and properties of the neuropeptide receptors in the developing AL. While nothing is known on the expression pattern of neuropeptide receptors during AL development, spontaneous electrical activity has been shown *in vivo* for the ORNs (starting at the beginning of phase II; Oland et al., 1996) and in cell culture for developing LNs and PNs (Mercer and Hildebrand, 2002). Furthermore, during phase II, application of the ORN transmitter ACh or of its agonist carbachol, and electrical stimulation of the antennal nerve elicits long lasting calcium transients as well as calcium oscillations in neurons of the LC (Lohr, 2003 and personal communication J. Schachtner). By means of calcium imaging and electrophysiological techniques we are currently examining how neuropeptides influence electrical activity in the developing AL.

The present study represents a detailed analysis of the cellular appearance and developmental expression of the neuropeptide Mas-AT in a defined brain area, the AL of *M. sexta*. A comparison of the time course of Mas-AT immunostaining and its neuronal localization with defined phases of AL development revealed parallels that are consistent with the hypothesis that Mas-AT is involved in certain aspects of AL development. Manipulation of the pupal 20E titer has further revealed that Mas-AT expression in different neuron types of the AL is under the developmental control of 20E. Double labeling experiments with antisera against GABA and two other neuropeptides revealed different populations of Mas-AT expressing neurons of the same morphological type, but with a different biochemical identity.

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