Antennal-lobe tracts in the noctuid moth, *Heliothis virescens*: new anatomical findings

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Abstract

As in other insects, three main tracts in the moth brain form parallel connections between the antennal lobe and the protocerebrum. These tracts, which consist of the antennal-lobe projection-neuron axons, target two main areas in the protocerebrum, the calyces of the mushroom bodies and the lateral horn. In spite of the solid neuroanatomical knowledge already established there are still unresolved issues regarding the antennal-lobe tracts of the moth. One is the proportion of lateral-tract neurons targeting the calyces. In the study presented here, we have performed both retrograde and anterograde labeling of the antennal-lobe projection neurons in the brain of the moth, *Heliothis virescens*. The results from the retrograde staining, obtained by applying dye in the calyces, demonstrated that the direct connection between the antennal lobe and this neuropil is maintained primarily by the medial antennal-lobe tract; only a few axons confined to the lateral tract were found to innervate the calyces. In addition, these staining experiments, which allowed us to explore the arborization pattern of labeled neurons within the antennal lobe, resulted in new findings regarding anatomical arrangement of roots and cell body clusters linked to the medial tract. The results from the anterograde staining, obtained by applying dye into the antennal lobe, visualized the total assembly of axons passing along the antennal-lobe tracts. In addition to the three classical tracts, we found a transverse antennal-lobe tract not previously described in the moth. Also, these staining experiments revealed an organized neuropil in the lateral horn formed by terminals of the four antennal-lobe tracts.

Introduction

The organization of the olfactory pathway seems to be extraordinarily well conserved across various species of the animal kingdom. In all organisms, the olfactory sensory neuron has a similar basic structure: a small bipolar neuron with a dendrite containing the receptor proteins and an unmyelinated axon conveying nerve impulses directly to the brain (reviewed by Hildebrand and Shepherd 1997; Ache and Young 2005). In the primary olfactory center of the brain − the antennal lobe in insects and the olfactory bulb in vertebrates − there is a striking similarity in synaptic organization, through sensory axon terminals making contact with second order neurons in characteristic spherical structures termed glomeruli (Shepherd 1974). Molecular biology experiments have demonstrated that the principle of odotopy guides the sensory axons from the periphery to the brain, meaning that sensory neurons expressing the same receptor type converge onto one or two glomeruli in the primary olfactory center (Vassar et al. 1994; Vosshall et al. 2000). Thus, specific sensory neuron types, randomly distributed in the periphery, project to distinct addresses in the antennal lobe/olfactory bulb. These findings are in full agreement with results from tracing of physiologically characterized receptor neurons in several moth species (Hansson et al. 1995; Berg et al. 1998, 2005; Lee et al. 2006).

Due to the male moth's amazing sense of smell, demonstrated by its ability to seek a potential mate from a distance of more than one kilometer (Priesner et al. 1986), a number of moth species have served as model organisms for exploring olfactory coding mechanisms responsible for reproductive behavior (reviewed by Sakurai et al. 2014; Berg et al., 2015). The sexual behavior is initially based on recognition of a few female-produced molecules by a distinct neuronal arrangement in the male. Thus, the male-specific olfactory pathway of moths offers the opportunity of investigating a neuronal network that is relatively simple and at the same time vital for reproduction and hence for survival of the species (Schneider 1992). In

addition, the availability of biologically relevant plant odors, as identified in particular moth species, enables exploration of chemosensory processing principles in general (reviewed by Mustaparta 2002).

As in other insects, the olfactory sensory neurons of the moth are situated on the antennae. The male-specific neurons, which are housed in long antennal sensilla, project to a small number of enlarged glomeruli positioned dorsally in the antennal lobe, the macro-glomerular complex (MGC), whereas the plant odor neurons, being present in both sexes and encapsulated in short sensilla, target the numerous ordinary glomeruli (reviewed by Anton and Homberg 1999). In the primary olfactory center, the information gathered from the external world is processed before being conveyed via second order neurons projecting to higher brain centers. As in most insect species, three parallel pathways in the moth brain connect the antennal lobe with higher processing sites in the protocerebrum (Homberg et al. 1988; Rø et al. 2007). These tracts are termed the medial, the medio-lateral, and the lateral antennal-lobe tract (mALT, mlALT, and lALT, respectively; for terminology, see Ito et al. 2014; previously named the inner, medial, and outer antenno-cerebral tracts, IACT, MACT, and OACT, respectively).The target regions of the tracts include two main areas: the calyces of the mushroom bodies and the lateral horn. The mALT, which is the most prominent tract, consists of projection neurons sending off collaterals to the calyces on its route to the lateral horn. In *M. sexta*, the number of axons confined to this tract is estimated at 398 in males and at 360 in females (Homberg et al. 1988). Many individual neurons confined to the mALT have been morphologically and physiologically identified in various moth species (*Agrotis segetum*: Hansson et al. 1994; *Bombyx mori*: Kanzaki et al. 2003; *Heliothis virescens*: Christensen et al. 1995; Berg et al. 1998; *Helicoverpa zea*: Christensen et al. 1991; Vickers et al. 1998; *Helicoverpa assulta*: Zhao and Berg 2010; *Manduca sexta*: Christensen and Hildebrand 1987; Hansson et al. 1991; *Spodoptera littoralis*: Anton and Hansson 1994).

Generally, the findings demonstrate that this tract includes a population of morphologically uniform projection neurons arborizing within one glomerulus. Medial-tract neurons innervating the MGC are reported to target a region both in the calyces and in the lateral horn that is distinct from the region innervated by plant odor neurons (Homberg et al. 1988; Kanzaki et al. 2003; Zhao et al. 2014).

While the mALT has been thoroughly described, the lALT and the mlALT remain less explored. The thinnest path, the mlALT, comprises about 120 multi-glomerular neurons (Homberg et. el., 1988), many of which contain GABA (Hoskins et al. 1995; Berg et al. 2009). This tract exits the antennal lobe together with the mALT, but then turns laterally and projects directly to the lateral horn (Homberg et al. 1988; Rø et al. 2007). The lALT is a considerably thicker bundle including more than 300 fibers (Homberg et al. 1988). This tract exits the antennal lobe more ventrally than the other two and consists of both uni- and multi-glomerular neurons. Like the mlALT, it projects laterally towards the lateral horn (Homberg et al. 1988; Rø et al. 2007). On its course, it extends one dorsally oriented side branch ending laterally to the mushroom body alpha lobe (Rø et al. 2007). The remaining lateral-tract fibers project to the lateral horn. Some of these are reported to innervate the calyces. In addition to the three main tracts mentioned above, two others have been reported in *M. sexta*: the dorso-medial ALT (dmALT) and the dorsal ALT (Homberg et al. 1988).

In general, the second order level of the olfactory pathway in moths appears to be relatively similar to that of other holometabolous insect orders (Galizia and Rössler 2010; Haupt et al. 2010; Martin et al. 2011). In the fruit fly, for example, odor information from the antennal lobe is conveyed to higher brain centers along three main ALTs largely corresponding to those identified in moths (Tanaka et al. 2012a; Ito et al. 2014). One additional path, termed the transverse ALT (tALT), was recently discovered in the fruit fly (Tanaka et al. 2012a). In hymenoptera, on the other hand, the ALTs include a dual pathway

consisting of a lateral and a medial tract targeting the higher brain centers in an opposite order and carrying odor signals from distinct divisions of the antennal lobe (Abel et al. 2001; Kirschner et al. 2006; Rössler and Zube 2011). This arrangement implies a lALT consisting of uni-glomerular projection neurons, all of which project to the calyces after ramifying within the lateral horn (Kirschner et al. 2006).

It might seem futile to study the neural connections between the antennal lobe and the subsequent processing centers of the moth, due to the solid neuroanatomical knowledge already established. However, one of several issues that need to be more thoroughly explored in this insect group is the arrangement forming a direct connection between the antennal lobe and the calyces. As previously pointed out by both Homberg et al. (1988) and Rø et al. (2007), axons confined to the lALT in moths include a morphologically heterogeneous assembly of projection neurons, some of which terminate in the calyces. So far, however, the proportion of lateral-tract neurons targeting the calyces in the moth has not been determined. In this study we have performed both anterograde and retrograde labeling of the antennal-lobe projection neurons for the purpose of mapping the entire second order pathway as well as exploring anatomical details concerning distinct tracts. The staining experiments including dye application in the calyces uncovered the direct connection between the antennal lobe and this neuropil in the moth brain. Also, the retrograde labeling, which allowed us to explore the arborization pattern of projection neurons within the antennal lobe, resulted in new findings concerning the organization of cell body clusters and internal roots. The anterograde labelling experiments revealed more than the three classical ALTs; they also showed one additional tract never previously described in the moth. Finally, these staining experiments uncovered a highly organized neuropil structure in the lateral horn, formed by terminal branches of the antennal-lobe projection neurons.

Materials and methods

Insects and preparation

Males of *Heliothis virescens* (Heliothinae; Lepidoptera; Noctuidae) were used for the experimental work. In addition to insects obtained from a laboratory culture maintained at the Norwegian University of Science and Technology, pupae were kindly delivered by Syngenta (Basle, Switzerland). Eggs for the lab culture were kindly supplied by Bayer CropScience AG (Mohnheim am Rhein, Germany). According to Norwegian law of animal welfare there are no restrictions regarding experimental use of Lepidoptera.

All pupae were sexed and kept in separate chambers at 24^{o}C on a phase-shifted LD 14-10 h. Adults 2-7 days old were used for the experiments. The moth was restrained inside a plastic tube with the head exposed and then immobilized with dental wax (Kerr Corporation, Romulus, MI). The head capsule and intracranial muscles were carefully removed to expose the brain. Saline solution (in mM: 150 NaCl, 3 CaCl2, 3 KCl, 25 Sucrose, and 10 N-tris (hydroxymethyl)-methyl-2-amino-ethanesulfonic acid, pH 6.9) was applied to the brain to provide nutrition and prevent desiccation. Following tracer application (see below), the preparation was kept for 2-3 hours at room temperature to allow transportation of the dye. The brain was then dissected from the head capsule, fixed in 4% paraformaldehyde for 1 hour at room temperature, and subsequently washed in a phosphate-buffered saline (PBS; in mM: 137 NaCl, 2.7 KCl, 10 Na2HPO4, 1.8 KH2PO4; pH 7.4). Finally, the preparation was dehydrated in an ascending ethanol series (50%, 70%, 90%, 96%, 2x100%; 10 min each) before being cleared in methyl salicylate (Sigma-Aldrich, Germany) and mounted into 0.5 mm thick aluminum slides.

Staining

The mass staining experiments included utilization of two different fluorescent dyes: Micro-Ruby (tetramethyl rhodamine and biotin, 3000MW, excitation/emission maxima ~555/580 nm; Life Technologies AS, Lot Number 1654904) and Micro-Emerald (fluorescein and biotin 3000 MW, excitation/emission maxima ~494/518 nm; Life Technologies AS, Lot Number 1485203). For staining the total assembly of antennal-lobe projection neurons, anterograde labeling was performed by applying dye into the antennal lobe. Thus, a micro needle (Fine Science Tools) was used to penetrate the neurolemma and then inject crystals of Micro-Ruby into the current brain region. The brain was subsequently covered by a piece of paper moistened with Ringer's solution and kept for 2-3 hours in darkness at room temperature. In order to trace the sub-population of antennal-lobe projection neurons linked to the calyces, retrograde labeling was performed by applying Micro-Ruby to the calyces. Optimal access to the calyces was achieved by removing the head of the moth from the thorax and placing it in melted wax with the frontal part pointing down. The caudal part of the head cuticula was then removed to expose the brain region of interest. Minor cuts were made on the calyx surface followed by application of the fluorescent dye. All preparations being stained only by Micro-Ruby were intensified by incubating the brain in fluorescent conjugate streptavidin-Cy3 (Jackson Immunoresearch, West Grove; PA, diluted 1:200 in PBS) for 2 h before dissection. After that, the preparations were treated as described above.

In order to visualize the relative number of lateral-tract projection neurons innervating the calyces, combined anterograde and retrograde labeling was performed in the same brain. Thus, Micro-Ruby crystals were first inserted into the antennal lobe followed by application of Micro-Emerald into the ipsilateral calyces.

Immunostaining

In order to identify neuropil regions in the moth brain, immune-staining with an anti-body marking synaptic regions, anti SYNORF1, was performed on five pre-stained brains. The anti SYNORF1 (Developmental Studies Hybridoma Bank, University of Iowa) was raised against fusion proteins composed of glutathione-S-transferase and the *Drosophila* SYN1 protein (SYNORF1; Klagges et al. 1996). The specificity of this antibody has been described by Klagges et al. (1996), and it is reported to detect synaptic neuropil in various insect species, heliothine moths included (Berg et al. 2002; Kvello et al. 2009; Zhao et al. 2015). After analyzing the mass-stained brains by confocal laser scanning microscopy, the preparation was rehydrated through a decreased ethanol series (10 min each) and rinsed in PBS. Then the brain was pre-incubated in 5 % normal goat serum (NGS, Sigma, St.Louis, MO, USA) in PBSX (PBS containing 0.5 % Triton-X 100, pH 7.4). The preparation was then incubated for 5 days at 4 °C in the primary antibody, anti SYNORF1(dilution 1:10 in PBSX containing 5 % NGS). After rinsing in PBS 6 x 20 min at room's temperature, the brain was incubated with Cy5-conjugated anti-mouse secondary antibody (Invitrogen, Eugene, OR; dilution 1:500 in PBSX) for 2 days at 4 °C. Finally, we rinsed, dehydrated, cleared, and mounted the brain in methyl salicylate.

Confocal microscopy and image processing

All preparations were scanned using a confocal laser microscope (LSM 510 META Zeiss, Jena, Germany) equipped with a Plan-Neofluar 20x/0.5 and C-Achroplan 40x/0.8W objective. The staining originating from Micro-Ruby (Ex_{max} 555 nm, Em_{max} 580 nm) was excited by the 543-nm line of a HeNe 1 laser (filter BP 565-615 IR) and the staining originating from Micro-Emerald (Ex_{max} 490 nm, Em_{max} 508 nm) by the 488-nm line of an Argon laser (filter KP685). The optical sections were scanned at distances of 2 to 4 µm. The immunostaining obtained by

the Cy5 (Ex_{max} 682 nm, Em_{max} 647 nm), was excited by the 633-nm line of a HeNe2 laser (filter 655-709). The image resolution was set to 1024x1024 pixels. Double- and triple labeled preparations were scanned by using two- and three-channel detection modules, respectively. The confocal images were further analyzed by means of the Zeiss LSM software. In addition to snapshots from single optical sections, projection views and stereo-images were made from confocal stacks. The image material was further edited using Adobe Photoshop CS6 for brightness and contrast adjustment. Finally, Adobe Illustrator CS6 was used for panel composition. All brain preparations are presented in either frontal or dorsal view with the same orientation of the brain midline, except for some of those included in the stereo images (here, rotation causes loss of the stereo effect). The orientation of all brain structures is indicated relative to the body axis of the insect, as in Homberg et al. (1988). The term medial in the figure legends means medial relative to the midline of the brain.

Results

Here we present new findings on the anatomical organization of the ALTs in the moth, including their projection patterns in the protocerebrum and arrangements within the antennal lobe. The results from the anterograde staining, obtained by applying dye into the antennal lobe, visualized the total assembly of projection neuron axons passing along the parallel ALTs whereas the retrograde technique, including dye inserted into the calyces, provided selective staining of the antennal-lobe neurons innervating this neuropil. Terminologies for the relevant brain areas in the present study are used in accordance with the newly established nomenclature of the insect brain (Ito et al. 2014). Even though some antennal-lobe projection neurons send fibers into the contralateral protocerebrum, we have analyzed the ipsilateral hemisphere only and present the data accordingly.

A new transverse tract

Dye application into the antennal lobe visualized three parallel tracts identified as the medial, the medio-lateral, and the lateral ALT. The overall projection pattern of these tracts is consistent with previous findings (Homberg et al. 1989; Rø et al. 2007). As shown in figures 1, 2a, and 3, the prominent mALT projects posteriorly and sends off side-branches to the calyces on its route to the lateral horn whereas the mlALT and the lALT project directly to the lateral horn. In addition to these classical ALTs, we discovered a novel tract. Like the mlALT it joins the mALT before turning laterally. Because of its similarity to a recently discovered fiber bundle in *Drosophila*, which was named the transverse ALT (tALT; Tanaka et al. 2012a), we have used the same terminology for the new tract in the moth brain. As demonstrated in figures 1b, 2a, and 3, the tALT tract deviates from the mALT more posteriorly than the mlALT. On its lateral course, it soon splits into two sub-branches. The first sub-branch targets two different regions, i.e. the base of the medial calyx and the posterior lateral protocerebrum (Fig. 3c-f). The second sub-branch projects slightly anteriorly and joins the mlALT before ending in the lateral horn (Fig. 3c, d). Here, the terminal projections from the two tracts seem to overlap. In addition, the second sub-branch of the tALT sends processes towards the base of the medial calyx, apparently terminating in the region innervated by the first sub-branch (Fig. 3e). The three classical ALTs occurred in all preparations successfully stained via the anterograde technique, whereas the new transversal tract appeared infrequently (Fig. 1a, b). Another ALT that appeared occasionally, projecting posteriorly along the dorsal surface of the protocerebrum, close to the brain midline, was identified as the dmALT (Fig. 2A).

The lALT – new findings

The retrograde staining technique demonstrated that only a few of the lateral-tract axons innervate the calyces. Typical examples of preparations stained from the calyces are presented in Figs. 2b and 4a; here, the mALT can be seen as a prominent fiber bundle whereas only a few fibers confined to the lALT are visible. A similar projection pattern occurred in all brains stained using the retrograde technique. As shown from confocal images of the antennal lobe, we clearly see that the stained lateral-tract fibers project ventrally in the antennal lobe, outside all antennal-lobe roots linked to the medial tract (Figs. 4a; 5). Neither the mlALT nor the tALT were stained by the retrograde technique. Double-labeling experiments including combination of the two staining techniques confirmed the findings by showing overlap of the two dyes only in a few lateral-tract axons (Fig. 5). In the mALT, on the other hand, there was complete overlap of the two dyes.

Otherwise, the identification of the lALT as a relatively thick fiber bundle projecting laterally from the antennal lobe ventrally of the mlALT, obtained by anterograde labeling, matches previous descriptions (Homberg et al., 1989; Rø et al., 2007). As demonstrated in Figs. 2a and 3, however, a substantial proportion of the lateral-tract axons soon changes course from lateral to dorsal whereas the remaining fibers continue laterally to the ventral part of the lateral horn. The dorsally projecting branch has a unique form characterized by a massively stained fascicle of neural processes lacking visible ramifications. The immune-stained preparations showed that this structure splits into two sub-branches: one thin turning laterally and terminating in the anterior ventro-lateral protocerebrum and one thick targeting the area between the anterior optic tubercle and the alpha-lobe of the mushroom bodies (Fig. 6). The presence of these sub-branches appeared intermittently in different preparations (Fig

1a and b). Notably, the dorsal part of the pillar formed structure terminates close to some of the numerous stained dendrites shown in figure 6d. These processes belong to an antennal-lobe centrifugal neuron confined to the dmALT (Zhao et al. 2013).

Anatomical arrangement of medial-tract neurons in the antennal lobe

The preparations that were treated using the retrograde technique offered the opportunity to study the anatomical organization of the stained projection neurons within the antennal lobe. The glomerular innervation pattern of this neuron population, including mainly medial-tract neurons, is organized according to two main roots: the dorsal root innervating the dorso-caudal hemi-lobe and the ventral root innervating the ventro-rostral hemi-lobe (Fig. 7). The somata of the neurons forming the relatively thick dorsal root are located in the medial cell body cluster (Figs. 2d and 7). Due to the density of stained cell bodies in this cluster, it appears that it is linked to the mALT exclusively. The considerably thinner ventral root is formed by neurons that have their somata located in the lateral cell body cluster (Figs. 2c, d and 7). The appearance of stained somata intermingled with numerous unstained somata demonstrates that this large cluster is connected to numerous neurons that are not confined to the mALT. In addition to the two main roots, a few scattered branches extending from the dorsal root are linked to somata in the anterior cell cluster (Figs. 2c, d and 7h). These fiber bundles, which pass transversely through the antennal lobe, innervate ventro-medially located glomeruli.

Close to the dorsal boundary of the anterior cell body cluster, the most successfully stained preparation displayed an assembly of approximately 10 somata (Fig. 4b), each having a mean diameter of 5.0 μm (SD 0,24; N=10). These tiny somata are connected to a loose bundle of thin fibers projecting along the outer part of the antennal lobe (Fig.4b). These fibers join the mALT between the dorsal and ventral root (data not shown).

An organized innervation pattern in the lateral horn

The lateral horn is described as an unstructured neuropil. However, in dorsally oriented brains, at a particular depth, a specific structure formed by axon terminals of the ALTs appears. This characteristic structure consists of two linked toroids (Fig. 3a, b). The inner diameter of each toroid is 41.2 µm (SD 1.0; N=6).

Discussion

In addition to mapping the classical antennal-lobe tracts of the moth, our study includes the following new discoveries: 1) a comprehensive mapping of the lALT including its inconspicuous connection to the calyces, plus the projection pattern of one prominent sub-branch in the protocerebrum, 2) the presence of the tALT, not previously described in the moth, 3) the presence of a highly organized neuropil in the lateral horn, not previously described in any insect species, 4) the presence of a new cell body cluster in the antennal lobe and 5) mapping of the antennal-lobe roots linked to the medial tract including totally four bundles, each linked to one of the four cell body clusters. A schematic overview of the ALTs including their sub-branches and target regions is provided in figure 8.

Methodological consideration

Different kinds of staining techniques are used for anatomical investigations of olfactory pathways, among which genetically controlled neural labeling of the *Drosophila* brain is one of the most promising (Wong et al. 2002, Tanaka et al. 2012a, Lai et al. 2008). However, genetic modifications may fail to detect pathways for which genetic labels have not yet been identified (Aso et al. 2009; Tanaka et al. 2012b). One advantage of the mass staining technique is that it offers the opportunity to map the complete neural circuit of interest. On the

other hand, a weakness of this method is the possibility of not visualizing relevant neural structures due to distinct innervations of their associated neurons at the site of dye injection. In the anterogradely stained preparations analyzed here, we observed three distinct structures appearing infrequently, i.e. the tALT, the dmALT, and the pillar-formed side branch of the lALT, presumably because of their non-distributed ramification patterns in the region for dye application. Regarding lateral-tract neurons innervating the calyces, we should obviously not ignore the possibility that we failed to stain some of these axons when using the retrograde technique. However, the fact that these neurons are reported to innervate the entire volume of the calyces (Homberg et al. 1988; Rø et al. 2007), plus the consistent finding of a few labeled fibers in the preparations stained via the retrograde technique supports our conclusions.

Only a few lALT fibers innervate the calyces

Previous studies of moths have described lateral-tract projection neurons as consisting of various morphological types, some of which are reported to innervate the calyces (Homberg et al. 1988; Rø et al. 2007). However, the proportion of lateral-tract neurons making a direct connection between the antennal lobe and the calyces has not previously been determined. As shown by the experiments performed here a minor proportion of the lALT axons terminate in the calyces. These findings indicate that the direct connection between the antennal lobe and the calyces is maintained primarily by the mALT in the moth. The relatively few lateral-tract fibers visualized in the preparations stained from the calyces in the study presented here probably belong to a category of formerly identified projection neurons; individual lateral-tract projection neurons passing through the lateral horn to the calyces have been reported in both *M. sexta* and *H. virescence* (Homberg et al. 1988; Rø et al. 2007). Based on the double-labeled preparations in this study, it seems that the lateral-tract axons targeting the calyces pass along the same pathway.

The difference between the lALT of lepidopterans, which consists of morphologically heterogeneous projection neuron types, and the lALT of hymenopterans, comprising one half of a dual system of homogeneous uni-glomerular projection neurons all of which terminate in the calyces, has been pointed out previously (Kirchner et al. 2006; Galizia and Rössler 2010; Martin et al. 2011). The fact that only a very small proportion of the lateral-tract axons in moths innervates the calyces further proves the essential distinction between the similarly named tracts of the two insect orders. Thus, rather than two equally prominent ALTs innervating the calyces, as found in the honeybee, the calyces are innervated predominantly by one tract, the mALT, in the moth. Actually, the main portion of the lALT, terminating in the lateral horn of the moth, appears to correspond closer with the most ventral of the three mlALTs of the honeybee (Kirschner et al., 2006). Generally, the arrangement in the moth seems to be more congruent with that of the fruit fly; here, a total of four lateral-tract neuron categories has been reported, one of which targets the calyces, i.e. the AL-lPN1 projection neurons (Tanaka et al. 2012a).

One of the striking features typifying the lALT in this study was the appearance of strongly stained axons that split from the classic course nearby the spur of the mushroom bodies and form a characteristic pillar-like structure extending dorsally. Though, similar fibers "making a dorsal turn away from the common tract and ending lateral to the alpha lobe" were described by Rø et al. (2007), the extraordinary prominence of this path, as demonstrated in figures 1b and 2a, has not previously been pointed out. The reason for the strong staining might be distinct morphological properties characterizing the individual axons forming this pillar-formed structure; thus, as reported by Homberg et al. (1988), projection neurons passing along this particular pathway, named POa, possess numerous short side branches extending from the axons. Actually, the POa neurons constituted the largest category of lALT neurons identified in *M. sexta*. Also, individual lateral-tract neurons confined to the

similar path in *H. virescens* were reported to extend short processes from the main fiber (Rø et al. 2007). As far as we know, a corresponding projection pattern associated with the lALT has not been reported in any other insect order.

Antennal-lobe organization of projection neurons associated with the medial ALT

Retrograde labeling from the calyces visualized distinct neural structures in the antennal lobe, the main proportion of which are associated with the mALT. The dendritic ramifications of the medial-tract projection neurons are separated according to two main roots, the dorsal and the ventral (Homberg et al. 1988; Løfaldli et al., 2010) in such a way that the former innervates the dorso-caudal hemi-lobe and the latter the ventro-rostral. Interestingly, this morphological segregation might be compared with the dual arrangement in the honeybee comprising two protocerebral tracts each innervating one hemi-lobe of the antennal lobe (Kirchner et al. 2006). Thus, from the anatomical point of view the ventral root of the mALT in the moth might actually be comparable with the lALT of the honeybee.

A new cell body cluster in the moth antennal lobe was discovered here. The reason why the assembly of somata located adjacent to the anterior cell group has not been described before is probably their unusually small size. The projection pattern of the fibers linked to these somata differs from those formed by the connections to the three classical cell clusters. Thus, the few thin neurites attached to the tiny somata pass along a peripheral path enveloping the glomerular layer whereas all the other roots extend in the aglomerular core of the antennal-lobe. So far, individual projection neurons with somata neither in the anterior cell cluster nor in the newly discovered cluster have been identified in heliothine moths.

The transverse ALT

Due to its morphological correspondence with the tALT in *Drosophila*, first identified by Tanaka and colleagues in 2012, we have chosen the same name for the newly discovered tract

in the heliothine moth. As in the fruit fly, a fiber bundle extending from the mALT posteriorly of the central body projects laterally and targets several regions in the ipsilateral protocerebrum of the moth brain. The occasional occurrence of the tALT in the stained preparations presented here indicates its origin in a specific subset of the antennal-lobe glomeruli. Interestingly, all four sub-categories of projection neurons forming the tALT in the fruit fly are reported to originate in ventrally located antennal-lobe glomeruli (Tanaka et al. 2012a). The reason why this tract has not previously been revealed in any moth species is probably its presumed connection with a distinct region of the antennal lobe. It seems unlikely that the infrequent staining of this tract is due to individual variations. As demonstrated in Fig. 1, the three classical tracts are visible in all the depicted hemispheres, while the tALT clearly appears in only one. It should be mentioned that this tract might have been shown in a previous study of the heliothine moth, however, in which it was identified as the mlALT (Løfaldli et al. 2012). Furthermore, some individual antennal-lobe projection neurons in *M. sexta*, named PIc, seem to project in a course similar to the tALT (Homberg et al. 1988), but were identified as belonging to the mALT. Generally, the multiple side branches of this tract, as described here, indicate that it includes morphologically distinct neurons, similarly to what has been shown in *Drosophila* (Tanaka et al. 2012a). Besides its correspondence with the tALT in the fruit fly, we have noticed that the transverse tract in the moth is comparable with one of the three mlALTs in the honeybee by innervating a region close to the initial peduncle, in addition to the lateral horn (Kirchner et al. 2006). As regards the cockroach, however, which is a hemimetabolous insect, we found no comparable tract among the three mediolateral ALTs identified in this species (Malun et al. 1993).

Anatomical organization in the lateral horn

Generally, previous reports have characterized the lateral horn as a diffuse unorganized neuropil (Martin et al., 2011). However, our findings revealed one highly organized region of

the lateral horn where input from all main ALTs forms a prominent structure of two linked toroids. The dorsal orientation of the preparation is obviously critical for the appearance of this neural construction. The distinct pattern predicts morphological features typifying dendritic arbors of subsequent neurons in the moth olfactory pathway; presumably, at least some of the third order elements possess dendrites forming a tuft − a feature which actually characterize certain lateral-horn neurons in the locust (Gupta and Stopfer 2012).

Conclusion

The results obtained here supplement previous findings on anatomical arrangements of the parallel ALTs in the moth brain. Generally, the lateral horn seems to be innervated by at least four of these tracts whereas the calyces receive input mainly from one. The numerous peculiarities formed by the antennal-lobe projection neurons imply the complexity of encoding mechanisms residing at the second order level of the moth olfactory pathway. The data from this anatomical study will improve the basis for future analyses of morphologically different antennal-lobe projection neurons and their putative roles. In addition, the results obtained here may contribute to considerations on anatomical arrangement of the central olfactory pathway in a comparative perspective.

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Conflict of interest statement

The authors declare no conflict of interest.

Role of authors

The research was designed by EI and BGB. The experimental work was carried out by AB, EI, and SCL. All authors contributed with the figure material and analysis of the data. The article was organized and written by EI and BGB.

Literature cited

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Figure legends

Fig. 1 Projection views of the antennal-lobe tracts (ALTs). **a**: Confocal image showing the three classic ALTs in each brain hemisphere, the medial ALT (mALT), the medio-lateral ALT (mlALT), and the lateral ALT (lALT). The prominent mALT projects to the calyces (Ca) before terminating in the lateral horn (LH) whereas the mlALT and the lALT project directly to the LH. **b:** Confocal image of one brain hemisphere showing, in addition to the three classical ALTs, one additional tract, the transverse ALT (tALT). The arrow points to a massively stained region of the lALT. CB, central body; A, anterior; P, posterior; M, medial. Scale bars: 100 µm.

Fig. 2 Confocal images showing the antennal-lobe tracts (ALTs) in a three-dimensional view. (Red/green or read/blue stereo spectacles with the red lens in front of the left eye should be used) **a:** Stereo image of the ALTs in a dorsal orientation, obtained by applying dye into the antennal lobe (made from the same confocal stack as 1B, but inverted). In addition to the three classical ALTs, i.e. the medial, the medio-lateral, and the lateral (mALT, mlALT, and

lALT, respectively), the transverse ALT (tALT) and the dorso-medial ALT (dmALT) can be seen. A massively stained side-branch of the lALT projecting dorsally from its lateral course is visible (arrow). **b:** Stereo image of one frontally oriented brain hemisphere that was stained by applying dye into the calyces. The mALT appears as a massively labeled bundle whereas only a few fibers of the lALT were stained. As shown, the mALT splits into three roots at its entrance into the AL (arrow). **c:** Stereo image of the AL in a dorsal view, obtained by applying dye into the calyces. The retrograde labeling shows the roots of the mALT and their connection to distinct cell body clusters enveloping the glomeruli. The ventral root (VR) connects to the lateral cell cluster (LC) and the dorsal root (DR) to the medial cell cluster (MC). Due to the selection of optical sections only a part of the MC is visible. Several thinner fiber bundles project through the AL and connect to the anterior cell cluster (AC; arrow) **d:** Stereo image of the AL in a frontal view, obtained by applying dye into the calyces (same brain preparation as in B). The dorsal and ventral roots of the mALT, linked to the MC and LC, respectively, are visible. In addition, the AC and its connection to several thinner fiber bundles extending from the mALT can be seen (arrow). Ca, calyces; LH, lateral horn; CB central body; MB, mushroom body lobes; P, posterior; M, medial; D, dorsal. Scale bars: 100 µm.

Fig. 3 Confocal sections of the antennal-lobe tracts (ALTs) in six different planes, obtained by applying dye into the antennal lobe (same confocal stack as 1b). The depth of each section, relative to the most dorsal part of the brain is indicated. **a:** The most dorsal section shows the medio-lateral ALT (mlALT) and a cross-section of one dorsally oriented side-branch linked to the lateral ALT (lALT). In the lateral horn (LH), terminal projections of the ALTs form a characteristic structure consisting of two fused toroids. **b-d:** The three consecutive sections, located more ventrally, visualize the prominent medial ALT (mALT) projecting along the anterior border of the calyces (Ca) before terminating in the LH, plus the transverse ALT

(tALT) splitting off from the mALT posteriorly of the mlALT. One sub-branch of the tALT projects to the LH and sends off collaterals to an area nearby the medial Ca (mCa). **e, f:** In the two most ventral sections, the laterally projecting axons of the lALT, which target the LH, can be seen. In addition, a second sub-branch of the tALT targets the posterior lateral protocerebrum (pLP) and extends processes in the area nearby the mCA. P, posterior; M, medial. Scale bar: 50 µm.

Fig. 4 Projection views of antennal-lobe tracts (ALTs) and antennal-lobe cell body clusters visualized by applying dye in the calyces (same confocal stack as 2c). **a:** As shown, the medial ALT (mALT) is massively stained whereas only a thin bundle of the lateral ALT (lALT) is visualized. **b:** The dashed ellipse indicates a small group of somata located adjacent to the anterior cell body cluster (AC). The assembly of tiny somata connects with a loose bundle of very thin fibers projecting along the peripheral part of the antennal lobe (AL; arrow). LC, lateral cell body cluster; MC, medial cell cluster; P, posterior; M, medial. Scale bars: 50 µm.

Fig. 5. Double-labeled preparation showing the small proportion of lateral-tract neurons linked to the calyces. **a:** Anterograde labeling obtained by applying Micro-Ruby into the antennal lobe (magenta). Both the medial antennal-lobe tract (mALT) and the lateral antennal-lobe tract (lALT) are massively stained. **b:** Retrograde labeling obtained by applying Micro-Emerald into the calyces (green). The mALT is heavily stained whereas the lALT appears as a thin path. **c:** Merged images showing complete overlap of staining in the mALT and limited overlap in the lALT. P, posterior; M, medial. Scale bar: 50 µm.

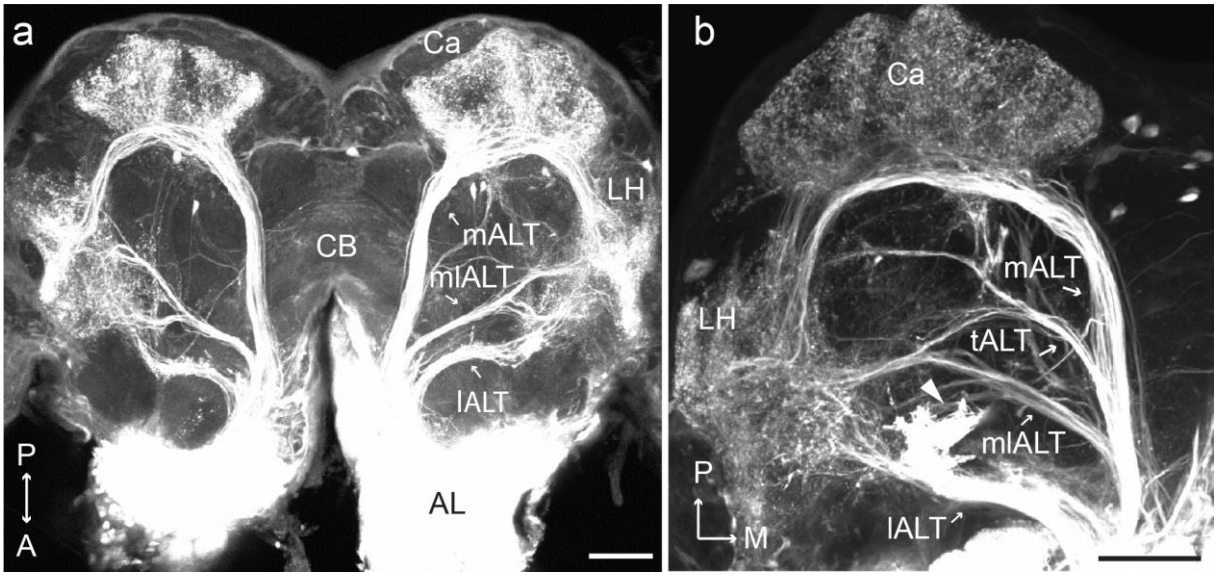
Fig. 6 Triple-labeled preparation showing the localization of distinct elements of the lateral antennal-lobe tract (lALT) relative to common neuropils in the moth brain (same prep as in figure 5). Magenta indicates projections labeled from the antennal lobe (anterograde labeling); green indicates projections labeled from the calyces (retrograde labeling); blue indicates anti-

synapsin staining. The four confocal images show optical sections from ventral to dorsal. The depth of each section is indicated. **a:** Ventral section showing the common path of the lALT projecting directly from the antennal lobe (AL) to the lateral horn (LH). **b**: More dorsal section showing the starting point of a dorsally oriented branch splitting off from the common path of the lALT. **c**: The dorsally oriented branch divides in two: one thin sub-branch projecting to the anterior ventro-lateral protocerebrum (AVLP). **d:** Most dorsal section showing the target region of the dorsally projecting sub-branch, positioned between the anterior optic tubercle (AOTU) and the alpha-lobe of the mushroom body (αL) The arrow-head points to the extensive dendrites of one centrifugal neuron confined to the dorso-medial antennal lobe tract (Zhao et al. 2013). mALT, medial antennal lobe tract; SPU, spur of the mushroom body; P, posterior; M, medial. Scale bar: 50 µm.

Fig. 7 Confocal sections in different planes (dorsal view) showing the internal organization of medial-tract neurons in the antennal lobe (AL) (same prep as shown in figure 4). The staining was obtained by applying dye into the calyces (same preparation as 2c). The depth of each section is indicated according to the most dorsal part of the AL. **a, b:** The two most dorsal sections show the macroglomerular complex (MGC) and the dorsal root (DR) which is connected to the medial cell body cluster (MC). **c-f:** In the consecutive sections, located more ventrally, the lateral cell body cluster (LC) and the anterior cell cluster (AC) appear. **g-i:** In the most ventral sections, the ventral root (VR), which is linked to the LC, appears, plus an assembly of fiber bundles connected to the AC (arrow in h). P, posterior; M, medial. Scale bar: 50 µm.

Fig. 8 Schematic overview of the antennal lobe tracts (ALTs) in the moth brain (dorsal view). The black arrow points to the pillar-formed side-branch of the lALT. One glomerulus indicates mainly uniglomerular projection neurons (PNs); four glomeruli – multiglomerular PNs and two glomeruli – both uni- and multiglomerular PNs. AL, antennal lobe; OL, optic

lobe; mALT, medial ALT; lALT, lateral ALT; mlALT, mediolateral ALT; tALT, transverse ALT; CB, central body; AOTU, anterior optic tubercle; αL, alpha lobe; Ca, calyces.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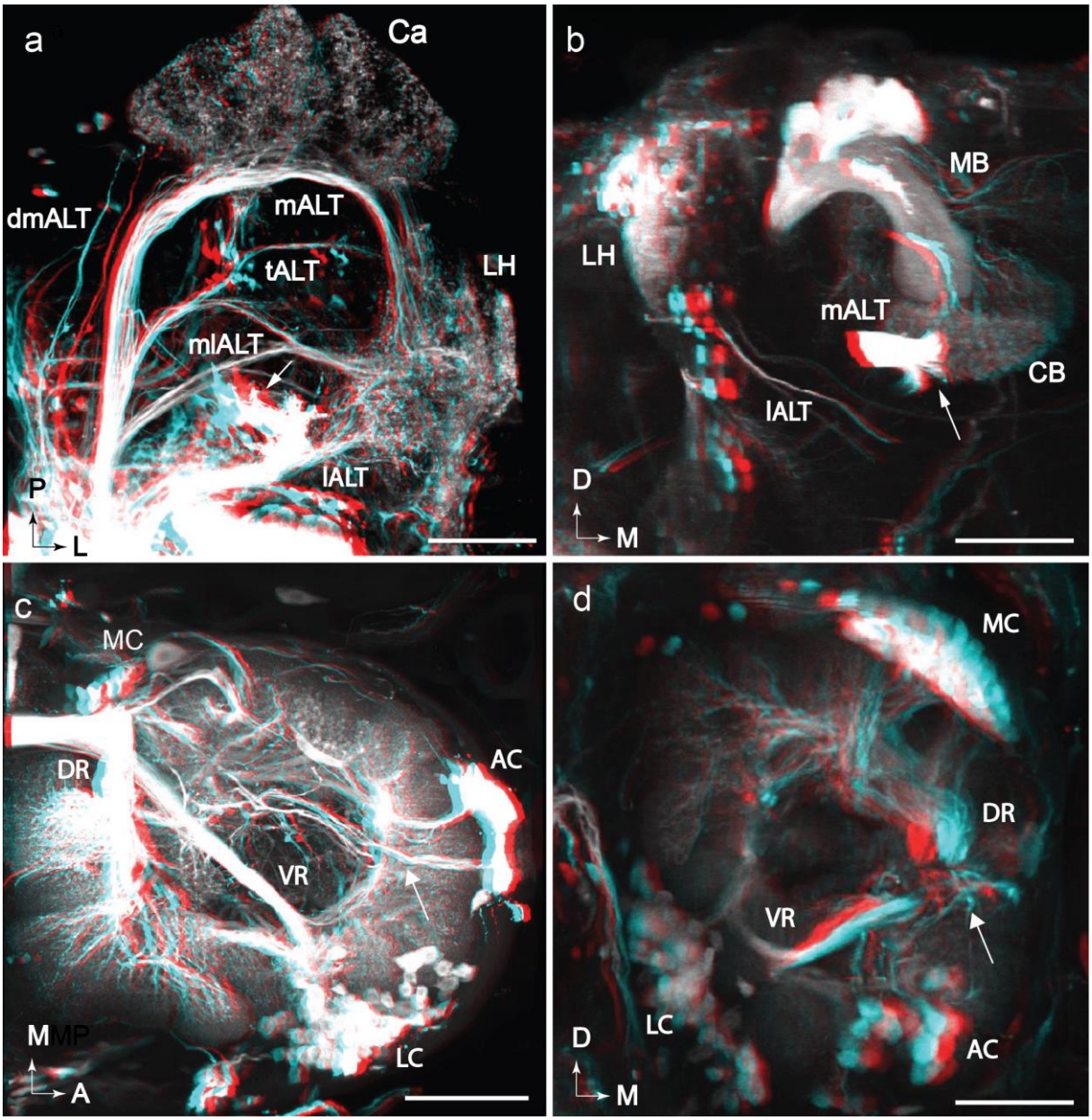
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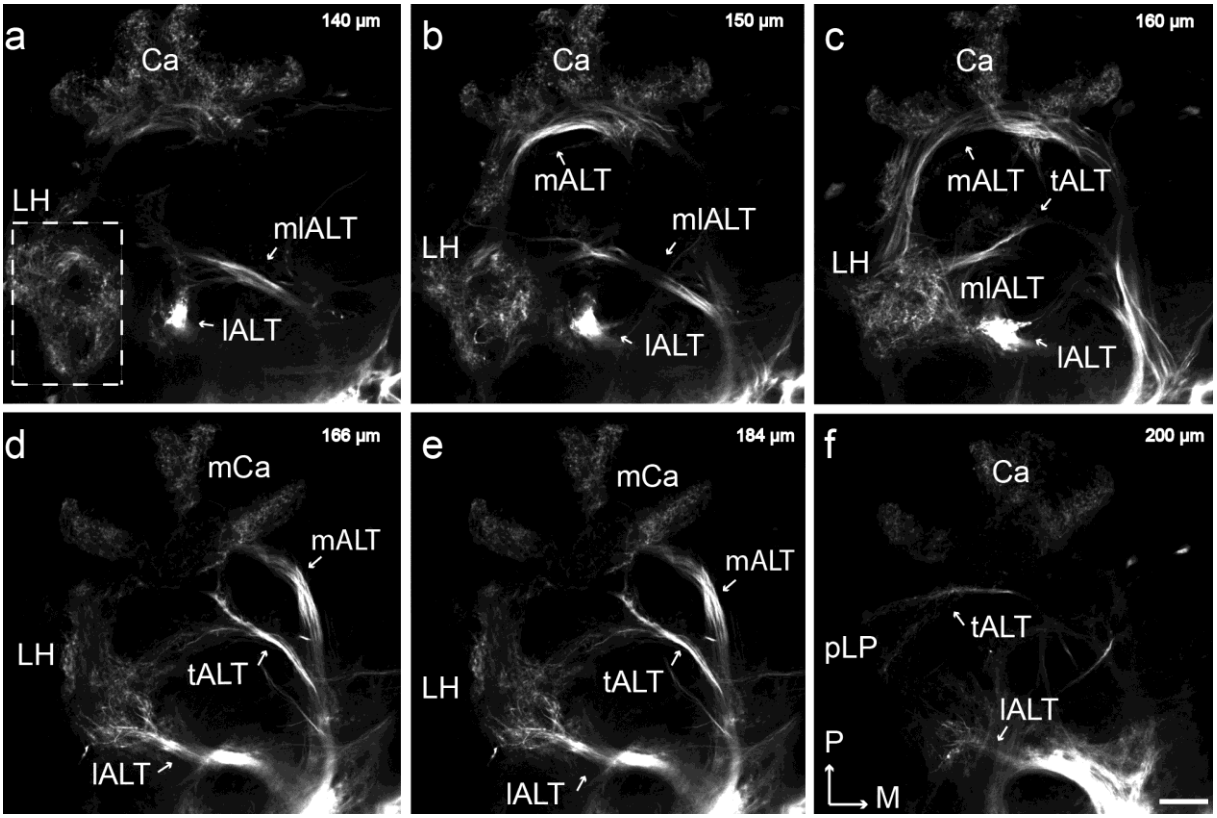
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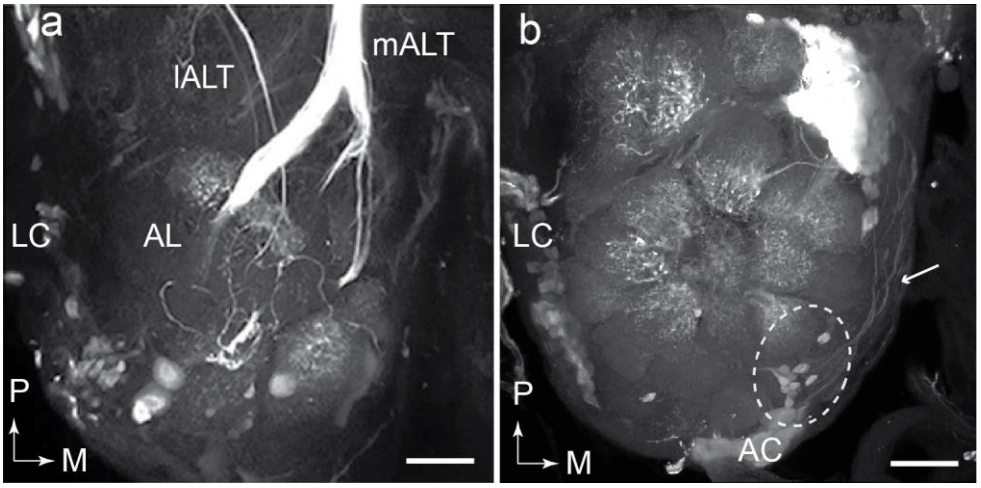
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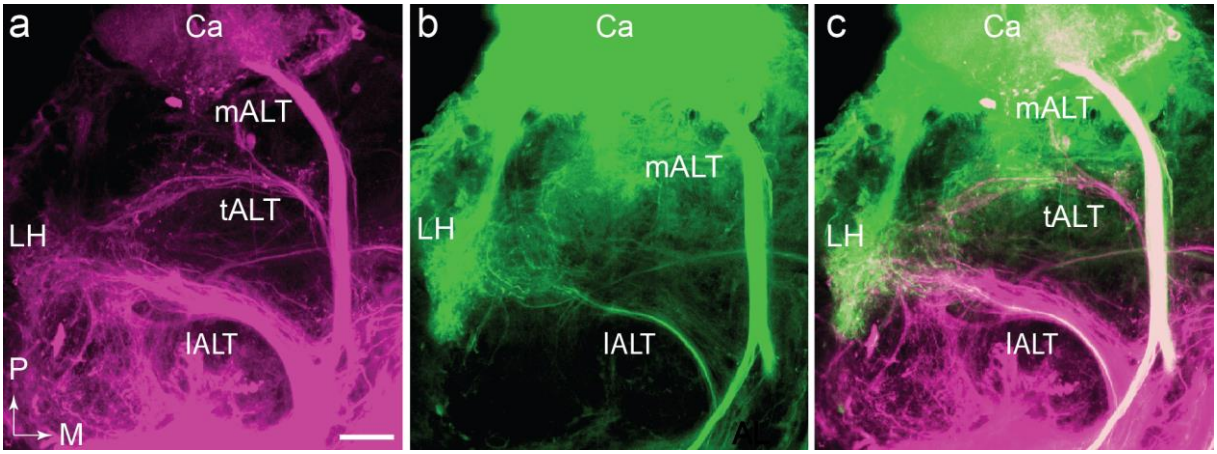
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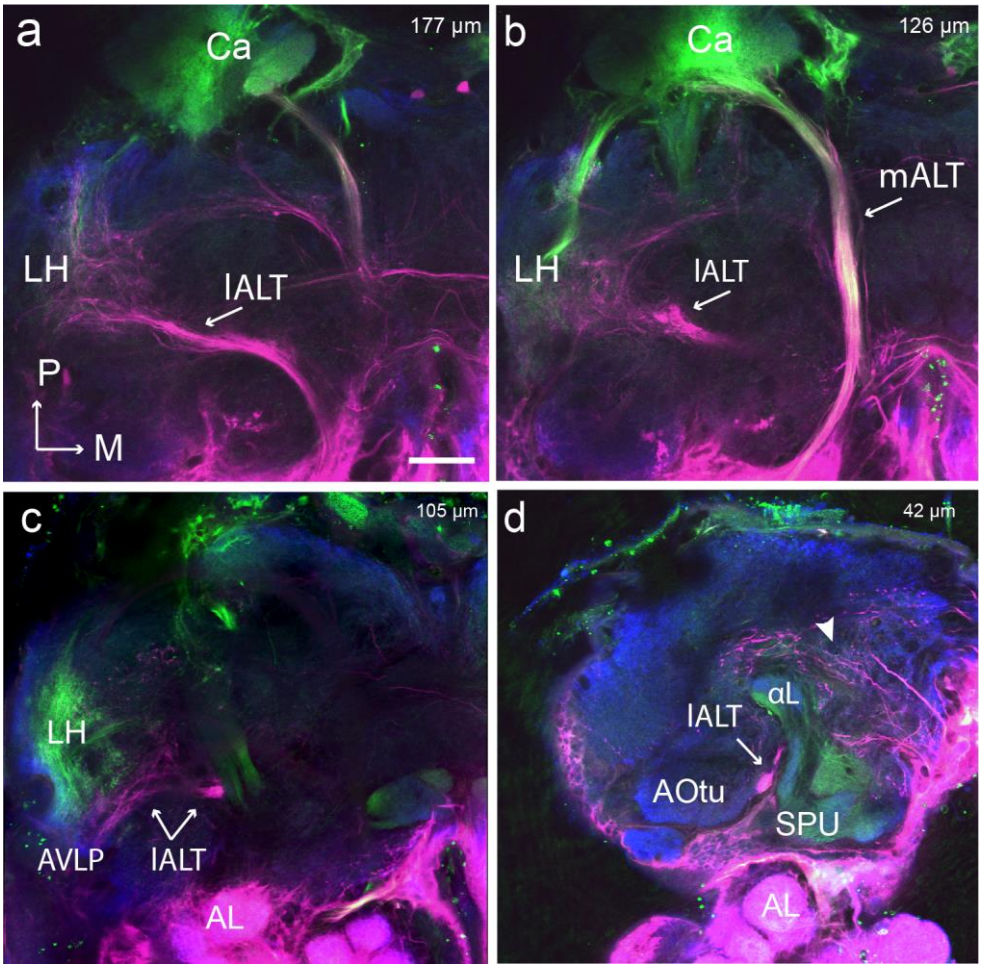
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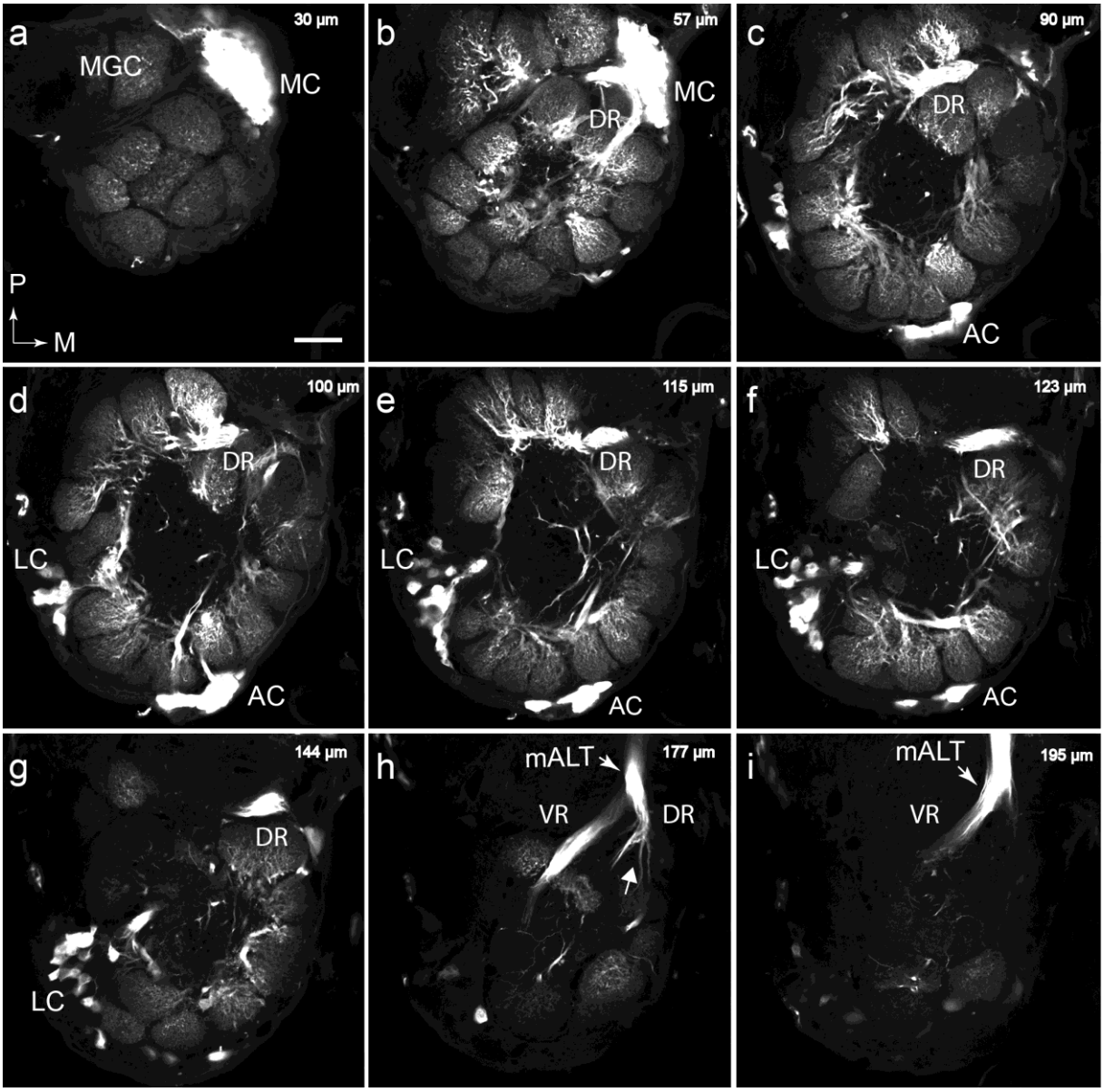
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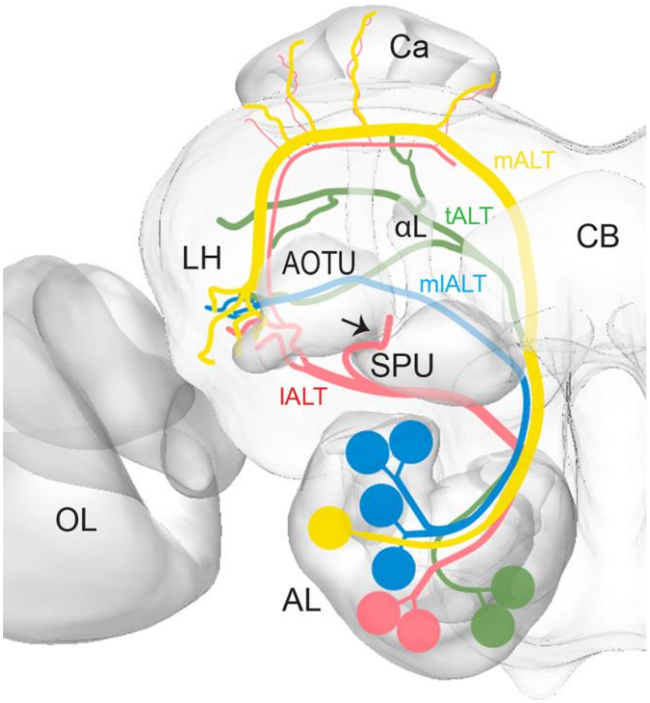
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