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REGULAR ARTICLE

Brief sensory experience differentially affects the volume of olfactory brain centres in a moth

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Abstract Experience modifies behaviour in animals so that they adapt to their environment. In male noctuid moths, *Spodoptera littoralis*, brief pre-exposure to various behaviourally relevant sensory signals modifies subsequent behaviour towards the same or different sensory modalities. Correlated with a behavioural increase in responses of male moths to the female-emitted sex pheromone after pre-exposure to olfactory, acoustic or gustatory stimuli, an increase in sensitivity of olfactory neurons within the primary olfactory centre, the antennal lobe, is found for olfactory and acoustic stimuli, but not for gustatory stimuli. Here, we investigated whether anatomical changes occurring in the antennal lobes and in the mushroom bodies (the secondary olfactory centres) possibly correlated with the changes observed in behaviour and in olfactory neuron physiology. Our results showed that significant volume changes occurred in glomeruli (olfactory units) responsive to sex pheromone following exposure to both pheromone and predator sounds. The volume of the mushroom body input region (calyx) also increased significantly after pheromone and predator sound treatment. However, we found no changes in the volume of antennal lobe glomeruli or of the mushroom body calyx after pre-exposure to sucrose. These findings show a relationship of antennal lobe sensitivity changes to the pheromone with changes in the volume of the related glomeruli and the output area of antennal lobe projection neurons elicited by sensory cues causing a behavioural change. Behavioural changes observed after sucrose pre-exposure must originate from changes in higher integration centres in the brain.

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Introduction

Insects, like all animals, are confronted with an ever-changing environment during their lifetime. An important means of adapting to such changes in the environment is to profit from previous experience. Various forms of learning have been described in insects, from simple forms such as habituation and sensitization to associative learning and even social learning (Kandel 2001; Menzel 2001; Giurfa 2007). Much is known about the neural mechanisms underlying these forms of learning, including changes at the anatomical, physiological and molecular levels (Menzel 2001; Busto et al. 2010). More recently, a form of sensitization following brief pre-exposure has been described in a noctuid moth. Males of *Spodoptera littoralis* briefly exposed to various behaviourally relevant sensory stimuli during early adult life respond better to suboptimal doses of the female-emitted sex pheromone 24 h later (Anderson et al. 2003, 2007; Minoli et al. 2012). These behavioural changes in sex pheromone responses occur after pre-exposure to odours including an optimal dose of the pheromone itself and to diverse flower-emitted volatiles (linalool and geraniol), but also after pre-exposure to an attractive (sucrose) or aversive (quinine) gustatory stimulus and to an evasive-manoeuvre-eliciting predator sound (Minoli et al. 2012; Anton et al. 2011). Physiological investigations indicate that the population of pheromone-sensitive olfactory sensory neurons slightly, but significantly, increases its sensitivity after pheromone pre-exposure (Guerrieri et al. 2012). Central olfactory neurons in the antennal lobe, the primary olfactory centre, also increase their sensitivity by five orders of magnitude 24 h after pheromone pre-exposure (Anderson et al. 2007). These sensitivity changes in peripheral and central olfactory neurons are accompanied by an increase in the relative volume of the cumulus, the largest glomerulus of the pheromone-processing macroglomerular complex (MGC; Guerrieri et al. 2012). Interestingly, pre-exposure to a predator sound also leads to an increase in the sensitivity of antennal lobe neurons 24 h later to the sex pheromone, as does exposure to plant-related odours by one to two orders of magnitude (Anton et al. 2011). On the other hand, pre-exposure to sucrose does not alter the sensitivity of antennal lobe neurons to the sex pheromone 24 h later but results in increased behavioural responses to the sex pheromone (Minoli et al. 2012).

Although the literature on cross-modal anatomical changes in sensory-deprived humans and other mammals is extensive (e.g. Fox and Wong 2005), evidence of cross-modal experience-induced modifications under physiological conditions is scarce and, to our knowledge, is limited to behavioural or physiological responses in the absence of structural data (e.g. Landgrebe et al. 2008; Yu et al. 2009). However, with its easily accessible centres and its modular organization and well-characterised functional properties, the insect olfactory system provides an interesting model system for studying the cellular and molecular mechanisms of this type of plasticity.

Here, we sought anatomical correlates of the observed sensitivity changes in the primary and secondary olfactory centres of the brain following pre-exposure with a variety of sensory stimuli, previously shown to affect orientation behaviour towards the sex pheromone. As certain cues affected the sensitivity of antennal lobe neurons, but not others, we hypothesized that the volume changes of glomeruli within the primary olfactory centre might correlate with such changes, whereas cues eliciting a behavioural change without any increase in antennal lobe neuron sensitivity might cause volume changes in higher olfactory centres. We determined the volumes of five glomeruli within the antennal lobe, three being part of the pheromone-processing MGC and two involved with the processing of plant-related odours, and the volume of the calyx of the mushroom body in male moths pre-exposed 24 h earlier to the sex pheromone, a bat sound or sucrose and compared them to the respective control treatments (hexane, a non-pulsed tone or water).

Materials and methods

Insects

Males and females of *Spodoptera littoralis* Boisduval 1833 (Lepidoptera: Noctuidae) were reared separately under a 16h/8h light:dark cycle in a rearing chamber at 22–23 °C and 65 % relative humidity (Anderson et al. 2003). Freshly hatched adults were collected daily and males were maintained in plastic containers without feeding.

Pre-exposure procedures

Males were pre-exposed to olfactory, gustatory or auditory signals 2 to 5 h into the scotophase of the first day after hatching (day-1), which corresponds to the time of maximal activity. For all pre-exposure procedures, males were transferred to the respective experimental chamber before the beginning of the scotophase and pre-exposure to the various stimuli was performed under red light illumination. Pre-exposure procedures closely matched the procedures used in previous behavioural and neurobiological investigations (Anton et al. 2011; Minoli et al. 2012; Guerrieri et al. 2012).

For olfactory pre-exposure, the wings were cut on the day of emergence (day-0) and the day after, males were delicately positioned on a locomotion compensator (LC300, Syntech,

Kirchzarten, Germany) and submitted to a constant charcoal-filtered humidified airflow (17 mLs) directed at the antennae of the moth. A second airflow (7 mL/s) was directed through a Pasteur pipette with a filter paper containing either 1 female equivalent of a female pheromone extract (for preparation, see Anton et al. 2011) or 10 μL of the solvent hexane as a control and inserted into the constant airflow for 1 min as described by Minoli et al. (2012).

For gustatory pre-exposure, males were placed in a cut plastic pipette tip with the head and antennae protruding. The antennae of each individual were gently touched for 10 s with a toothpick soaked with a 1 M sucrose solution or distilled water as a control (Minoli et al. 2012).

For auditory pre-exposure, males were placed individually in mesh wire cages and placed in a marked area in front of a loudspeaker emitting either a pulsed bat-like sound or a tone. The bat-like sound consisted of 20 trapezoid shaped pulses of 4.7 ms length with a carrier frequency of 30 kHz and an intensity at 102 dB SPL (sound pressure level). The pulse trains were repeated ten times resulting in a total exposure time of 940 ms. The tone signal consisted of a continuous 940 ms 30 kHz frequency at 102 dB SPL as a control (Anton et al. 2011). After exposure, males were transferred into plastic containers and kept in a rearing chamber until the next day. Brains were dissected after 24 to 28 h following all types of pre-exposure.

Histology and microscopy

Brains were dissected from the head capsule under phosphate-buffered saline (PBS) and then fixed for 24 to 48 h at 4 °C in 4 % paraformaldehyde in PBS with 0.2 % Triton C-100 (PBS-T). Staining was performed as described previously (Guerrieri et al. 2012). Briefly, brains were rinsed in PBS-T and then incubated for 5 days at 4 °C in anti-synapsin antibody (1:50 in PBS-T; SYNORF1, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa, USA). After being rinsed, they were incubated for 4 days at 4 °C in the secondary antibody (Alexa-Fluor-546-conjugated anti-mouse, 1:150 in PBS-T; Invitrogen, Abingdon, UK). Brains were then rinsed again, dehydrated, cleared in methyl salicylate and stored at room temperature until observation. Brains were mounted on perforated aluminium slides with glued glass coverslips to allow observation from both sides. Preparations were observed and optically sectioned with a laser-scanning microscope (Nikon A1 or Leica SP2) with a 20×0.75 water immersion objective. Because of the thickness of the brains, preparations were turned between the detailed scans of antennal lobes and the mushroom body calyces. Preparations were excited with 561 nm light and fluorescence was detected between 570 and 620 nm. Stacks of optical sections (512×512 pixels) with a 4× frame average were acquired for each antennal lobe and mushroom body calyx at intervals of 1 μm.

Volume analyses

The stacks were imported into Amira 3.1.1 (Visualization Sciences Group, Mérignac, France). Reconstructions were performed blind to the treatment by manually tracing their outlines with the computer mouse over the sections of the image stack every three frames and interpolating the surfaces of the intermediate sections. The surface of each reconstructed neuropil was generated with the "SurfaceGen" tool of the software to obtain a volume estimation from the drawn serial surface by using the "Measure" tool of the software (Guerrieri et al. 2012 and references therein). The volume of the same glomeruli as in a previous study was determined in each antennal lobe (Guerrieri et al. 2012): three glomeruli belonging to the macroglomerular complex (MGC: 17, 18 and 37) and two plant-odour-processing glomeruli (4 and 11) according to the antennal lobe atlas by Couton et al. (2009; Fig. 1a). The volume of the calyces of the mushroom body was determined by the same procedure (Fig. 1b). For each brain, only one antennal lobe and one mushroom body calyx were analysed, with sample sizes ranging from 17 to 20 for each treatment. Mann–Whitney tests were used to compare the mean volumes of each structure between each pre-exposed group and its corresponding control group.

Results

To reveal potential modifications in the anatomy of the primary and secondary olfactory integration centres following pre-exposure with various sensory stimuli, we determined the volume of pheromone-processing versus plant-odour-processing glomeruli within the antennal lobe and the volume of the calyx (input region) of the mushroom body. Volumes of the brain structures in pre-exposed male moths were compared statistically with those of the respective control-treated male. The summed volume of the five reconstructed glomeruli increased significantly after pre-exposure to pheromone and bat sound (data not shown; $U=64.0$, $P<0.001$ and $U=63.0$, $P<0.05$, respectively). Additionally, the size of individual glomeruli varied differently according to sensory pre-exposure (Fig. 2). Glomerular sizes increased significantly for two out of the three analysed MGC glomeruli after pheromone (Fig. 2a) and bat sound (Fig. 2b) pre-exposure. The volume of the cumulus (glomerulus 18) increased significantly after pheromone pre-exposure ($U=67.0$, $P<0.05$) as previously shown (Guerrieri et al. 2012), as did that of MGC glomerulus G17 ($U=58.0$, $P<0.001$). By contrast, MGC glomerulus 37 was unaffected ($U=104.0$, $P>0.05$). We observed similar specific changes following bat sound pre-exposure: significant increases in volume were found for the cumulus and MGC glomerulus 17 in bat-sound-exposed male brains ($U=77.0$, $P<0.05$ and $U=69.0$, $P<0.01$, respectively), but not in
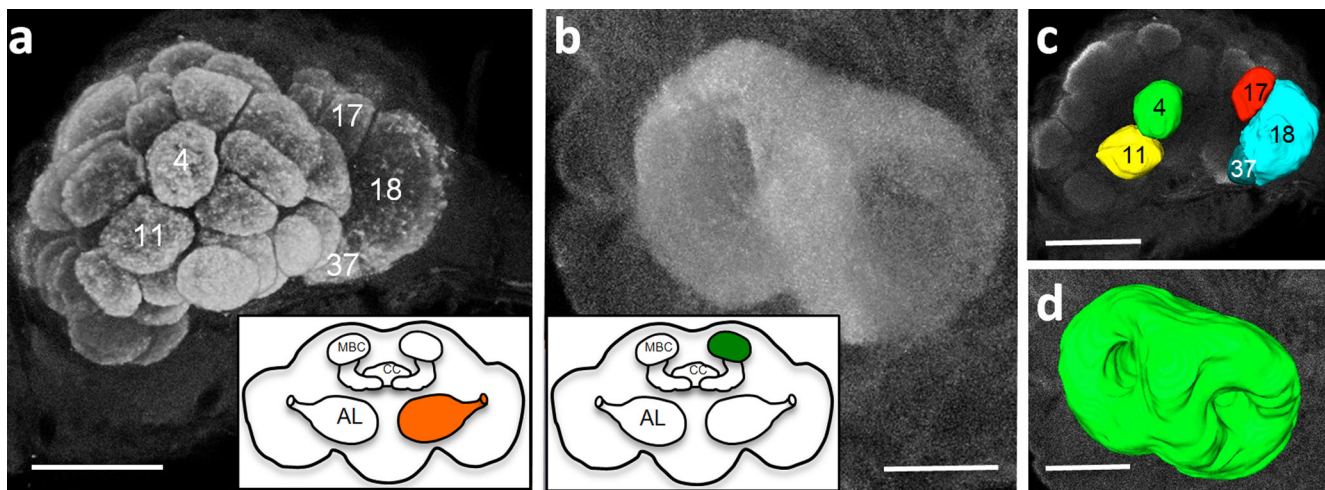
Fig. 1 **a** Frontal view of the three-dimensionally reconstructed left antennal lobe of a male *Spodoptera littoralis* with the reconstructed glomeruli studied here identified by their numbers: glomeruli 17, 18 (cumulus) and 37 are part of the macroglomerular complex, whereas glomeruli 4 and 11 are plant-odour-processing glomeruli. **b** Frontal view of the three-dimensionally reconstructed calyx of the left mushroom body. **c** Three-dimensional (3D) manual reconstructions of the glomeruli studied here, numbered as in **a**. **d** 3D manual reconstruction of the calyx of the left mushroom body as in **b**. *Insets* Representations of the moth brain in a frontal view, indicating the position of structures in **a**, **b** (*AL* antennal lobe, *CC* central complex, *MBC* mushroom body calyx). *Bars* 100 μm

MGC glomerulus 37 ($U=109.0$, $P>0.1$). Interestingly, the plant-odour-processing glomerulus 4 increased in volume in bat-sound-exposed male brains ($U=73.0$, $P<0.05$). Sucrose exposure (Fig. 2c), on the other hand did not lead to any significant anatomical modification within the antennal lobe ($U>153.0$, $P>0.1$ in all cases).

The volume changes of the mushroom body calyces largely reflected the changes observed within three of the five measured glomeruli: the calyx volume increased significantly after either pheromone ($U=65.0$, $P<0.01$) or bat sound ($U=78.5$, $P<0.01$) pre-exposure, but not after exposure to sucrose ($U=149.0$, $P>0.1$; Fig. 3).

Discussion

In the present study, our aim has been to identify potential anatomical correlates of previously observed changes in physiological and/or behavioural olfactory responses at 24 h after pre-exposure with sensory signals of various modalities. We reveal anatomical modifications in the primary and secondary olfactory centres of the male moth brain: a brief pre-exposure to either the sex pheromone or a predator sound elicits a significant increase in volume of certain antennal lobe glomeruli and of the calyces of the mushroom bodies 24 h later. An investigation of other brain regions was out of the scope of the present study, although other sensory input and integration areas might be affected by exposure to different sensory signals. We therefore focus our discussion on the herein-studied olfaction-related areas within the brain. The observed changes are remarkable with respect to the short duration of the pre-exposure and the relatively short delay before anatomical

analysis. As in vertebrates (Grubb and Thompson 2004), anatomical modifications of brain structures after experience have been found after multiple or long-term exposure to sensory stimuli in insects (Devaud et al. 2001, 2003; Sachse et al. 2007; Groh and Meinertzhagen 2010; Stieb et al. 2010; Jones et al. 2013; Scholl et al. 2014). However, the fast anatomical effects of this brief exposure on the antennal lobe and calyces of the mushroom bodies in male *S. littoralis* can only be compared with the rapidly occurring effects of associative learning in the honey bee, *Apis mellifera* (Hourcade et al. 2009, 2010). Several attempts have been made to investigate the mechanisms underlying the observed anatomical changes in insects. Volume increases of antennal lobe glomeruli in honey bees and *Drosophila melanogaster* through experience or adult development/hormone action have been shown to originate from an increase in synaptic connections (Devaud et al. 2003; Brown et al. 2004). Increased volumes of mushroom body calyces during the transition from nursing to foraging in honey bee workers have also been shown to be correlated with an increase in neuron branching (Farris et al. 2001) and in the number of synaptic contacts (Groh et al. 2012). On the other hand, the calyces did not increase in size after associative odour learning, whereas the density of mushroom body microglomeruli (synaptic boutons) increased in the honey bee (Hourcade et al. 2010).

With regard to structure-function correlations, the observed anatomical modifications are clearly related to previously described physiological changes within the primary olfactory centre, namely the antennal lobe. As mentioned above, we do not hypothesize that such modifications would be restricted to the olfactory centres. Nevertheless, they are clearly specific in the sense that not all the neuropiles measured here show
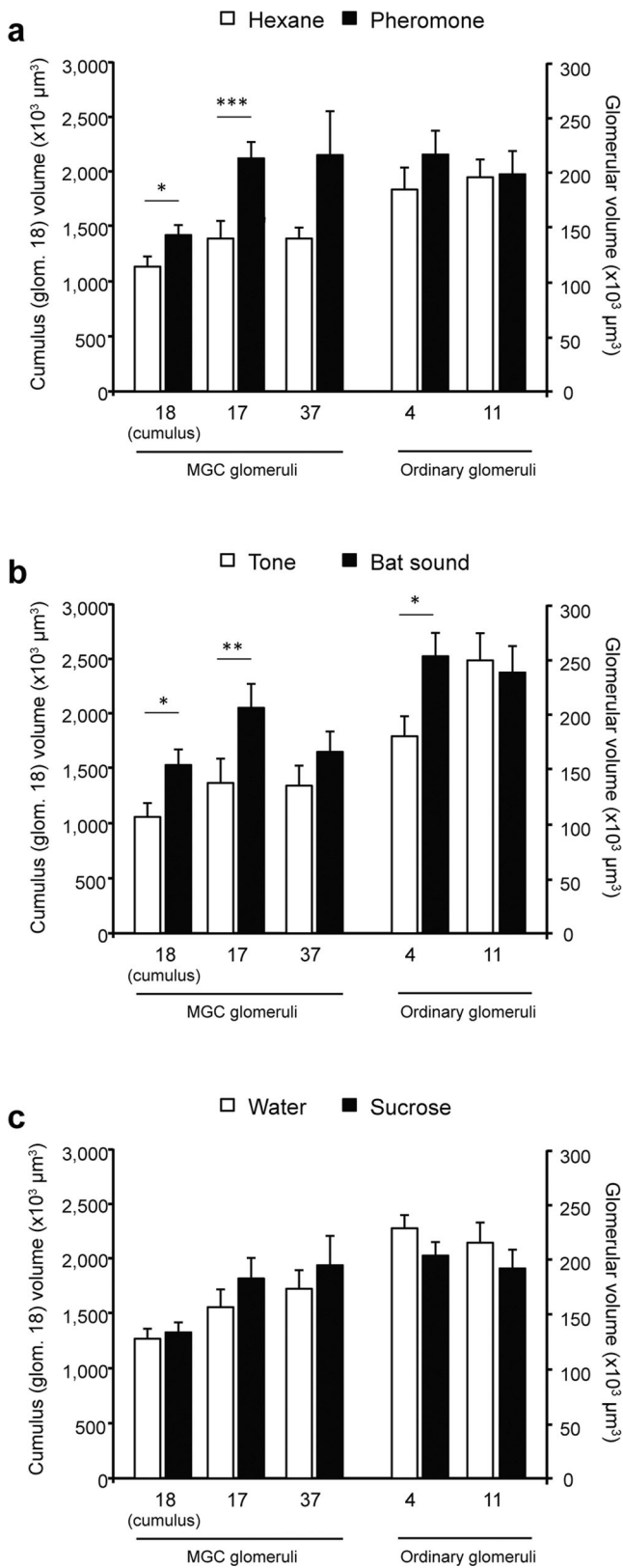
◀ **Fig. 2** Volumes of antennal lobe glomeruli in male *S. littoralis* as a function of pre-exposure to (**a**) pheromone, (**b**) a bat sound and (**c**) sucrose. Note that two glomeruli of the macroglomerular complex (18, 17) increased their volume significantly after pre-exposure to the sex pheromone and to the predator sound, whereas one plant-odour-processing glomerulus increased in size only after predator sound exposure. On the other hand, sucrose pre-exposure did not modify the size of the analysed glomeruli. Note that the volumetric scale is different for glomerulus 18 (cumulus), which is much larger than the others. *Asterisks* indicate significance levels: * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *Error bars* denote SEM

increase of sensitivity to the sex pheromone after sex pheromone and bat sound pre-exposure, but also an increase in the sensitivity to plant compounds (Anderson et al. 2007; Anton et al. 2011). The increased volumes of glomeruli processing sex pheromone information and even of one plant-odour-processing glomerulus thus nicely reflect the observed physiological changes at the antennal lobe input level. Whether the differences in the effects of bat sound pre-exposure on glomeruli 4 and 11 are related to different responses from those of plant odorants is unknown but the available functional data from studies of male and female *S. littoralis* indicate variable physiological state-dependent plasticity in the sensitivity to diverse plant odorants (Saveer et al. 2012; Kromann et al. 2015). Our present results confirm the results of an earlier study in which an increase in the size of the cumulus had been detected after pre-exposure to the sex pheromone (Guerrieri et al. 2012) but now show additional modifications not found earlier. Interestingly, similar volume changes have also been found at the projection neuron output area, i.e. the mushroom body calyces. On the other hand, the lack of
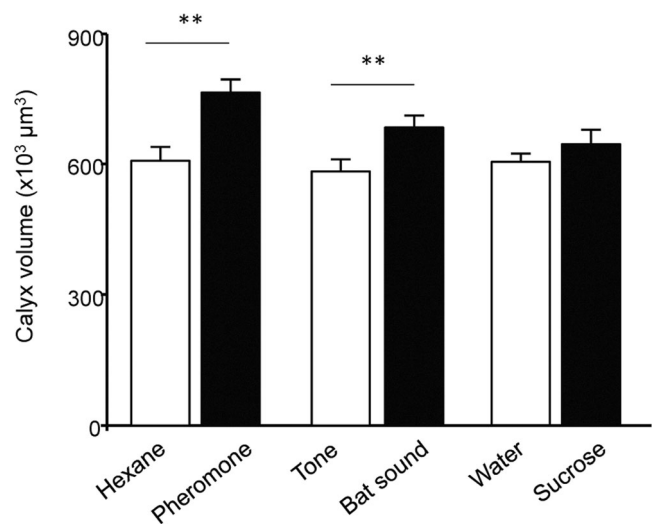

Fig. 3 Volume of mushroom body calyx as a function of pre-exposure treatment. The same treatments eliciting volume changes in certain MGC glomeruli also caused an increase in the volume of the mushroom body calyx as compared with the respective control treatments. Significance levels as in Fig. 2. Error bars denote SEM

identical trends and, thus, they cannot be attributed to general brain size variations across groups. Intracellular recordings of antennal lobe projection neurons have revealed not only an

physiological changes in the antennal lobe after pre-exposure to an attractive gustatory stimulus, i.e. sucrose, is correlated with an absence of anatomical changes in the antennal lobe and the calyces of the mushroom bodies (Minoli et al. 2012). We had, in this last case, expected a lack of change within the antennal lobe but had hypothesized that behavioural effects of pre-exposure with sucrose might originate from modifications within the secondary olfactory centre, namely the mushroom bodies. The absence of a correlation between anatomical and behavioural modifications after sucrose pre-exposure indicates that either modulation occurs at a different integration centre or that modifications are too subtle to be detected by the methods used here.

Taken together, the anatomical modifications reported here and the related functional and behavioural changes mentioned above clearly show that olfactory centre organisation can be modulated not only by olfactory input, but also by auditory stimuli. What could be the anatomical substrates for such cross-modal plasticity in moths? Both peripheral and central gustatory neurons (Popescu et al. 2013; Jorgensen et al. 2007; Kvello et al. 2010) and central auditory neurons (Pfuhl et al. 2014) project next to the antennal lobes. However, no direct connections have been described. Although mushroom bodies receive input from olfactory centres in most insects investigated so far and visual input in certain hymenoptera (Gronenberg 2001), no direct evidence of auditory and gustatory input has been found to our knowledge. Putative gustatory input to the mushroom bodies has, however, been described in the honeybee (Schröter and Menzel 2003). Moreover, some indications have been found for the integration of mechanosensory/ auditory information with olfactory input. Direct mechanosensory input reaches the antennal lobe in the moth *S. littoralis* (Han et al. 2005) and a sound-sensitive neuron in a heliothine moth probably responds to the species-specific sex pheromone, for example (Pfuhl et al. 2014). By contrast, unlike in other insect species, such as the honey bee or fruit fly (Hammer 1993; Liu et al. 2012; Huetteroth et al. 2015), evidence is lacking for possible sucrose-sensitive neurons modulating olfactory processing in moths. Such neurons are nevertheless likely to occur in the protocerebrum, because moths, like honey bees and fruit flies, are able to associate a sucrose reward with olfactory signals (Fan et al. 1997; Hartlieb et al. 1999). Now that multimodal interactions have been shown to occur at the behavioural, physiological and anatomical levels and given the evidence of high levels of the plasticity provided by this and other work, we suggest that moths are a suitable model for further studies regarding the way that such interactions occur and the manner in which the different sensory systems are interconnected within the brain.

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