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Early olfactory experience induces structural changes in the primary olfactory center of an insect brain

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Abstract

The antennal lobe (AL) is the first olfactory center of the insect brain and is constituted of different functional units, the glomeruli. In the AL, odors are coded as spatiotemporal patterns of glomerular activity. In honeybees, olfactory learning during early adulthood modifies neural activity in the AL on a long-term scale and also enhances later memory retention. By means of behavioral experiments, we first verified that olfactory learning between the fifth and eighth day of adulthood induces better retention performances at a late adult stage than the same experience acquired before or after this period. We checked that the specificity of memory for the odorants used was improved. We then studied whether such early olfactory learning also induces long-term structural changes in the AL consistent with the formation of long-term olfactory memories. We also measured the volume of 15 identified glomeruli in the ALs of 17-day-old honeybees that either experienced an odor associated with sucrose solution between the fifth and eighth day of adulthood or were left untreated. We found that early olfactory experience induces glomerulus-selective increases in volume that were specific to the learned odor. By comparing our volumetric measures with calcium-imaging recordings from a previous study, performed in 17-day-old bees subjected to the same treatment and experimental conditions, we found that glomeruli that showed structural changes after early learning were those that exhibited a significant increase in neural activity. Our results make evident a correlation between structural and functional changes in the AL following early olfactory learning.

Introduction

To what extent early experience determines long-lasting changes in physiological, structural and behavioral processes remains a subject of intensive study and debate. Insects, with their relatively simple and accessible brain, and the possibility of manipulating early experience in the laboratory, offer a suitable model to answer this question. Several studies have documented that early sensory experience plays a role in shaping neuronal circuits and behavior in adult insects (Gascuel & Masson, 1987; Heisenberg *et al.*, 1995; Barth & Heisenberg, 1997; Devaud *et al.*, 2001; Stieb *et al.*, 2011). For instance, in the fruit fly, *Drosophila melanogaster*, morphological changes in the primary olfactory neuropile – the antennal lobe (AL) – were detected after non-associative odor exposure a few days after emergence (Devaud *et al.*, 2001, 2003). In addition, prolonged exposure to CO_2 during an early period of the fly's life induces a reversible volume increase in a region of the AL devoted to CO_2 processing (the CO_2-specific glomerulus; Sachse *et al.*, 2007). Besides simple exposure to sensory cues, young animals are also capable of learning relationships between sensory cues and their outcome in their environment. Yet, little is known about the consequences of early associative learning on the functional and structural properties of adult neural networks, especially if such learning occurs at a moment when the nervous system is not yet fully developed (Carman *et al.*, 2002; Manrique *et al.*, 2005; Schäble *et al.*, 2007).

In honeybees, odors are detected by receptor neurons located on the antennae. These neurons project to the AL, which is constituted of spherical subunits termed glomeruli. In the AL, olfactory information is encoded as specific spatiotemporal patterns of glomerular activation (Joerges *et al.*, 1997; Galizia *et al.*, 1998, 1999; Sachse *et al.*, 1999; Sachse & Galizia, 2002; Deisig *et al.*, 2006, 2010), which are subject to change after associative learning in adult bees (Faber *et al.*, 1999; Sandoz *et al.*, 2003; Rath *et al.*, 2011). Recently, we showed that early odor learning during young adulthood, i.e. 5–8 days after emergence, also modifies odor-evoked

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activity in the AL. Early learning enhances odor-induced neural activity in the AL of 17-day-old honeybees, both for the learned odor and for structurally similar odors (Arenas *et al.*, 2009a). Furthermore, associative olfactory learning during this early specific period leads to better retention performances in 17-day-old bees, compared with when the same experience was acquired earlier (i.e. first–fourth day of adult age) or later (i.e. ninth–12th day of adult age; Arenas & Farina, 2008). Lastly, learning during this putative sensitive period improves the retention of newly-learned odorants later in life (Arenas *et al.*, 2009b). Taken together, these results indicate that early odor-rewarded experience induces a long-term, stable reorganization of olfactory circuits, which may have profound effects on the bees' olfactory learning abilities. To what extent such reorganization translates into structural changes within the AL remains to be determined. Odor learning taking place in adult honeybees (~2 weeks of age) leads after 3 days to the formation of a long-term memory, coinciding with modifications of the volume in a subset of AL glomeruli (Hourcade *et al.*, 2009). We therefore asked whether early olfactory exposure may modulate later behavior based on a similar mechanism.

In the present study, we ask whether memories acquired at young adult ages modify the AL architecture in a long-lasting manner. To answer this question, we measured the volume of 15 identified glomeruli in 17-old-day bees, which were previously subjected to olfactory learning between the fifth and eighth day of adult age and had thus established long-lasting olfactory memories as a consequence of that experience. Data from treated bees were compared to naïve bees without such experience. We then compared our volumetric data with the results of calcium-imaging recordings of AL activity obtained in identical conditions (17-day-old bees that had been subjected to olfactory learning between the fifth and eighth days of adulthood) in a previous study (Arenas *et al.*, 2009a). In this way, we could determine whether early olfactory learning induces correlated structural and functional modifications in the AL network.

Materials and methods

Animals

European honeybees (*Apis mellifera*) were obtained in the laboratory from sealed brood frames. Frames containing cupped brood were placed in an incubator at 36 °C, 55% relative humidity and darkness. Newly emerged workers (0–1 day old) were collected in groups of 60 and caged in wooden boxes (10 × 10 × 10 cm). Caged bees were kept in the incubator for 17 days at 36 °C, 55% relative humidity and darkness, and were fed with 1.8 M sugar solution, water and pollen *ad libitum*.

Early olfactory experiences

To allow early olfactory learning, scented sucrose solution was offered within the wooden boxes for a defined period after emergence. The scented solution was obtained by adding 50 μL of pure odor per liter of 1.8 M sucrose solution. Odorants 1-nonanol (1-NON) or 1-hexanol (1-HEX), both obtained from Sigma Aldrich, were used to this end. The scented solution was offered in plastic tubes (10 mL volume) with a small opening at the tip (1 mm diameter), which hanged inside the rearing cages (for details, see Arenas & Farina, 2008). The scented solution was offered for four consecutive days inside the cages and was the only source of sugar.

For behavioral experiments the scented sucrose solution was made available during days 1–4 (T1–4), 5–8 (T5–8) or 9–12 (T9–12) of adult age (Fig. 1A). For anatomical experiments, it was made available during days 5–8 (T5–8).

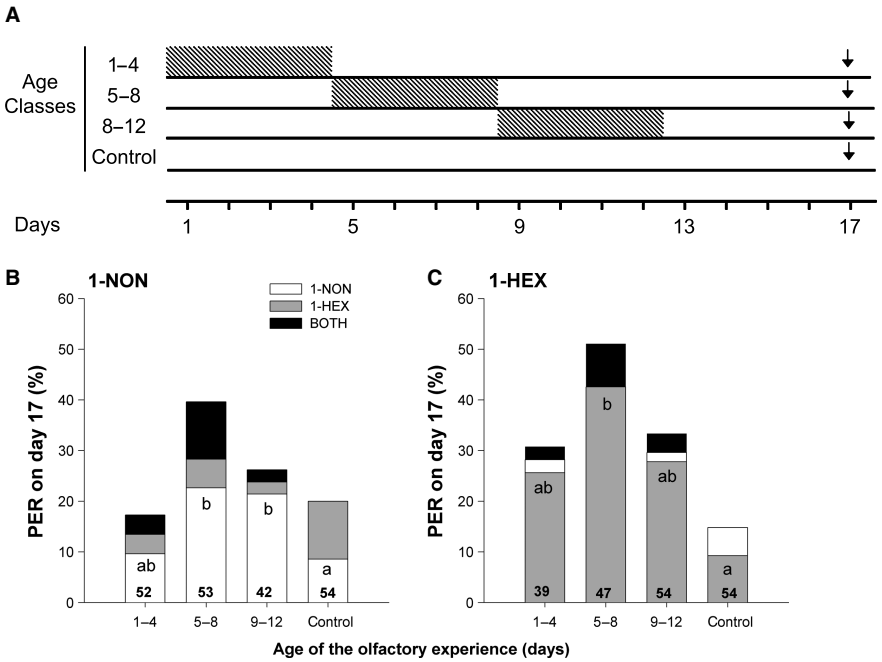

FIG. 1. Schematic schedule for the behavioral experiment. (A) Caged bees reared throughout their adult lifespan in incubators were offered a scented sugar solution during four consecutive days (gray boxes). The solution was either scented with 1-nonanol (1-NON) or 1-hexanol (1-HEX). At 17 days of age (black arrow) olfactory memory retention was tested. (B) Percentages of 17-day-old bees showing PER in response to 1-NON only (the CS, white), 1-HEX only (the novel odor, gray) or to both (black) following exposure to 1-NON in sucrose solution. (C) Percentages of 17-day-old bees responding to 1-NON only (the novel odor, white), 1-HEX only (the CS, gray) or to both (black) when bees had been exposed to 1-HEX diluted in sucrose solution. Groups with different letters are significantly different at $P < 0.015$ after applying G-test on PER proportions (corrected threshold = 0.016). Sample sizes are presented at the bottom of each bar.

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Behavioral experiments

We first verified that early olfactory learning between the fifth and eighth day of adulthood determines better retention performances at a later adult stage (Arenas & Farina, 2008; Arenas *et al.*, 2009b). Our previous work demonstrated this significant effect using linalool and phenylacetaldehyde as early-conditioned odors (Arenas & Farina, 2008; Arenas *et al.*, 2009b). Here we studied whether improvement of retention performances can also be found if 1-HEX and 1-NON are used as early-conditioned odors between the fifth and eighth day of adulthood. To assess the specificity of this period, retention performance of bees that experienced these odorants paired with reward between the first and fourth, and between the ninth and 12th days of adulthood were also quantified (Fig. 1A). In all cases, we quantified memory retention for the conditioned odor (conditioned stimulus or CS, the odor associated with the sucrose solution) at the age of 17 days, when workers commonly initiate foraging tasks (Rösch, 1925; Lindauer, 1952). As a control, we quantified responses to a novel (non-conditioned) odor (1-HEX for bees that had experienced 1-NON associated with sucrose and vice versa).

Seventeen-day old bees were harnessed after chilling them at 4 °C, and were kept in their individual holders within an incubator (32 °C, 55% relative humidity and darkness) for 3 h. Only bees that showed PER to 1.8 M sucrose solution applied onto the antennae (unconditioned response) 20 min before the odor presentations were kept for retention tests (> 80% of bees). In such tests, the CS and the novel odor were delivered by means of an automated device that sent a continuous clean air flow (50 mL/s) to the bee's head. Controlled odor pulses were injected through a secondary air flow (6.25 mL/s) into the continuous clean air flow. An air extractor was positioned behind the bee to prevent residual odor contamination. For each odor presentation, the bee was positioned in front of the device and was exposed to the clean airflow for 15 s. In this way, the insect was familiarized with the experimental context and it was possible to verify that it did not present any PER to the context or to the mechanical stimulus of the clean airflow. Only a few bees (< 5%) performed so and were discarded. Then, the odor (the CS or the novel odor) was presented for 6 s and the occurrence of PER was noted. After odor offset, the bee remained in the setup for another 19 s and was then replaced by another bee. Each bee experienced two odor presentations (the CS and the novel odor) in a random order.

We distinguished three classes of responses during the retention test – the bee could respond to the CS only (CS-specific responses); to the novel odor only; or to both odors. Bees having associated the conditioned odor with the sucrose solution at young adult ages were expected to exhibit PER to the conditioned but not to the novel odor at a late adult stage (Takeda, 1961; Bitterman *et al.*, 1983).

Brain preparation

For structural analyses of AL architecture following early olfactory learning, 17-day-old bees were caught from the experimental cages, killed on ice and mounted individually in metal tubes to dissect their brains. The head was fixed with low-temperature-melting wax (Deiberit 502; Böhme & Schöps Dental GmbH, Goslar, Germany) and the head capsule was opened. All glands, membranes and trachea covering the brain were carefully removed to expose both ALs. The brain was bathed with a protease solution (from *Bacillus licheniformis* in propylene glycol; Sigma-Aldrich) for 20 min, rinsed in bee saline (in mM: NaCl, 130; KCl, 6; $MgCl_2$, 4; $CaCl_2$, 5; HEPES, 10; glucose, 25; sucrose, 160) and stained in 4% neutral red for 20 min (Michrome no226; Edward Gurr). The whole head was then placed overnight in paraformaldehyde at 4 °C for fixation (4% in phosphate-buffered saline; Sigma-Aldrich). On the next day, the brains were dissected, dehydrated with increasing concentrations of ethanol (50, 70, 90, 95 and 100%) and clarified using xylene and 100% ethanol. Preparations were then kept in methyl salicylate (Sigma-Aldrich) for clarification, at −20 °C from 2 to 6 days until observation under the microscope.

Confocal microscopy

Preparations were mounted in specially designed slides that could contain the whole brain structure (Hourcade *et al.*, 2009). Each brain was viewed under a confocal laser-scanning microscope (TCS2; Leica) with a 40× oil-immersion objective (Leica HPX PL apochromate, N.A. 1.25). Preparations were excited with a He/Ne laser at 543 nm, and fluorescence was detected between 555 and 650 nm. Within each bee, the rostro-ventral part of each AL (Strausfeld, 2002) was scanned (512 × 512 pixels) at intervals of 2 μm with 4× frame average.

Glomerulus reconstruction and volume measurement

The complete image stacks were imported into the Amira 3.0 software (Mercury Computer Systems). Glomeruli were individually reconstructed by manually tracing their contours on each image. AMIRA 3.0 provided a reconstruction of the glomerular structures and estimated their volume from the drawn serial surfaces. Individual glomeruli were identified using the standardized AL atlas (Galizia *et al.*, 1999) according to their shape, size and relative position in the AL. A total of 15 glomeruli that could be reliably identified across individuals were reconstructed in all bees, most of which had been similarly analysed in a previous study (Hourcade *et al.*, 2009). The whole procedure was blindfold with respect to treatment. Because measurements were done in the left or the right AL indistinctly, we checked for differences between sides. In some analyses (see below), we aimed to exclude inter-individual size differences, and therefore divided the absolute volume of each glomerulus by the sum of the volumes of the 15 identified glomeruli, thus obtaining relative volume values.

Statistics

Comparisons of PER levels among ages within the three response categories (CS only, novel odor only or both odors) were performed by means of a G-test using the simultaneous-test procedure of the unplanned-test of homogeneity for goodness of fit (Sokal & Rohlf, 1995). To reduce the risk of type I error, significance thresholds were corrected using the Bonferroni method ($\alpha' = \alpha/k$), with $\alpha = 0.05$ and $k = 3$. Thus, our significance threshold was $\alpha' = 0.016$.

For the analysis of volume data, a two-way analysis of variance (ANOVA) with repeated measures was applied, with 'treatments' as a between-group factor (three levels: T5–8/1-NON; T5–8/1-HEX; and Control) and 'glomerular volume' as the repeated measure. Simple effects were used for describing interactions detected in the ANOVA (Quinn & Keough, 2002). Tukey tests were performed for *post hoc* comparisons between T5–8/1-NON, T5–8/1-HEX and control bees.

We determined whether morphological glomerular changes correlated with odor-evoked activity ($\Delta F/F\%$) by means of Pearson's r correlation analysis. When data did not satisfy the assumptions of this method, we applied Spearman's r correlation analysis. Calcium-imaging recordings were obtained from a previous study (Arenas *et al.*, 2009a). In a first analysis, we correlated the percentage of volume change in each glomerulus with odor-evoked activity in this

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glomerulus in naïve honeybees, i.e. bees that had not been subjected to any early olfactory learning (data from Arenas *et al.*, 2009a). Because negative values in calcium green signals are generally associated with bleaching of the preparation rather than a glomerulus inhibitory activity, odor-evoked responses were clipped to 0 before calculations. Volume change was calculated as the difference between the relative glomerular volumes of T5–8 bees and control bees. In a second analysis, we correlated the volume change in each glomerulus with the 'change' in odor-evoked activity (with respect to naïve bees) observed in 'experienced' bees, i.e. bees that were subjected to olfactory learning between days 5 and 8 of adult age (Arenas *et al.*, 2009a). For better visualization of these results we established maps of glomerular volume change, maps of neural activity and maps of changes in glomerular activity (response). For that, the volume change of each glomerulus was normalized by setting the largest change to 100% and scaling the volume changes of the other 14 glomeruli accordingly. Similarly, changes in activity were normalized by setting the largest change at 100% and scaling the other responses accordingly.

Results

Early olfactory learning between the fifth and eighth day determines better retention performances at a later adult stage

After exposing young bees for 4 days to an odor (1-NON or 1-HEX) associated with sucrose reward (the conditioned odor or CS), we measured the percentage of CS-specific responses, PER to the novel odor only, or to both odors in bees that were 17 days old (Fig. 1B and C). These responses were compared statistically between different stimulation periods and against untreated control bees.

Irrespective of the odorant learned, CS-specific responses in 17-day-old bees (Fig. 1B – white bars for bees having experienced 1-NON; Fig. 1C – gray bars for bees having experienced 1-HEX) differed between the four experimental groups: (i) bees that experienced the rewarded odor between the first and fourth day of adulthood (T1–4); (ii) between the fifth and eighth day (T5–8); (iii) between the ninth and 12th day (T9–12); and (iv) untreated controls (G-test: $G_{h\ 1\text{-}NON} = 13.113$, $P = 0.004$, $N = 201$, df = 3; $G_{h\ 1\text{-}HEX} = 15.779$, $P = 0.001$, $N = 194$, df = 3). Heterogeneity in PER levels was due to the fact that CS-specific responses varied with the period during which learning occurred – for both odorants, T1–4 bees showed CS-specific responses that did not differ from those of control bees (G-test: $G_{h\ 1\text{-}NON} = 3.23$, $P = 0.36$, $N = 106$, df = 3; $G_{h\ 1\text{-}HEX} = 4.45$, $P = 0.22$, $N = 94$, df = 3); on the contrary, T5–8 bees showed CS-specific responses that were significantly higher than those of control bees ($G_{h\ 1\text{-}NON} = 10.58$, $P = 0.0142$, $N = 201$, df = 3; $G_{h\ 1\text{-}HEX} = 15.78$, $P = 0.001$, $N = 194$, df = 3); finally, CS-specific responses in T9–12 bees were (1-NON) or not (1-HEX) higher than those of control bees ($G_{h\ 1\text{-}NON} = 10.58$, $P = 0.0142$, $N = 201$, df = 3; $G_{h\ 1\text{-}HEX} = 3.50$, $P = 0.065$, $N = 194$, df = 3).

An analysis of responses to the novel odor (Fig. 1B – gray bars for bees having experienced 1-NON; Fig. 1C – white bars for bees having experienced 1-HEX) and to both odors (Fig. 1B and C – black bars) showed no significant differences between the four experimental groups (novel odor: $G_{h\ 1\text{-}NON} = 1.53$, $P = 0.675$, $N = 201$, df = 3; $G_{h\ 1\text{-}HEX} = 3.32$, $P = 0.35$, $N = 194$, df = 3; both odors: $G_{h\ 1\text{-}NON} = 8.81$, $P = 0.031$, $N = 201$, df = 3; $G_{h\ 1\text{-}HEX} = 5.68$, $P = 0.13$, $N = 194$, df = 3). Note that the tendency to exhibit higher responses to both odors in the T5–8 group conditioned to 1-NON ($P = 0.031$) was considered not significant given the corrected alpha level ($P = 0.016$).

Taken together, our behavioral data show that olfactory learning between the fifth and eighth day of adulthood induces better retention performances at a late adult stage than the same experience acquired before (first–fourth day) of after (ninth–12th day) this period.

Early olfactory learning induces odor-specific increases in glomerular volume

We aimed at determining whether olfactory learning between the fifth and eighth day of adulthood results in long-term structural changes in the AL, visible at a late adult stage (i.e. when bees are 17 days old). We compared bees that experienced a rewarded odor (1-NON or 1-HEX) between the fifth and eighth day of adulthood ($n = 20$) with control bees ($n = 15$). Based on their recognizable shape and respective position, 15 glomeruli were systematically identified in the confocal microscopy data and were reconstructed in three dimensions, allowing volumetric measurement.

The sum of the 15 measured glomerular volumes ('total volume') differed notably between the three groups, T5–$8_{1\text{-}HEX}$, T5–$8_{1\text{-}NON}$ and controls (one-way ANOVA: $F_{2,32} = 10.42$; $P < 0.001$), due to the unexpected presence of overall larger glomeruli in bees having experienced 1-HEX (mean overall volume ± SEM: $6.28 \pm 0.53 \times 10^5$ μm^3; $N = 10$) compared with bees having experienced 1-NON ($4.50 \pm 0.58 \times 10^5$ μm^3; $N = 10$) and control bees ($4.39 \pm 0.42 \times 10^5$ μm^3; $N = 15$). In theory, this difference may be related to the treatment or may result from an already existing heterogeneity in brain sizes across groups due to inter-individual variability (see Discussion). In a conservative approach and to avoid possible biases due to inter-individual size variations that were not related to early learning, we normalized individual glomerular volumes to the total volume of each animal. In other terms, for each individual bee, the volume occupied by a given glomerulus was expressed as a percentage of the total volume considered. By doing so, we looked for experience-related changes in the relative volume of the 15 glomeruli, independently of general size differences.

The distributions of normalized glomerular volumes differed between the three groups, T1–$5_{1\text{-}HEX}$, T1–$5_{1\text{-}NON}$ and controls (Fig. 2), as indicated by a significant Group × Glomerulus interaction

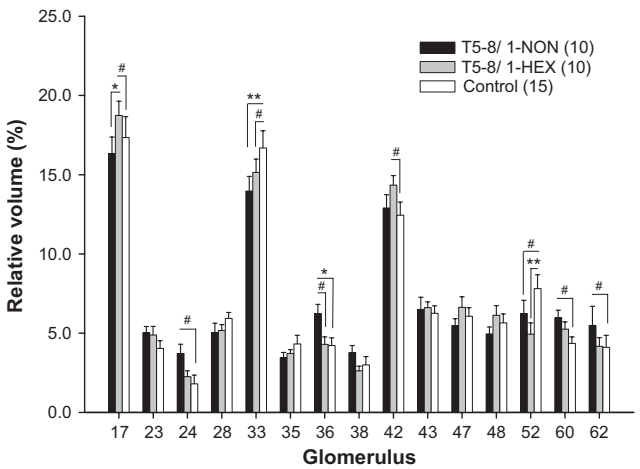

FIG. 2. Relative volumes of glomeruli from 17-day-old bees that were offered scented sugar solutions between the fifth and eighth day of their adult lifespan (T5–8). The relative volume (mean ± SEM, see Materials and methods) is presented for 15 identified glomeruli of the AL in early conditioned bees that experienced 1-hexanol (1-HEX) or 1-nonanol (1-NON), and for control bees. Significant differences in relative volume compared with controls are labeled with $^{**}P < 0.01$, $^{*}P < 0.05$, $^{\#}P < 0.10$.

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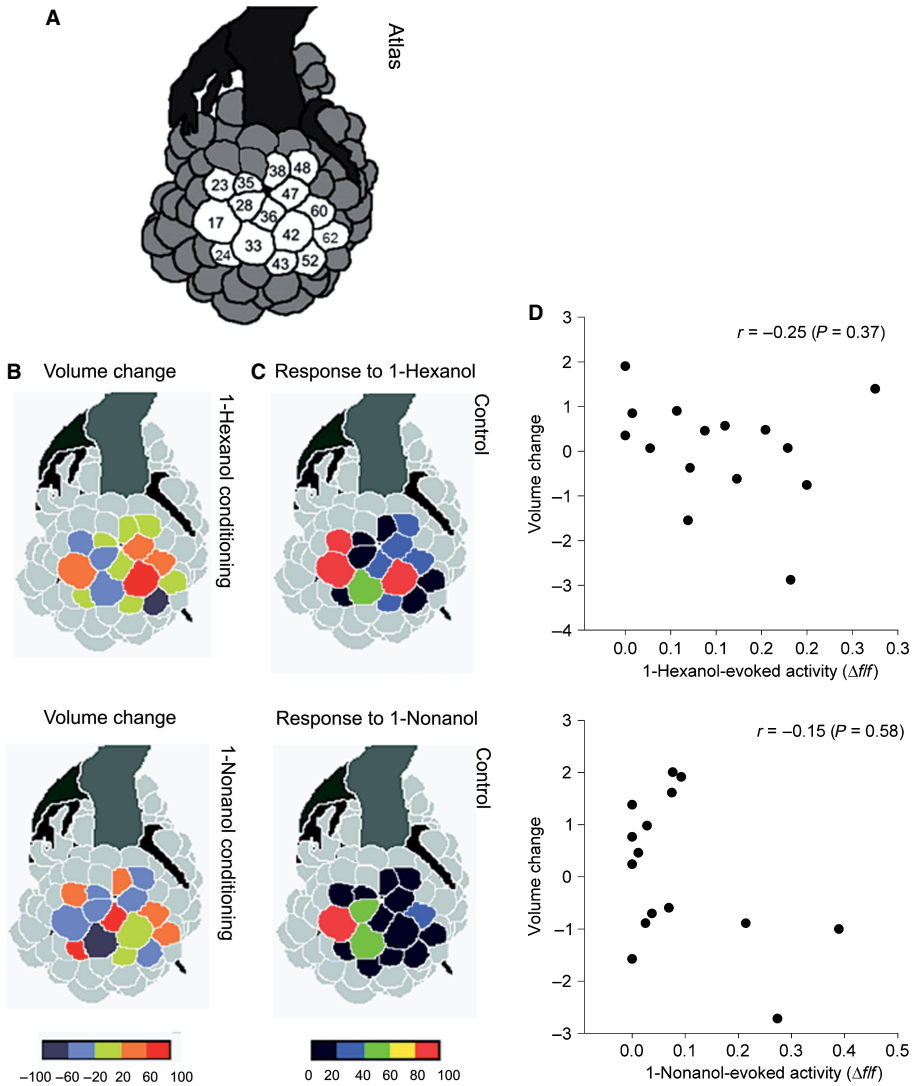

FIG. 3. Glomerular morphological plasticity in 17-day-old bees that were offered scented sugar solutions between the fifth and eighth day of their adult lifespan (T5–8). (A) Schematic representation of the honeybee AL showing the 15 identified glomeruli of the AL (Atlas). (B) Schematic representation of the relative volume change observed between controls and early experienced bees (T5–8) (top – 1-hexanol; bottom – 1-nonanol). Volume change was categorized in five equal bins from −100 to 100%. (C) Spatial patterns of glomerular activity as averaged from control 17-day-old bees responding to 1-hexanol and 1-nonanol, left (data from Arenas *et al.*, 2009a). Response intensity was categorized in five equal bins from 0 to 100% (see Galizia *et al.*, 1999; Hourcade *et al.*, 2009). (D) Glomerulus-wise correlation between the volume change (percent of volume change relative to naïve 17-day-old bees) and 1-hexanol-evoked activity (the panel above) and 1-nonanol-evoked activity (the panel below) in naïve 17-day-old bees.

(two-way, repeated-measure ANOVA: $F_{28,448} = 2.26$; $P < 0.001$). Simple effect analyses revealed that relative volumes varied significantly between groups in the case of glomeruli 17 ($F_{2,480} = 3.71$; $P = 0.025$), 33 ($F_{2,480} = 5.81$; $P = 0.003$), 36 ($F_{2,480} = 3.54$; $P = 0.03$) and 52 ($F_{2,480} = 6.47$; $P = 0.002$). While some glomeruli tended to decrease their relative volume with respect to that of controls irrespectively of the odorant experienced between days 5 and 8 of adulthood (e.g. glomeruli 33 and 52; see Fig. 2), other glomeruli significantly changed volumes in an odor- and experience-specific manner (e.g. glomeruli 33 and 36 for bees having experienced 1-NON). In addition, the volumes of some glomeruli were affected specifically without reaching the significance level (e.g. glomeruli 24 and 62 for bees having experienced 1-NON, and 17 and 42 for bees having experienced 1-HEX). At least in one case (glom. 17), the volume significantly differed between the two treated groups (Fig. 2).

The glomerular variations shown in Fig. 2 can be depicted for each odor-treated group with respect to controls in AL maps (Fig. 3A). Such maps show in a false color scale the amplitude of volume variation for each of the 15 glomeruli measured in our work (Fig. 3B), and suggest upon observation that changes in glomerular volumes differed between bees that experienced 1-HEX and bees that experienced 1-NON. To confirm odor specificity between bees having experienced 1-HEX and 1-NON we performed additional analyses excluding control bees. There, when we just compared odor-treated animals, we found a significant Group × Glomerulus interaction (two-way, repeated-measure ANOVA: $F_{14,252} = 2.00$; $P = 0.021$) that stated that the glomerular changes are odor-specific.

Early olfactory learning determines odor-specific volume increases in glomeruli that are newly activated after learning

To determine the relationship between glomeruli that exhibit significant odor- and experience-dependent variations in their volume and glomeruli that are activated during odor presentations, we compared

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our volumetric measurements with calcium-imaging measurements of glomerular activity obtained under identical experimental conditions (i.e. 17-day-old bees that had experienced a rewarded odor between the fifth and eighth day of adulthood). Measures of glomerular activity were acquired in a previous study by means of calcium-imaging recordings upon olfactory stimulation (Arenas *et al.*, 2009a).

We first asked whether the glomeruli exhibiting significant changes in their volume after early olfactory learning were those responding to the odors provided during the T5–8 period, irrespective of any learning. We thus compared the maps of glomerular volume change shown in Fig. 3B with maps representing the neural activity patterns evoked by 1-NON and 1-HEX in naïve 17-day-old bees (Fig. 3C). The latter maps were obtained from calcium-imaging recordings of AL activity in our previous study in which glomerular responses to 1-HEX and 1-NON were recorded in 17-day-old naïves bees (Arenas *et al.*, 2009a). Upon simple visual inspection, no clear correspondence can be seen for either odorant between the two kinds of maps. This observation was confirmed by glomerulus-wise correlations between volume changes and odor-evoked activity levels ($\Delta F/F\%$) in naïve 17-day-old bees (Fig. 3D) – the analyses showed no significant correlations (1-HEX: $r = -0.25$, $P = 0.37$, df = 13, Pearson correlation; 1-NON: $r = -0.15$, $P = 0.58$, df = 13, Spearman correlation).

We then asked if glomerular volume changes after early learning took place in the same glomeruli that showed a change in their odor-evoked activity. We thus compared glomerular volume changes and changes in neural AL activity in 17-day-old bees that had been subjected to early learning of 1-NON. AL activity data in 17-day-old bees subjected to learning of 1-HEX were not tested in our previous study, so that the comparison is now restricted to 1-NON. Figure 4A shows the glomerular maps of calcium responses to 1-NON in 17-day-old control bees (naïve bees) and in 17-day-old bees subjected to early learning of 1-NON. Differences between the response patterns of naïve and experienced bees can be better visualized in a map of relative response change (as the percentage of activity in naïve bees – Fig. 4B, left). In such a map, it is possible to appreciate that the main variation was the additional activation of four glomeruli (23, 24, 36 and 62) in experienced bees (Fig. 4A and B, left). Observation of the map of volume change in 17-day-old bees subjected to early learning with 1-NON (Fig. 4B, right) shows that the four newly recruited glomeruli were those that exhibited the largest volume increases after learning of 1-NON (see Fig. 2). Consistent with this observation, we found a significant positive correlation between structural and functional changes ($r = 0.52$, $P = 0.045$, df = 13, Pearson correlation; Fig. 4C).

Because an early experience might induce changes in the overall responsiveness of the glomeruli, affecting responses to all odorants including those not experienced previously by the animal, we also correlated glomerular volume changes and changes in glomerular responsiveness. Glomerular responsiveness (Hourcade *et al.*, 2009) was calculated as the sum of the response obtained for each glomerulus in the four odorants tested in Arenas *et al.* (2009b) (1-NON, 1-HEX, hexanal and nonanal). We found no correlation between structural and functional changes for glomerular responsiveness ($r = 0.19$, $P = 0.36$, df = 13, Pearson correlation).

Discussion

Our results demonstrate the impact of early associative olfactory experiences on the architecture of the AL, the primary olfactory center of the insect brain. We first verified that early odor-rewarded experiences with 1-HEX and 1-NON between the fifth and the eighth day of adulthood determine a significant improvement of memory retention at late adult ages. We then showed that early olfactory learning results in long-lasting structural and functional modifications of the AL network in the form of glomerular volume variations and on the activation of new glomeruli upon olfactory stimulation with the odor that has been learned. In adult bees, changes in glomerular volume accompany the consolidation of associative olfactory memory following the repetitive pairing of an odor with sucrose reward in the protocol of olfactory PER conditioning (Hourcade *et al.*, 2009). We therefore reasoned that young bees experiencing a scented sucrose solution in the first days of adulthood might display a similar structural reorganization of AL connectivity, which would accompany the formation of long-term memories retrievable when they are 17 days old. Our study shows that both structural and functional modifications of the AL network occur when long-term olfactory memories are established between the fifth and eighth day of adulthood. This suggests the existence of a sensitive period in which olfactory experience leads to pronounced modifications of the AL. This finding confirms the AL as a site in which structural and functional plasticity can be observed following the formation of long-term olfactory

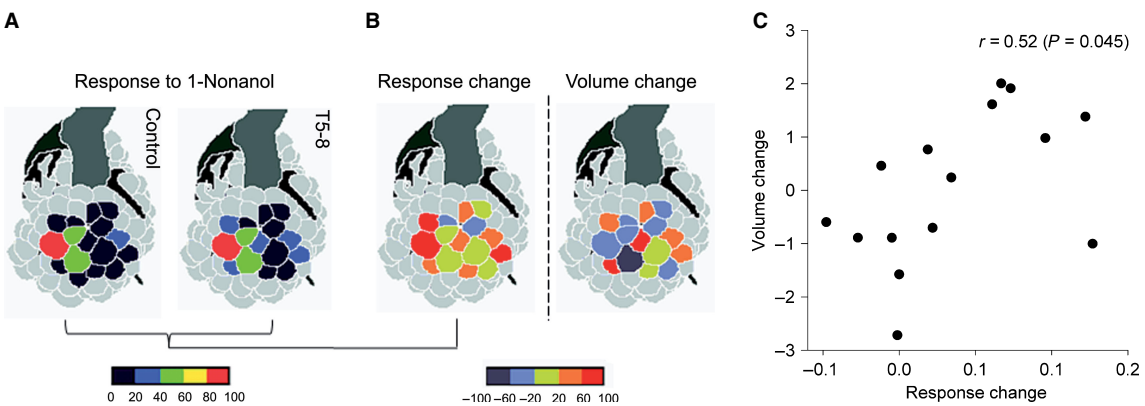

FIG. 4. Morphological and functional glomerular plasticity in 17-day-old bees that were offered scented sugar solutions between the fifth and eighth day of their adult lifespan (T5–8). (A) Spatial patterns of glomerular activity in response to 1-nonanol in naïve 17-day-old bees (left); and in T5–8 bees (right). (B) Schematic representation of the change in odor-evoked activity (left) after early olfactory experience and relative volume change obtained between control and experienced bees (right). Response intensity and volume changes were categorized as in Fig. 3. (C) Glomerulus-wise correlation between the volume change and the change in 1-nonanol-evoked activity between T5–8 and control bees.

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memories (Grünbaum & Müller, 1998; Müller, 2000; Hourcade *et al.*, 2009).

The effect of early olfactory learning on late memory retention

In agreement with previous studies in which other odorants were used (Arenas & Farina, 2008; Arenas *et al.*, 2009a,b), we found that experiencing food scented with 1-HEX- or 1-NON between the fifth and eighth day post-emergence induces strong behavioral responses to these odorants (the CS) at maturity. This finding thus confirms the importance of the fifth–eighth day period for the establishment of robust olfactory memories retrievable at late adult ages (Arenas & Farina, 2008; Arenas *et al.*, 2009a,b; this work). Moreover, it extends this conclusion to two odorants, 1-HEX and 1-NON, which differ from those used in our previous accounts of this early-learning effect (linalool and phenylacetaldehyde; see Arenas & Farina, 2008; Arenas *et al.*, 2009b), thus revealing the general nature of this phenomenon.

Our findings also show that besides this general effect, odorants may induce specific effects on memory retention. Though we had previously shown that both 1-HEX and 1-NON induced odorant-specific memories after exposure between the fifth and eighth days of adulthood (Arenas *et al.*, 2009a), here we show that the specificity of such memories was improved compared with other early ages. Yet, experiencing 1-NON rewarded during the ninth–12th day period also resulted in the same level of CS retention as that induced by the fifth–eighth day period. These results suggest that olfactory experience acquired during the period between the fifth and eighth day post-emergence is critical for the development of olfactory memories.

Early olfactory learning and the maturation of olfactory circuits

Olfactory experiences between the fifth and eighth day of adult life could be important for the correct maturation of olfactory circuits. It is indeed plausible that the maturation of the young worker bee's olfactory system requires the presence of chemosensory stimuli in the environment, like odors in the food or in the hive, thereby tuning the system for ensuring efficient learning and retention at older ages (Masson & Arnold, 1984, 1987; Winnington *et al.*, 1996; Arenas *et al.*, 2009b). The period between the fifth and eighth day of adulthood coincides with the time at which sensory neurons already exhibit mature responses to odorants (Masson & Arnold, 1984, 1987), while the AL still undergoes maturation (Winnington *et al.*, 1996). During this period the bees' olfactory system would thus be particularly sensitive to olfactory experiences, which would shape later adult behavior. This early sensitivity to incoming sensory input was first documented in the mammalian visual system by Hubel & Wiesel (1962), and seems to be a general property of sensory systems (Farbman *et al.*, 1988; Schmidt & Eckert, 1988; Cummings & Brunjes, 1997; Devaud *et al.*, 2003; Phan *et al.*, 2006; Keuroghlian & Knudsen, 2007).

Early olfactory learning differs from imprinting

Sensory experiences during early specific periods can dramatically shape later behavior. A good example is the process termed 'imprinting' by which an animal acquires a permanent reference pattern from a stimulus experienced during an early sensitive period (Lorenz, 1935), which impacts irreversibly onto adult behavior. Imprinting-like forms of learning have been suggested for honey bees in an olfactory context (Gascuel & Mason, 1987). For instance, imprinting might be fairly adaptive for bees as it would allow establishing a strong olfactory bond to the hive, thus facilitating orientation and recognition (Masson & Arnold, 1987).

In contrast with standard views of imprinting processes, early experiences with scented food as established in our work are subjected to extinction and might therefore not be related to imprinting. Indeed, successive non-rewarded presentations of the odorant paired with food during periods T1–4, T5–8 or T9–12 resulted in extinction of the early conditioned odor (Arenas *et al.*, 2009b), thus showing that the acquisition of olfactory information during early learning events does not lead to irreversible olfactory-driven preferences, which would be expected in an imprinting-like phenomenon. The results obtained in our work rather seem to reflect basic associative learning occurring in a period in which it induces profound remodeling of the olfactory circuits implicated in odor learning.

Structural and functional changes of olfactory circuits resulting from early and late olfactory learning

The importance of associative learning as a remodeling factor acting on the AL network has been emphasized in a recent work that studied changes in glomerular volume in bees subjected to controlled PER olfactory conditioning (Hourcade *et al.*, 2009). In this case, mature adult workers collected from the hive were trained with five pairings of odor and sucrose, a procedure that generates stable long-term odor-specific memories, retrievable 3 days after training (Menzel, 1999). These bees exhibited volume variations in a subset of glomeruli that were specific to the learned odorant. In our experiments, we did not control the number of rewarded experiences that caged bees had with the rewarded scent. Because each individual bee fed several times per day on the feeder that was placed inside the hive, it is highly probable that bees experienced multiple odor–sucrose associations leading to structural changes in certain glomeruli, similarly to what was observed in adult mature bees (Hourcade *et al.*, 2009). This hypothesis is supported by the presence of long-term olfactory memories that were specific for the CS in 17-day-old bees.

Yet, the processes triggered by associative learning in young and older adult bees are not identical, as shown by qualitative differences between our work and that of Hourcade *et al.* (2009). Although in both cases long-term CS-specific memories were formed, the relation between volumetric variations and activity patterns upon CS stimulation were different. In mature adult workers, no changes in glomerular activity patterns were found despite the presence of long-term CS-specific memories and volumetric glomerular variations (Hourcade *et al.*, 2009). It was then concluded that long-term memory is not accompanied by any significant change in odorant-induced calcium responses (at least at the AL input level). By contrast, using the same technique, we showed that bees subjected to early olfactory learning also developed long-term CS-specific memories but that this process was accompanied by an increase in glomerular activity in response to the CS (as well as to perceptually similar odorants) when presented at 17 days (Arenas *et al.*, 2009a). While Hourcade *et al.* (2009) explained their volumetric variations in terms of glomerular inhibition and suggested that the glomeruli that were the least inhibited by the CS showed the most important volume increase, our data did not show such relation between volume change and odor-induced inhibition in naïve bees (Pearson's correlation coefficient for 1-HEX: $r = -0.29$, $P = 0.31$, df = 13; 1-NON: $r = 0.17$, $P = 0.57$, df = 13; see Hourcade *et al.*, 2009 for calculations). Instead we showed that the glomeruli newly recruited by the CS in conditioned bees were those exhibiting the most significant variations in volume. Specifically, newly recruited glomeruli (23, 24, 36 and 62; see Fig. 4B) increased

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their volume, thus showing for the first time a positive correlation between changes in glomerular response and changes in glomerular volume. Still, this does not exclude a role for local inhibitory interneurons in setting synaptic changes in specific glomeruli. Interestingly, aversive associative learning leads to protein synthesis in specific glomeruli in *Drosophila* (Ashraf *et al.*, 2006), among which are those newly recruited after conditioning (Yu *et al.*, 2004). Further work will be needed to unravel the cellular mechanisms underlying these changes.

In any case, the results from both studies – ours on young bees and the study by Hourcade *et al.* (2009) on adult bees – go against the possibility that CS–unconditioned stimulus pairings inducing concomitant activation of the odor-signaling and sucrose-signaling pathways induce stronger plasticity in the glomeruli activated by the learned odor. This hypothesis is discarded in both studies by the fact that the glomeruli that respond highly to the CS are not those exhibiting significant volume variations. By contrast, structural plasticity might be related with changes in activity in inhibitory local interneurons that are postsynaptic to the sensory input (Hourcade *et al.*, 2009). In our case, inhibition of such neurons (disinhibition) could be responsible for the recruitment of new glomeruli experiencing volumetric increases. A role for such inhibitory neurons has been already demonstrated in the case of *Drosophila* where an increase of glomerular size following CO_2 exposure was related to increased activity in local interneurons involved in the processing of the CO_2 (Sachse *et al.*, 2007).

A comparative view of early olfactory experiences in insects – the case of Drosophila

The effect of olfactory experiences on later odor-driven behavior has also been found in the fruit fly *Drosophila melanogaster* in the case of non-associative odor exposures (Devaud *et al.*, 2001, 2003; Sachse *et al.*, 2007). In these studies, continuous exposure to an odor during the first days post-emergence reduced orientation towards it in mature flies. Yet, the same exposure occurring later had no effect. In addition, the early exposure to CO_2, a major component of *Drosophila* stress odor (Suh *et al.*, 2004), decreases the distance walked by the flies to avoid the stimulus (Sachse *et al.*, 2007). Besides affecting behavior, such early exposures also shape the structure of the fruit fly AL. While exposure to an odor decreased the volume of some glomeruli (Devaud *et al.*, 2003), prolonged exposure to CO_2 during an early period of the fly's lifespan resulted in increased glomerular size probably due to an increase in the activity of local interneurons involved in the processing of CO_2 (Sachse *et al.*, 2007). Taken together, these results indicate that in fruits flies, like in bees, a sensitive period exists in which odor exposures (non-associative in cited studies in *Drosophila*) induce long-lasting changes in behavior, as well as structural and functional modifications of the AL network. Similarly to bees, such a period corresponds to the phase in which the fruit fly olfactory system undergoes final maturation (Devaud *et al.*, 2003).

Besides this timing factor, the biological meaning of the odor itself (such as CO_2 or a floral component) and the manner in which it was proposed (associated with food or simply delivered in the environment) also appears to be important for determining how the structure of the AL might change. Within a certain glomerulus different population of neurons might be selectively or differentially activated by the stimulus and their synapses could be strengthened or weakened according to the nature of the odorant and of the context in which it was presented (appetitive, aversive, etc.). Therefore, it is difficult to predict how olfactory experience may shape the AL. Certain stimulations (such as passive odor exposure) could decrease the volume of a set of glomeruli (Devaud *et al.*, 2001, 2003), whereas others (such as CO_2 passive-exposure; Sachse *et al.*, 2007) could drastically increase their size.

Early olfactory learning in an ecological context

As we measured increased responses to an early experienced odorant at the age of 17 days, which corresponds to the period in which worker bees commonly initiate foraging (Rösch, 1925; Lindauer, 1952; Seeley, 1982), our results suggest that the decisions of foragers searching for food in the field may be shaped by early olfactory experiences within the hive (Arenas *et al.*, 2007, 2008). Foragers might prefer to search for flowers whose scent would be reminiscent of food odors experienced at young adult ages. This might indeed relate with the concept of 'innate search image' proposed by Menzel (1985), which refers mainly to an innate template, which would later guide the animal's decisions, thereby biasing them towards profitable food sources (e.g. Giurfa *et al.*, 1995). If, however, the odor that was early learned within the hive is no longer available when the bees initiate their foraging activities in the field, the remarkable and fast behavioral and neural plasticity of bees (Menzel, 1999; Giurfa, 2007) would allow for extinction of the information acquired through early learning and prompt reversal towards new rewarding floral types. Adult bees experience different rewarding sources and such experiences modify in turn the AL network (Hourcade *et al.*, 2009). How successive olfactory experiences modify the AL, and whether potential consecutive modifications of the AL compensate each other to keep global AL volume relatively constant remains to be determined.

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Abbreviations

1-HEX, 1-hexanol; 1-NON, 1-nonanol; AL, antennal lobe; CS, conditioned stimulus; PER, Proboscis Extension Response.

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