Associative Conditioning Tunes Transient Dynamics of Early Olfactory Processing

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Odors evoke complex spatiotemporal responses in the insect antennal lobe (AL) and mammalian olfactory bulb. However, the behavioral relevance of spatiotemporal coding remains unclear. In the present work we combined behavioral analyses with calcium imaging of odor induced activity in the honeybee AL to evaluate the relevance of this temporal dimension in the olfactory code. We used a new way for evaluation of odor similarity of binary mixtures in behavioral studies, which involved testing whether a match of odor-sampling time is necessary between training and testing conditions for odor recognition during associative learning. Using graded changes in the similarity of the mixture ratios, we found high correlations between the behavioral generalization across those mixtures and a gradient of activation in AL output. Furthermore, short odor stimuli of 500 ms or less affected how well odors were matched with a memory template, and this time corresponded to a shift from a sampling-time-dependent to a sampling-time-independent memory. Accordingly, 375 ms corresponded to the time required for spatiotemporal AL activity patterns to reach maximal separation according to imaging studies. Finally, we compared spatiotemporal representations of binary mixtures in trained and untrained animals. AL activity was modified by conditioning to improve separation of odor representations. These data suggest that one role of reinforcement is to “tune” the AL such that relevant odors become more discriminable.

Introduction

Odor stimulation evokes spatial patterns of activity in the insect antennal lobe (AL) and mammalian olfactory bulb (OB) that covary with odorant molecular structure (Sachse et al., 1999; Wang et al., 2003; Lei et al., 2004; Johnson and Leon, 2007), mixture composition (Giraudet et al., 2002; Duchamp-Viret et al., 2003; Heinbockel et al., 2004; Tabor et al., 2004; Silbering and Galizia, 2007) and concentration (Johnson and Leon, 2000; Meister and Bonhoeffer, 2001; Sachse and Galizia, 2003; Stopfer et al., 2003; Hallem and Carlson, 2006; Silbering et al., 2008). However, activity also consists of fast and slow temporal patterns that correlate to odorant features (Kashiwadani et al., 1999; Laurent et al., 2001; Laurent, 2002; Daly et al., 2004a; Friedrich et al., 2004). Several studies have shown that temporal processing can make initially similar activity patterns more distinct over longer sam-

pling times (Stopfer et al., 1997; Fdez Galán et al., 2004; Friedrich and Laurent, 2004; Mazor and Laurent, 2005). This processing sets up a series of activity patterns, or “transients,” that could improve odor discriminability (Rabinovich et al., 2008). Thus, in principle, more specific information should be available as sampling times increase.

Previous studies aimed at evaluating tradeoffs between sampling time and decision accuracy have shown that longer sampling times can improve performance (Abraham et al., 2004; Rinberg et al., 2006; Wright et al., 2009). However, animals are also capable of making reasonably accurate decisions even with short sampling times (Ditzen et al., 2003; Uchida and Mainen, 2003). Therefore, the issues of specifically when and how time is relevant for odor identification require further investigation.

Furthermore, there is now growing evidence that AL and OB neural activity may be altered by plasticity (Davis, 2004). Repeated nonassociative exposure to an odor increases the coherent spiking of projection neurons (PNs) in the locust AL (Stopfer and Laurent, 1999) and changes the receptive fields of mitral cells in the OB (Vanderwolf and Zibrowski, 2001; Fletcher and Wilson, 2003). Several studies in insects have reported that neural activity in the AL is altered by classical conditioning (Faber et al., 1999; Müller, 2000; Sandoz et al., 2003; Daly et al., 2004b; Yu et al., 2004; Thum et al., 2007), and conditioning alters oscillations in specific frequency bands in the mammalian OB (Ravel et al., 2003; Martin et al., 2004; Beshel et al., 2007).

Our study investigated how associative plasticity affects spatiotemporal activity patterns and how both impact behavioral decisions. We combined a robust olfactory conditioning paradigm in the honeybee for evaluating odor perception (Smith et al., 2006; Giurfa, 2007) and *in vivo* imaging of odor driven activity

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in the AL (Sachse and Galizia 2002). We used binary mixtures that differ in ratios of components at low concentration, which makes discrimination difficult. We found that conditioning increased the separation between the spatiotemporal patterns evoked by the binary mixtures in the AL. Furthermore, the time needed for maximal separation of odor representations corresponded to the time at which honeybees transition from sampling time-dependent to sampling time-independent odor discrimination.

Materials and Methods

Animals. Forager honeybees (*Apis mellifera carnica*) were collected in the morning at the entrance of the hive, shortly cooled, and restrained in individual harnesses. After recovering from cooling, bees were fed 2 μ l of a 1.0 M sucrose solution and allowed to remain undisturbed for 2 h at room temperature until conditioning took place.

Odor stimulation. Odors used for stimulation were the aliphatic alcohol 1-hexanol and the ketone 2-octanone (both TCI America, 98%), alone or combined in binary mixtures in the ratios 9:1, 7:3, 5:5, 3:7, and 1:9 (1-hexanol: 2-octanone molar ratio). Odors were diluted from purity in mineral oil (Sigma-Aldrich) and mixed to obtain in all cases a final concentration of 0.02 M. The output of the odor delivery device, either in behavioral or imaging experiments, was positioned 2 cm away from the bee's head and targeted toward the antennae. During the experiment a continuous charcoal filtered air stream (25 ml/s) ventilated the antennae. Five centimeters behind the bee an exhaust continuously removed the air from the arena. The odor cartridges consisted of 1 ml glass syringe containing a filter paper strip (0.5 $\times$ 4 cm) loaded with 10 μ l odorant solution. Four cm upstream from the cartridge, a three-way valve (LFAA1200118H; The LEE Company) controlled the onset of the airflow through the odor cartridge.

In the behavioral experiments, opening of the valve was triggered by a programmable controller (Automation-Direct) and the durations used were: 200, 500, 1000, 2000, or 4000 ms. In the imaging experiments, the valve was synchronized with the optical recordings directly from the imaging acquisition software TILLVision (TILL Photonics) and valve opening lasted 1000 ms except in the experiment plotted in figure S5C. When the valve was open, the odor-laden air from the cartridges was pushed into the continuous air stream in a mixing chamber 2 cm before the output of the odor delivery device. The odor device had eight independent and identical channels composed of a valve and an odor cartridge. Calculation based on the geometry of the odor delivery device and the airflow yielded an estimated delay of 15 ms between opening of the valve and the odor reaching the antennae. The valve response time provided by the manufacturer was as fast as 0.25 ms. Between experiments, odors were rotated across channels to balance any differences between channels.

Olfactory conditioning. A differential conditioning protocol was used to train bees and examine their ability to perceive differences among odors. Bees were trained as described by Smith (1998). Each bee received differential conditioning to two odorants in the following pseudorandomized 12 or 16 trial sequence: ABBABAABABAB or ABBABAABABABABABA respectively, where A is the rewarded odor and B is the unrewarded one. The intertrial interval was 6 min. In the rewarded trials (CS+), odor was paired with the unconditioned stimulus (US), which consisted of first touching the antennae with a 2 M sucrose solution and followed by feeding with 0.4 μ l during extension of the proboscis. In unrewarded trials (CS−), bees were stimulated with odor but sucrose was omitted. Duration of odor stimulation ranged from 200 to 4000 ms depending on the experiment (see Figs. 1–6). The US was omitted on tests trials and the odorants were presented as above. To avoid extinction of the conditioned response, a retraining trial was offered every 2 test trials. During the retraining trial, the CS+ was paired with the US whereas the CS− was not, in the same way as was done during the training phase.

On every acquisition trial, the response of each subject was recorded as a positive response if the subject extended its proboscis during odor stimulation and before the US presentation. In the experiments in which

the odor sampling time was 4000 ms, the sucrose US was presented 3 s after the start of odor stimulation, which allowed for 1 s overlap between odor and the US. In the experiment with variable sampling time (see Fig. 6) the US was presented in all cases 1 s after odor onset. Thus, there was no CS–US overlap for 200 and 500 ms, there was 1 s overlap for 2000 ms and, in the case of 1000 ms, the start of the US coincided with the odor shut off. These data were plotted as the percentage of subjects that responded to the odor on each trial. Proboscis extension was recorded as a binary variable—extension response or not—during the training trials. Each test trial was also recorded using a digital camcorder for offline quantification of the response duration. Duration of the proboscis extension was used as a response measure because it has been shown that it is more sensitive for revealing differences in response among treatment groups than is a percentage response (Smith and Menzel 1989, Smith 1997). Duration is defined as the elapsed time that the proboscis is extended beyond the line connecting the tips of the opened mandibles (as the subject is viewed along the longitudinal axis).

Statistical analysis. Duration of proboscis extension was analyzed by a two way repeated measures ANOVA using a generalized linear model (GLM) (SPSS) and GLM a priori contrasts. For the timing experiment, we used specific pairwise comparisons (CS+ vs CS−) by Wilcoxon-matched pairs test.

Calcium imaging. Bees were restrained into Plexiglas stages suited for proboscis extension response (PER) conditioning and optical recordings. The head was fixed to the stage with soft dental wax (Kerr, Sybron Dental Specialties) in a way they could freely move antennae and proboscis. Nine to ten hours after behavioral conditioning, PNs were stained by backfilling with the calcium sensor dye fura-dextran (potassium salt, 10,000 MW, Invitrogen). A window was cut in the head capsule dorsal to the joints of the antennae and rostral to the medial ocellus. The glands were carefully moved until the α -lobes (Rybak and Menzel, 1993) in the brain were visible, which is easily recognizable and serves as spatial reference for the staining (Sachse and Galizia, 2002). The tip of a glass electrode coated with fura-dextran prepared in 3% bovine serum albumin solution (Sigma-Aldrich) was inserted into both sides of the deutocerebrum dorsolateral to the α -lobes, aiming for the lateral antennocerebral tracts (l-ACT) that contains the axons of a subset of uniglomerular PNs (Abel et al., 2001). The dye bolus dissolved into the tissue in 3–5 s and the window was immediately closed using the same piece of cuticle that was previously removed. Eicosane was used to glue and seal the cuticle. The dye was left to travel along the tracts for 8–12 h.

Before imaging, the antennae were fixed pointing toward the front using eicosane (Sigma-Aldrich) and body movements were prevented by gently compressing the abdomen and thorax with a piece of foam. The brain was rinsed with Ringer solution (130 mM NaCl, 6 mM KCl, 4 mM MgCl₂, 5 mM CaCl₂, 160 mM sucrose, 25 mM glucose, 10 mM HEPES, pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich), and glands and trachea covering the antennal lobes were removed. A second hole was cut ventrally to the antennae and the compact structure of muscles, esophagus, and supporting chitin was lifted and put under slight tension to prevent movements of the brain (Mauelshagen 1993). Thus, stabilization was accomplished without damage to the brain. After surgery and before measurements bees were allowed to recover for 20 min. The antennal lobes were examined for appropriate staining and only the one that presented the more homogenous staining was selected for the measurements.

Calcium imaging was done using a CCD camera (SensiCamQE, T.I.L.L. Photonics) mounted on an upright fluorescence microscope (Olympus BX-50WI, Japan) equipped with a 20 $\times$ dip objective, numerical aperture = 0.95 (Olympus), 505 DRLPX dichroic mirror, and 515 nm LP filter (TILL Photonics). Monochromatic excitation light provided by a PolichromeV (TILL Photonics) alternated between 340 and 380 nm. Fluorescence was detected at a sampling rate of 8 Hz. Spatial resolution was 172 $\times$ 130 pixels, after a binning of 8 $\times$ 8 on a chip of 1376 $\times$ 1040 pixels, resulting in a spatial sampling of 2.6 μ m per pixel side. Exposure times were 8 and 2 ms for 340 and 380 nm respectively.

The entire image analyses were done using custom software written in Interactive Data Language (IDL; Research Systems) created by Giovanni

Galizia and Mathias Ditzen (University of Konstanz, Konstanz, Germany). Each data set or measurement consisted in a double sequence of 80 fluorescence images, obtained at 340 and 380 nm excitation light (Fi_{340} , Fi_{380} , where i is the number of images from 1 to 80). Calcium signals were subsequently calculated using a ratiometric method: For each pair of images Fi we calculated the ratio $Ri = (Fi_{340} \text{ nm}/Fi_{380} \text{ nm}) \times 100$ and subtracted the background R_b , obtained by averaging the Ri values 1 s immediately before the odor onset [$R_b = 1/8 (R_{16} + \dots + R_{23})$]. Resulting values represent percentage of change from the reference window (R_{16} – R_{23}) and are proportional to the changes in the intracellular calcium concentration. The analysis was based on the time course of calcium signals in each identified glomerulus. For this aim, glomeruli were identified on the basis of their morphology and relative position using the digital atlas of the honeybee AL as a reference (Galizia et al., 1999). The visualization of glomeruli is possible observing the raw fluorescence images obtained at 380 nm excitation light. An additional tool written and provided by Mathias Ditzen was used to confirm glomerular shape and position. This tool calculates images representing the degree of correlation between neighboring pixels. Since glomeruli respond as functional units, pixels stemming from the same glomerulus are highly correlated over time. In contrast, pixels from different glomeruli are uncorrelated. This provides images in which glomeruli are clearly visible and clearly separated by contrasting boundaries.

Seventeen glomeruli were identified across all animals and were used in the present analysis. All glomeruli were located in the dorsostral side of the AL and correspond to a subset of glomeruli innervated by the antennal nerve tract 1 (Kirschner et al., 2006). We calculated activity in glomeruli 17, 23, 24, 25, 28, 29, 33, 35, 36, 37, 38, 42, 43, 47, 48, 49, and 52 according to the nomenclature previously established (Flanagan and Mercer, 1989; Galizia et al., 1999). Glomerular activation was calculated by averaging activity in a square area of 9×9 pixels that correspond to $23.4 \times 23.4 \mu\text{m}$ and fits well within the boundaries of the glomeruli. Glomerular activity in the present study refers to activity of the uniglomerular PNs in each glomerulus, because only these neurons were stained, and therefore only this particular population of cells was measured in each glomerulus.

For visualization of the data, we reduced the 17 dimensions using a principal component analysis (PCA) that identifies orthogonal axes (factors) that explains maximum variance in the data, and thus projects the data into a lower-dimensionality space. The first two factors that explain most of the observed variance were further subjected to Varimax rotation (SPSS). Such rotational strategy maximizes the variance accounted for by the calculated factors, while minimizing the variance around them.

Statistical analysis was based on euclidean distances (EDs) among pairs of odors calculated in a 17-dimensional space in which each axis represents the activation of one glomerulus ($ED_{\text{hex-oct}} = [(R_{17\text{hex}} - R_{17\text{oct}})^2 + \dots + (R_{52\text{hex}} - R_{52\text{oct}})^2]^{1/2}$, where for example $R_{17\text{hex}}$ means the response of glomerulus 17 during stimulation with 1-hexanol). This spatial relationship provided us a measure of difference or similarity between the neural representations of two odors. ED was calculated for each possible pair of odors and for the 80 time intervals that lasted each measurement. This provided a matrix of 21×80 EDs per animal. Since ED between two neuronal representations may be affected by the general strength of the calcium signals, all EDs obtained for each animal were normalized by dividing by the ED obtained for the 10:0–0:10 (hexanol–octanone) pair 500 ms after odor onset. The normalization based on EDs used the global activity in the AL and avoids normalizing to activity of specific glomeruli that eventually may be not reliable. The latter alternative was, however, considered and is further explained in the supplemental Figure S3, available at www.jneurosci.org as supplemental material, as a complementary analysis. All statistical analysis of EDs was performed using Mann–Whitney U test at each time point for each odor pair.

Results

Transitions in ratios of binary mixtures define smooth transitions in odor perception

The goal of this first series of experiments was to establish whether smooth transitions among ratios in binary mixtures

would generate a smooth transition in generalization among the odors. The pattern of response—“generalization”—from conditioned odors to novel, unexperienced odors depends on the specific conditioning procedure (Wright et al., 2008). Therefore, high levels of generalization cannot be equated with lack of discriminability, per se. However, for any given conditioning procedure generalization is a function of perceptual similarity, and hence it is a measure of discriminability (Smith and Menzel, 1989).

We used differential conditioning in which animals were conditioned to two ratios; one ratio (the CS+) was reinforced with sucrose, whereas the other (CS−) was not. We increased the difficulty of the conditioning task in three ways. First, we used low odor intensities that compromise odor discrimination (Wright and Smith, 2004). Second, instead of using pure odorants that differ in molecular structure, we manipulated the ratios of components in binary mixtures (Ditzen et al., 2003; Uchida and Mainen, 2003; Abraham et al., 2004; Rinberg et al., 2006). Third, we selected mixture components (1-hexanol and 2-octanone) that show partially overlapping patterns of activation in the honeybee AL (Sachse et al., 1999).

Figure 1A (left) shows the mean response probabilities of the PER during conditioning to 1-hexanol (i.e., 10:0) as the CS+ and 2-octanone as the CS− (0:10 mixture) and vice versa (as a convention, 1-hexanol is always shown left of the colon and 2-octanone right). In both experiments, some animals responded spontaneously to the CS+ on the first trial, which is normal for PER experiments (Menzel, 1990). Response to the CS+ increased in each case on subsequent trials, which reflects associative conditioning (Bitterman et al., 1983). The response on the first trial with the CS− (trial 2 of the sequence) was high because of generalization from the previously reinforced trial. On subsequent trials the response becomes more specific to the CS+.

Figure 1A (right) shows the duration (see Materials and Methods) of the response during the test phase. Bees responded longer to the CS+ than to CS−. Therefore these odors (albeit pure odors in this case) are discriminable. There was also a smooth generalization from pure odors to the mixture ratios 1:9 and 9:1. The strongest response was to the mixture closest to the CS+. Repeated measures ANOVA revealed significant interaction between treatments (interaction $p < 0.001$), which suggests bees were able to discriminate accurately between 1-hexanol and 2-octanone when they each were the CS+.

In a different set of experiments, we differentially conditioned bees to 9:1 and 1:9 mixtures as the CS+ and CS−. Figure 1B (left) shows the mean response probabilities during conditioning. The response to the CS+ and CS− diverged over trials, and the response to the CS+ was consistently higher. In both cases, there was significant divergence in response on the last two trials, which occurred as retraining trials during the testing phase. This pattern indicates that the mixtures are discriminable, but bees have greater difficulty in discriminating these odor ratios that they did in the previous experiment with pure odors.

During the test phase (Fig. 1B, right), the duration of the proboscis extension response was higher to the CS+ than to the CS−, which substantiates that the mixtures are discriminable. As before, the response to all of the intermediate test ratios (7:3, 5:5, 3:7) showed a gradient of decreasing responses from the CS+ to the CS−. The opposite slopes of the two gradients ($p < 0.01$) indicates that bees differentiated between CS+ and CS− in both cases.

Excitation and inhibition interact along a perceptual gradient

The previous set of experiments revealed that discrimination declines as the CS+ and CS− are made more similar in regard to the molecular composition of the mixtures. This implies that the excitation produced to the CS+ by its association with sucrose reinforcement smoothly declines with progressively larger alterations of the mixture composition. The same applies to a form of “inhibition” generated to the CS−. Therefore, when the CS+ and CS− are made more similar they should summate in such a way that the strongest response will be to a mixture that is not the CS+ and which is farther away from the CS−. This phenomenon is called “peak shift” (Spence, 1937; Mackintosh, 1983), and we use it here as a means to imply that transitions across ratios of binary mixtures produce reasonably smooth changes in the perceptual qualities they produce (Daly et al., 2001).

We performed a set of experiments in which bees were differentially conditioned to a 5:5 ratio as the CS+ and to either a 1:9 or 9:1 ratio as the CS− (supplemental Fig. S1, available at www.jneurosci.org as supplemental material, shows acquisition curves). They were then tested as before with the range of ratios: 1:9, 3:7, 5:5, 7:3, 9:1. After conditioning to 5:5+ versus 9:1−, the strongest response (longest mean duration) was to the 3:7 test ratio ($p < 0.05$) (Fig. 2, dashed lines). The direction of the peak shift is consistent with the interaction of excitation and inhibition along a gradient of similarity in the neural codes defined by the test ratios. That is, the response shifted from the CS+ in a direction away from the CS−. However, a peak shift was not evident after conditioning to 5:5+ versus 1:9− (Fig. 2, solid lines). The effect in the former condition but not in the latter probably reflects an asymmetry in the coding for these odor ratios (Daly et al., 2001). It is unlikely that the asymmetry was attributable to a preference for 3:7, because a preference for that ratio was not evident with conditioning of 9:1 versus 1:9. A more extensive analysis of peak shift in olfactory conditioning of honeybees can be found in the study by Wright et al. (2009b).

In summary, experiments 1 and 2 support the following conclusions, which are important for interpretation of the imaging data below. First, gradations in the ratio of two odors in a mixture produce a smooth generalization from the CS+ through the CS−. Second, further support of the graded nature of similarity of mixtures is provided by the peak shift in the response away from 1:9− after 5:5+/1:9− conditioning. The next set of experiments was designed to evaluate how coding for mixtures that grade into one another might be reflected in spatiotemporal patterns set up in the AL.

Figure 1. Transitions in ratios of binary mixtures define smooth transitions in odor perception. **A**, Top row of boxes represents a schematic of the experimental protocol. Bees were trained to the CS+ = 10:0 (pure 1-hexanol) and CS− = 0:10 (pure 2-octanone) or vice versa over the indicated number of trials. During training (white boxes) bees were reinforced with 0.4 μ l of 2 M sucrose on every CS+ trial; CS− trials were unreinforced. The two graphs to the left show percentage of proboscis extension at each trial during training. The top and bottom graphs show two different groups in which the odors were counterbalanced as the CS+ and CS−. During testing (gray boxes to the right), different ratios of odors (1-hexanol: 2-octanone) were presented unreinforced in a randomized order. The graph shows the duration of the proboscis extension response (see Materials and Methods) during testing (mean $\pm$ SE) as a function of different test ratios. Dotted versus solid lines represent responses from the two groups shown to the left. Two-way repeated measures ANOVA was performed for the following factors: ratio, NS; conditioning treatment, NS; interaction ratio $\times$ treatment, $p < 0.001$, $N = 19$ bees for 10:0+, 19 bees for 0:10+. **B**, top row, Schematic of experimental protocol for bees trained either CS+ = 9:1, CS− = 1:9 or vice versa. White boxes intermingled with test trials indicate retraining trials (see Materials and Methods). Left graphs represent percentage proboscis extension during training; retraining trials performed in between recall testing (right) are separated by the gray line. Right graphs represent duration of proboscis extension response during testing (mean $\pm$ SE) as a function of different test ratios. Two-way repeated measures ANOVA was performed as above: ratio NS; conditioning treatment, NS; ratio $\times$ treatment $p = 0.006$, $N = 28$ bees for 9:1+, 32 bees for 1:9+. In all cases, sampling time (odor delivery time) was 4000 ms for both conditioning and testing.

Transient activity patterns show a gradient of activation along different ratios of binary mixtures

We measured the responses in the AL to binary mixtures along a gradient of ratios from 1-hexanol (10:0) to 2-octanone (0:10). Odor elicited activity in the AL was measured by backfilling PN axons with the calcium indicator fura-2 (see Materials and Methods) (Sachse and Galizia, 2002). The shape and relative position of the glomeruli in the AL is conserved across individuals allowing the identification of individual glomeruli from one animal to the other (Galizia et al., 1999). Since PNs from the l-ACT are stained, the maps of activity obtained with this technique specifically represent the output of an identified set of glomeruli on the dorsal surface of the AL. Calcium activity was recorded in 125 ms time steps. Although imaging calcium activity is slow relative to electrophysiological measurements (Daly et al., 2004a; Lei et al., 2004; Mazar and Laurent, 2005) and is an indirect indicator of electrical activity, a strong correlation between calcium imaging

Figure 2. Excitation and inhibition interact to produce a peak shift in the response. Top, Schematic of experimental protocol for bees trained to the CS+ using a 5:5 mixture and CS- either 1:9 or 9:1. Test odors were presented in random order and intermingled with retraining trials. Bottom, Duration of the proboscis extension response for 5:5+ versus 9:1- (dotted line) and 5:5+ versus 1:9- (full line) as a function of different 1-hexanol:2-octanone test ratios. Sampling time (odor delivery time) was 4000 ms for both conditioning and testing. Two-way repeated measures ANOVA was performed for the following factors: test ratio, NS; (GLM a priori contrasts between 3:7 and 9:1; $p = 0.01$); conditioning treatment, NS; interaction ratio $\times$ treatment, $p = 0.02$; (GLM a priori contrasts between 3:7 and 9:1; $p = 0.0004$). (GLM = general linear model). $N = 36$ bees for 5:5+ versus 1:9-; and $N = 39$ bees for 5:5+ versus 9:1-. Acquisition curves are shown in supplemental Figure S1, available at www.jneurosci.org as supplemental material.

and spike rate has been reported using the same calcium sensor (fura-2) and in the same honeybee PN that we recorded in the present work (Galizia and Kimmerle, 2004).

Figure 3A, left panel, shows an example of the raw fluorescence (380 nm excitation; >510 nm emission) of an AL 8 h after staining the ipsilateral I-ACT axons with fura-2. The homogeneous intensity in the raw fluorescence images is an indication of homogenous staining. The bright spots on the medial side of the AL (left) belong to a cluster of the I-ACT somas (Kirschner et al., 2006) and are also an indication of specific staining. Figure 3A, right panel, represents the correlated image of the same AL used to identify glomeruli (see Materials and Methods). Note that dark areas (i.e., low correlation values) do not mean a lack of staining, which is controlled in the raw fluorescence image. Instead, glomeruli in this area were not activated by any of the odors presented to the bee.

Figure 3B shows images representative of activity patterns induced by the seven odors tested. The activity patterns recorded from the different mixtures involved a combination of the PNs (glomeruli) activated by each component, as was previously shown in measurements using a calcium imaging technique in honeybees that most likely reveals activity in olfactory receptor neurons (Deisig et al., 2006). Each odor was measured three times per animal, and the AL response was consistent across these repetitions (see supplemental Fig. S2A,B, available at www.jneurosci.org as supplemental material). A qualitative analysis of the example shown in Figure 3 shows that the PNs differentially excited by one of the pure components were also excited in the mixtures with the highest proportion of that component. Excitation of odor-specific PNs dropped when the relative concentration of that odor decreased. For example, glomerulus 38 (Fig. 3B, upper white circle) is excited by 10:0 (pure 1-hexanol), 9:1 and 7:3. Its activity decreased in 5:5 and vanished in 3:7, 1:9 and 0:10 (pure 2-octanone). Glomerulus 52 (Fig. 3B, lower white circle) is

strongly activated by 0:10, 1:9, 3:7, and 5:5, the activation gradually diminishes until 10:0.

A similar analysis in a population of 9 bees showed that, from 17 identified glomeruli, 9 significantly increase activity with increasing concentrations of 2-octanone in the mixture (i.e., glom 17, 33, 36, 42, 43, 47, 48, 49, 52), one significantly increases its activity with increasing concentrations of 1-hexanol (i.e., glom 38), and 7 remain invariable (i.e., glom 23, 24, 25, 28, 29, 35, 37) (see examples in Fig. 3C). Therefore, the spatially defined neural representation of binary mixtures among the PNs showed a gradient of activation along the different ratios. At the individual level, some of the glomeruli eventually suggested synergistic or antagonistic interactions along the gradient. For example, visual inspection of Figure 3B shows glomeruli that seem to be more activated by some of the mixture ratios than by the pure components. However, when averaged across animals, none of the glomeruli activated by any of the mixtures showed an obvious and consistent effect of nonadditive interaction.

PN response patterns evolve through time showing a smooth transition along ratios from one pure component to the other

We analyzed the temporal evolution of PN activity patterns across the range of odor ratios used in the behavioral experiments. Figure 4A shows the temporal course of an odor signal in selected glomeruli for the ratios 9:1 and 1:9 in an individual animal. Typically, a response is clearly evident by first recorded 125 ms frame after odor onset. As shown in the example in Figure 4A, different glomeruli showed odor specific amplitudes and different temporal profiles, hence contributing to an odor-specific spatiotemporal response. Most active glomeruli show their peak in activity ~ 250 ms after odor onset; however, some glomeruli start to respond later in the odor presentation (e.g., compare glomeruli 36, 48, and 52). Interestingly, some glomeruli may respond early for a given odor and later for another (see glomerulus 28 for ratios 1:9 and 9:1). The activity decreased abruptly and stabilized back to baseline few hundred milliseconds after odor offset. The bottom panel in Figure 4A shows the temporal evolution of the Euclidean distance (ED) between the odors 1:9 and 9:1 calculated from the examples above, but based on all 17 glomeruli. The distance is maximal at ~ 500 ms in this example, which is after the peak activation of many glomeruli at or just after odor. Therefore, the temporal pattern contributes to the separation of the trajectories.

We then analyzed the activity of the 17 identified glomeruli over 125 ms time steps for each animal. We represented the spatiotemporal patterns as trajectories in a two-dimensional space using Principal Component Analysis. The temporal evolution of the response to each odor is then represented by a trajectory through this space (Fdez Galán et al., 2004; Mazor and Laurent, 2005). Figure 4B, left, shows examples of trajectories for the range of odor-ratios presented to two different individuals (bees C1 and C2). Since this pattern was fairly constant among animals (7 of 9 bees showed the same pattern), we averaged the activity of each of the 17 glomeruli along each time point across animals. PCA-based trajectories for the averaged data are shown in Figure 4B (right). After stimulus onset, trajectories depart from rest near the origin and evolve through time (thick line indicates odor exposure). They do not evolve at a constant speed but start to slow down after 500 ms, which is consistent with previous measurements by Fdez Galán et al. (2004). At that point the trajectories start to return although the odor is still being presented. After odor stimulation (dotted line), trajectories return slowly to the

Figure 3. Neural representation of binary mixtures 1-hexanol: 2-octanone in PNs of the AL showed a gradient of activation along different ratios. **A**, Anatomical view (raw fluorescence) of the antennal lobe (left) and correlated image (right) (see Materials and Methods) showing the 17 glomeruli identified in this study. **B**, Color-coded odor response patterns across the dorsal glomeruli in the AL from a representative individual honeybee. PNs were labeled by backfilling with fura 2-dextran. All the ratios from 1-hexanol (10:0) to 2-octanone (0:10) are shown. The maps are represented according to a common intensity scale (right). Each image represents an average of the glomerular activity between 375 and 625 ms after odor onset. **C**, Glomerular response delta (340/380) percentage of selected individual glomeruli (mean $\pm$ SE, $N = 9$) to different ratios of test odors. Mean of glomerular activity between 325 and 625 ms after stimulus onset is shown. In all cases asterisks indicate significant differences by repeated measures ANOVA ($*p < 0.05$, $***p < 0.001$).

Figure 4. Spatiotemporal response patterns show a smooth transition along ratios from one single component to the other. **A**, Top and middle panel, Odor responses (% delta 340/380) in eight representative glomeruli to ratios 9:1 (top) and 1:9 (bottom) over 125 ms time steps from just before odor delivery through 2750 ms for a control bee. Different colors correspond to different glomeruli (see legend). Stimulus duration (1000 ms) is marked by the shaded area. Bottom panel, Euclidean distance 9:1 to 1:9 over time for the same control bee. **B**, Left, Odor specific trajectories for two control bees; seven of nine bees follow the same general pattern. To generate this figure the original 17-dimensional space has been projected onto the first two principal components for each bee. Under these conditions, 86.2–97.5% of the variance is explained for each bee. All ratios were presented to each bee and Ca^{2+} transients were recorded at fixed time intervals (125 ms). Accordingly, the distance between different colored data points represents the divergence of the odor representations over time. Trajectories depart rapidly from baseline and slow down when they approach odor-specific regions. PC1 and PC2 indicate principal component factors 1 and 2 respectively. Right, PCA of odor-evoked activity patterns for a control “average” bee obtained by averaging 9 control bees by the activity of 17 glomeruli along 27 125 ms-time intervals. The response of each glomerulus was used as a dimension for the analysis. **C**, Euclidean distances between ratio 10:0 (pure 1-hexanol) and all the other test ratios based on a 17-dimensional space over 125 ms time steps from just before odor delivery through 3000 ms after odor onset. Stimulus duration (1000 ms) is marked by the shaded area.

Figure 5. Differential conditioning increases the distance between spatiotemporal patterns. Worker honeybees were differentially trained to 9:1+ and 1:9−. Nine hours after conditioning, brains were treated as above with fura-2 and 8–12 h later were imaged as described in Materials and Methods. These bees were evaluated in parallel to the untrained “control” bees reported in Figure 4. PCA analysis was done jointly for untrained and trained animals, allowing a direct comparison of the two groups. **A**, Left, Odor specific trajectories for two trained bees. Seven of eight bees follow the same pattern. The PCA of odor-evoked activity patterns account for 88.2–95.4% of the variance for each bee. PC1 and PC2 indicate principal component factors 1 and 2 respectively. Right, PCA of odor-evoked activity patterns for a trained average bee ($N = 8$). The relative positions of odor representations between trained and control groups (compare with Fig. 5B, right) suggest the modification of odor representation through conditioning. The first two components account for 97% of the whole variance of the trajectories. **B**, Relative euclidean distances between ratios 9:1 and 1:9 (i.e., CS+ vs CS−) based on a 17-dimensional space over 125 ms time steps from just before odor delivery through 3000 ms after odor onset. Stimulus pulse (1000 ms) is marked by the shaded area. Close and open symbols indicate respectively trained and untrained bees. Asterisks indicate significant differences between trained and control bees (Mann–Whitney U test, $p < 0.05$).

resting state following a path that is slightly different from the path during stimulation (Mazor and Laurent, 2005).

In addition, the spatiotemporal patterns change in an ordered way as the mixture ratio changes from one extreme to the other. The pure odors (0:10 and 10:0) correspond approximately to each of the PC axes. As the mixture ratio changes from 10:0 to 9:1 to 7:3 through the other pure odor, the angle of the loop through PC space gradually changes (Fig. 4B). To quantify the relationships between odors along time, we calculated EDs for each time point in the 17-dimensional space represented by the different glomeruli (Fig. 4C). Each ED obtained for each time point in each animal was normalized to its ED obtained between 10:0–0:10 at 500 ms (see Materials and Methods) (supplemental Fig. S3, available at www.jneurosci.org as supplemental material, also shows a different method for calculating ED).

In summary, after odor onset the distance between trajectories for 10:0 and different ratios begins to depart from baseline by 125 ms and it is significantly different by 250 ms (repeated measures ANOVA between 0 and 250 ms, time factor $p < 0.001$). The maximum odor separation was reached by 375 or 500 ms (Fig.

4A). Because the temporal evolution of responses differs in different glomeruli and contributes to the separation, this separation is the temporal decorrelation referred to by Mazor and Laurent (2005). The distance then slowly decreased showing no sharp boundary when the odor was shut off at 1000 ms. The distance stabilizes back to baseline levels by ~800 ms after odor shut off. The distances between trajectories become progressively greater as the difference in ratios increases from 10:0–9:1 through 10:0–0:10 (Fig. 4C). The gradient in these temporal patterns of PN responses therefore correlates closely to what we observed in the behavioral assays.

Differential conditioning increased the decorrelation between AL responses to odor mixtures

The imaging data summarized above comes from untrained honeybee workers. However, all of the previously reported behavioral responses were from trained honeybees. Therefore, we also evaluated AL responses to odors in honeybees that had been differentially conditioned to odor mixtures. We show examples of odor trajectories of two trained individuals (bees T1 and T2) (Fig. 5A, left) as presented before for control animals (Fig. 4B). Again, since this pattern was fairly constant among animals, we averaged the activity of each of the 17 glomeruli along each time point across trained animals. (The PCA in Figs. 5A, right, and 4B, right, was done jointly for untrained and trained animals to allow a direct comparison.) Trajectories for the average are shown to the right of Figure 5A. This visualization of odor trajectories suggests a shift in odor trajectories to the left in trained bees (Figs. 5A, right,

4B, right, compare same color lines). Interestingly, the relative distance among odor trajectories for the different ratios showed qualitative changes after differential conditioning. The trajectory for 9:1 (CS+) moved away from 0:10, 1:9 (CS−), 3:7, and 5:5 and became more similar to 10:0, the single component 1-hexanol, which is the major component in the rewarded ratio.

Figure 5B shows the Euclidean distances from −250 to 3000 ms (125 ms interval) for the comparison between CS+ and CS− (9:1 vs 1:9) in trained and untrained animals. The pattern for trained animals (closed symbols) was similar in its time course to that for control, untrained animals (open symbols), but it was different in magnitude. As before, distances departed from baseline by 125 ms. However, the distances were significantly larger in trained than in untrained animals. This difference was significant by 250 ms, and it remained significant at each time point between 375 and 1000 ms after odor onset (Mann–Whitney U test, $p < 0.05$) (see also supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Therefore, the reference frame for

Figure 6. Training times <500 ms lead to poor discrimination when test time does not match training time. **A**, Schematic of experimental protocol for bees trained to CS + 9:1 and CS – 1:9 (ratio 1-hexanol: 2-octanone). t_{TR} indicates odor sampling time during training (either 200, 500, 1000, or 2000 ms). During testing trials, the odors were presented in a random sequence. SV indicates stimulation by solvent. Thus, each set of honeybees was trained using one sampling time and then tested with each of the four times. Acquisition curves for each set are shown in supplemental Figure S2, available at www.jneurosci.org as supplemental material. Sample sizes were: $N = 37$ for 200 ms, $N = 30$ for 500 ms, $N = 39$ for 1000 ms, and $N = 40$ for 2000 ms. **B**, Duration of the proboscis extension response when odor sampling time during training matched test time, i.e., honeybees were trained and tested with the same sampling time. Hatched bars indicate response levels during the test with $10 \mu\text{l}$ of solvent (mineral oil). **C**, Duration of the conditioned response for all combinations of training and testing times (i.e., bees were trained with a specific stimulus length and tested with the same or with a different one). Gray bars denote data already shown in Figure 3B (presented here for comparison). In all cases, asterisks indicate significant differences by Wilcoxon matched pairs test between 9:1 and 1:9 ($*p < 0.05$, $**p < 0.005$).

the PN coding space was shifted in trained bees in a way that improves the separation between the CS+ and CS–.

Changes in activity across glomeruli were complex. As a result of differential conditioning, some glomeruli, for example 25, became slightly more excited, and other glomeruli (e.g., 17, 33, 36, 48, and 52) showed a slight reduction in activity (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). However, all of these changes were subtle and not significant in any case, suggesting that differences in EDs as shown in Figure 5B are not dominated by the differential activation of one or two specific glomeruli.

Training times <500 ms increase generalization when the test time does not match the training time

We have shown that the representation of odors in the AL is dynamic and depends on sampling time (Fdez Galán et al., 2004; Mazar and Laurent, 2005). We therefore performed a series of experiments to evaluate how temporal information may be involved in odor recognition. We also included a dimension not yet tested in olfactory conditioning experiments, which consists of matching or mismatching sampling times during training and testing. If the pattern at a particular time is stored in memory during training, then animals may or may not be capable of recognizing stimuli given different sampling times during recall tests.

In this experiment, we used the same odor ratios 9:1+ versus 1:9– and four different stimulus delivery times (200, 500, 1000, and 2000 ms) (Fig. 6A). These delivery times are realistic times

that honeybees might encounter when flying upwind in an odor plume in the field (Vickers, 2006). Sensory afference from the antennae, as measured by electroantennogram responses, reaches a maximum by 200 ms (supplemental Fig. S5A,B, available at www.jneurosci.org as supplemental material), which indicates that the different sampling times that we used are probably not perceived as different odor concentrations (Stopfer et al., 2003). Furthermore, short stimulation times produced activity patterns across glomeruli in the AL that are nested within the patterns produced by longer stimulation (supplemental Fig. S5C, available at www.jneurosci.org as supplemental material). Therefore, different sampling times are likely represented as truncated trajectories in the 17-dimensional space (Mazar and Laurent, 2005). In most cases, honeybees began to respond more to the 9:1+ ratio than to the 1:9– by the third or fourth trial, and this pattern carried through the testing phase (supplemental Fig. S6, available at www.jneurosci.org as supplemental material).

Figure 6B shows the duration of proboscis extension when training time matched testing time. The mean duration was higher for the CS+ than for the CS– in all cases ($p < 0.05$; Wilcoxon matched pairs test), indicating that honeybees were able to discriminate test ratios even with only 200 ms exposure. However, two patterns indicate that exposure time is important for discriminability. First, the duration of response (mean of the CS+ and CS– response levels) increased from 3.3 s or 3.4 s in the 200 and 500 ms stimulation groups, to 5.5 s in the 1000 ms group (one-way ANOVA, $p = 0.02$). This increase in response likely indicates that long sampling times increase the detectability of

odor over background, as was already reported by Wright et al. (2009a). Another important indication of a changing pattern of response comes from comparison to test responses to the solvent blank. At 500 ms or longer training/test exposures, the responses to the CS+ were on average longer than the mean responses to the solvent blanks (Fig. 6B, hatched bars) ($p < 0.001$). However, at 200 ms the response to the blank was equal to the CS+ and qualitatively longer than the responses to the CS−. This change in pattern suggests that honeybees may adopt different response strategies depending on sampling time. For example, the CS− may become discriminable from the blank much more quickly than the CS+. Regardless of the underlying cause, this result suggests that sampling time is a determinant of the response topology and decision strategy.

Further evidence that sampling time is important came from testing with times that were different—either shorter or longer—than training times (Fig. 6C). When honeybees were trained with 200 ms exposure, bees did not differ in response to the CS+ and CS− when odors were delivered at any of the times longer than 200 ms. After training to 500 ms, the highest generalization occurred at the shortest (200 ms) and at the longest (2000 ms) test exposures. The only significant indication of discrimination occurred when there was a match (500–500 ms) or a slight mismatch (500–1000 ms) between training and test exposures. Finally, discrimination was evident at all testing times with training exposures of 1000 or 2000 ms.

Animals that responded to odorants did so with approximately the same latency. The median latencies for the CS+ and CS− were 600 ms. Means were, respectively, 740 ms (± 50 ms, SE) and 810 ms (± 70 ms, SE). These values did not differ significantly across sampling time ($p > 0.05$ for all comparisons among 200, 500, 1000, and 2000 ms).

Discussion

We have shown that the transient dynamics in the PN output of the AL represent gradations of odor mixtures that correlate to behavioral generalization among those mixtures. Discrimination conditioning of binary mixtures produced smooth generalization during testing with intermediate ratios. The response to a test odor was directly proportional to its similarity to the CS+ or CS−. Furthermore, conditioning odor ratios that were similar produced a peak shift in the response in a direction away from the CS−. All of the behavior data suggest that smooth changes in mixture ratio produce smooth changes in the perceptual properties and, hence, in the neural representations of those mixtures. Therefore, responses in the AL should also grade smoothly with systematic variation in the ratio of components. Such smooth gradations in spatial response patterns have been shown in several studies of molecular features of monomolecular odorants (Sachse et al., 1999; Daly et al., 2004a; Lei et al., 2004; Hallem and Carlson, 2006; Johnson and Leon, 2007). Smooth gradations to changes in odor mixture ratio have been shown in the mammalian OB (Khan et al., 2008) and insects (Carlsson et al., 2007). However, little work has been done to investigate spatiotemporal coding using systematic changes in ratios of mixtures.

We show that the orientation of the transient's trajectory changes systematically with the composition of the mixture. As the ratios became more distinct the loops became farther separated, which correlates to generalization among odors in behavioral studies. The mechanism through which spatiotemporal patterns generate this gradation remains to be investigated. Several studies of spatial coding in sensory afferents have described mixture coding in honeybees (Deisig et al., 2006), fruit

flies (Hallem and Carlson, 2006; Silbering and Galizia, 2007) and moths (Carlsson et al., 2007). In general, mixture-induced activity patterns include patterns of each component. Complex mixtures largely activate patterns that contain component patterns, but with some suppression possibly because of global inhibitory interactions among glomeruli (Deisig et al., 2006).

Our studies investigated mixture coding in the output of AL. We found no obvious evidence of synergism or suppression. The lack of significant interaction between glomeruli activated by each of the odors could explain the smooth, almost additive transition of spatiotemporal pattern that we observed. This finding is consistent with a recent report on mixture coding in the mammalian OB (Khan et al., 2008). In contrast, Silbering and Galizia (2007) reported mixture suppression in PNs in *Drosophila*. Activated glomeruli suppressed other glomeruli through network-level inhibitory connections (Leitch and Laurent, 1996; Wilson and Laurent, 2005; Sachse et al., 2006; Mwilaria et al., 2008). Our study was not designed to specifically reveal interaction. Thus, a more specific, detailed analysis of mixture interaction will be necessary to establish how changes in mixture composition account for smooth changes in spatiotemporal activity.

Several studies have shown associative plasticity in the OB and AL (for review, see Davis, 2004). The most frequent change is in recruitment of units into a preexisting pattern (Kendrick et al., 1997; Faber et al., 1999; Ravel et al., 2003; Martin et al., 2004; Yu et al., 2004). Other studies have reported more complex changes involving many or all of the possible types of switches in activity between no response, excitation, and inhibition (Kay and Laurent, 1999; Daly et al., 2004b). The changes reported have been transient, lasting a few minutes in support of a form of short-term memory (Yu et al., 2004); they have lasted up to 3 h in support of an intermediate form of memory in the AL (Daly et al., 2004b); or they appear to be stable for much longer periods of time (Brennan and Keverne, 1997; Kendrick et al., 1997; Ravel et al., 2003; Sandoz et al., 2003; Martin et al., 2004). It is unclear whether these differences reflect choice of animal model, conditioning protocol, or recording technique.

We found that activity patterns of PNs are more distinct in trained than in control animals, which suggests that the AL is becoming tuned to best discriminate odors that are currently important (Smith et al., 2006). The change lasted at least 24 h, or it developed over that time, because animals were conditioned on the day before imaging studies. Thus a component of associative olfactory memory is consolidated in the AL, which is consistent with biochemical analyses of honeybee and fruit fly AL (Müller, 2000; Ashraf et al., 2006).

In a recent work in honeybees, Peele et al. (2006) used the same recording technique that we have used—backfilling of PNs with fura-dextran. However, they could not identify traces of associative plasticity in the projection neurons of the same tract that we studied here (I-ACT). There are several significant differences between our study and theirs that may explain the different outcomes and provide important insight into how plasticity operates in the antennal lobes. First, Peele et al. (2006) evaluated glomerular activity patterns up to 23 min after conditioning. We evaluated the activity patterns 24 h after conditioning. Memory traces at these two time points require different biochemical mechanisms (Müller, 2000) and may involve different cellular substrates (Erber et al., 1980; Hammer and Menzel, 1995, 1998). Our results would be consistent with those of Peele et al. (2006) if the presence of a memory trace in the I-ACT PNs emerges as memory consolidates and is either not present or not easily measurable using calcium imaging during short-term memory.

Our study also differed from the one by Peele et al. (2006) in the way in which reinforcement was delivered. They conditioned honeybees after PNs had been stained and the animals had been prepared for imaging. In this situation, the US is delivered to the sucrose receptors on the antennae and proboscis, but the animal may not be able to fully consume the sucrose. Stimulation of taste receptors is enough to induce short-term memory, but consumption of sucrose, as honeybees were allowed to do in our study, is an important condition for induction of long-term memory formation (Wright et al., 2007).

Instead of using pure odors that are easily to discriminate, we required animals to differentiate between binary mixtures that differ in the ratio of the two components. Use of mixtures under these conditions might prolong the time needed to effectively differentiate different odors. For example, Krofczik et al. (2008) used pure odors and found that maximal separation occurred circa 100–200 ms after onset of odor. Furthermore, the two components in our experiments were chosen because they activate a partially overlapping set of glomeruli (Sachse et al., 1999). These odor mixtures induce in the AL complex patterns that are similar in untrained animals. Learning different predictive values of two mixtures constitutes a difficult conditioning task, because successful discrimination requires more trials than when conditioning to relatively distinct pure odors (Smith and Menzel, 1989). We propose that in this situation the AL extracts different features of a mixture that more reliably predict reward, making both representations more different than in untrained animals (Smith et al., 2006). This phenomenon may be less evident between pure odors that have little or no overlap in their AL activity patterns.

We also established that stimulus duration is important for discriminability, as measured in generalization gradients. However, it is not a simple rule, such as that more time means better discrimination. If we had only tested stimulus durations that matched training, we might have concluded that stimulus duration is not relevant to odor discrimination, since animals showed discrimination even with very short (200 ms) stimulus pulses. We found a threshold for stimulus durations below which matching between training and testing is necessary for robust odor discrimination. For short training stimuli of 200 ms, odors were discriminable only when there was a match between training conditions, under which the memory is formed, and test conditions under which the stimulus is compared with the memory template. For longer training stimuli of 1000 or 2000 ms, discriminability became independent of stimulus duration. When training stimuli lasted 500 ms, we observed a transition in the matching requirement, because discrimination was possible with testing stimuli of 500 and 1000 ms. This shift in memory content—from time dependent to time invariant—occurs between 500 and 1000 ms after odor onset, which is approximately the time it takes for the spatiotemporal patterns to become maximally separated in the imaging analysis.

In natural environments the duration of odor pulses may be short and stochastic (Murlis and Jones, 1981), and it is likely that this would produce variable length pulses that do not match memory. However, animals may behaviorally compensate for this limitation. When allowed a choice, freely flying honeybees fly over an odor source for of 690 ms regardless of the difficulty of the task (Ditzen et al., 2003). We also found that the median latency to response to the CS+ and CS− was just over 700 ms. According to our imaging measurements, this is beyond the time necessary for the odor representation to reach maximal distinctiveness, and it is likely beyond the

time at which odor discrimination becomes time invariant. This suggests that bees make decisions only after having obtained enough information to generate a more reliable encoding of the stimulus.

Finally, the plasticity we have described is consistent with predictions of recent computational models of the AL (Linster and Smith, 1997; Borisjuk and Smith 2004). In those models, reinforcement may modify local inhibitory (GABAergic) synapses. Further work is now needed to test for this specific connectivity in the AL, particularly in regard to disruption of octopamine receptor pathways in the honeybee AL (Farooqui et al., 2003, 2004).

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