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INVERTEBRATE LEARNING AND MEMORY

Hosted by
Martin Giurfa, Jean-Marc Devaud
and Jean-Christophe Sandoz

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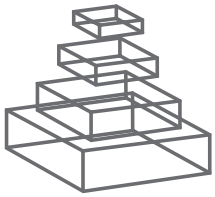

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INVERTEBRATE LEARNING AND MEMORY

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Associative learning is a capacity present in all animals with a nervous system, which allows extracting the logical structure of the world by evaluating the coincidental order of events. It leads to the generation and storage of memories, which can be retrieved in appropriate circumstances to provide adaptive responses to a changing environment. Invertebrates, with their less complex and accessible nervous systems have been pivotal organisms to understand learning and memory at the behavioral, cellular and molecular level. Not only do they exhibit different forms of associative learning, from Pavlovian to operant, from elemental to non-elemental ones, but their memory is also organized following basic principles common to vertebrates. These phenomena could be traced at the circuit and molecular level, thus yielding fundamental insights into the biological basis of learning and memory. Here we will provide an across-species dissection of these capacities focusing on various invertebrate models - from mollusks to insects. We will discuss evolutionary components and extract universal principles underlying learning and memory organization.

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Appetitive and aversive visual learning in freely moving *Drosophila*

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To compare appetitive and aversive visual memories of the fruit fly *Drosophila melanogaster*, we developed a new paradigm for classical conditioning. Adult flies are trained *en masse* to differentially associate one of two visual conditioned stimuli (CS) (blue and green light as CS) with an appetitive or aversive chemical substance (unconditioned stimulus or US). In a test phase, flies are given a choice between the paired and the unpaired visual stimuli. Associative memory is measured based on altered visual preference in the test. If a group of flies has, for example, received a sugar reward with green light in the training, they show a significantly higher preference for the green stimulus during the test than another group of flies having received the same reward with blue light. We demonstrate critical parameters for the formation of visual appetitive memory, such as training repetition, order of reinforcement, starvation, and individual conditioning. Furthermore, we show that formic acid can act as an aversive chemical reinforcer, yielding weak, yet significant, aversive memory. These results provide a basis for future investigations into the cellular and molecular mechanisms underlying visual memory and perception in *Drosophila*.

Keywords: behavioral assay, classical conditioning, chemical reinforcement, vision, method

INTRODUCTION

The fruit fly *Drosophila melanogaster* is an excellent model to study the genetic and neural bases of associative memory (McGuire et al., 2005; Pitman et al., 2009). Visual memories of the fly have been intensely studied using various aversive learning assays (Quinn et al., 1974; Spatz et al., 1974; Menne and Spatz, 1977; Lepot and Médioni, 1986; Wolf and Heisenberg, 1991; Le Bourg and Buecher, 2002; van Swinderen et al., 2009). In contrast to the relative abundance of aversive learning assays, only two behavioral paradigms for appetitive visual learning in *Drosophila* have been reported to date (Heisenberg, 1989; Gerber et al., 2004; see Fukushi, 1976, 1985, 1989 for studies on other fly species). However, appetitive and aversive visual memories have never been compared in the same setup. This is partially due to the limited compatibility of reinforcement application (i.e., exchangeable reward or punishment).

Few conditioning paradigms in insects are versatile enough to succeed in the direct comparison of mechanisms underlying appetitive and aversive memories. For example, in olfactory learning of *Drosophila*, paired presentation of an odor with sugar reward or electric shock punishment under otherwise same experimental conditions leads to approach or avoidance of the odor, respectively (Tempel et al., 1983; Schwaerzel et al., 2003; Gerber and Hendel, 2006; Honjo and Furukubo-Tokunaga, 2009). These opposite memories differentially recruit the two biogenic amines octopamine and dopamine, which respectively mediate appetitive and aversive reinforcements (Schwaerzel et al., 2003; Honjo and Furukubo-Tokunaga, 2009, but see Kim et al., 2007b; Selcho et al., 2009). Also in the olfactory conditioning of the sting extension reflex of the honey bee (Vergoz et al., 2007; Giurfa et al., 2009; Roussel et al., 2009) and in visual and olfactory learning in the cricket (Unoki et al., 2005, 2006; Mizunami et al., 2009; Nakatani et al., 2009), appetitive and aversive memories can be directly compared in the same setup. Such comparisons yielded similar results as in *Drosophila*: appetitive and aversive reinforcement is mediated by octopamine and dopamine, respectively.

In order to understand and compare the mechanisms underlying visual appetitive and aversive memories in *Drosophila*, we sought to establish a new behavioral paradigm for visual associative learning in adult flies. This assay should: (1) produce reproducible associative memory, (2) be simple to set up and maintain, and (3) accommodate the application of different stimuli. We developed a classical conditioning protocol using a setup in which various visual and chemical stimuli can be simultaneously presented. We utilized an LCD screen to generate spectrally different visual stimuli that illuminate flies in a cylindrical arena. Chemical stimuli were presented on the bottom of the arena. We analyzed the effect of critical parameters for the formation of memories such as training repetition, order of reinforcement, interval between conditioned stimuli (CSs), motivation, and the impact of appetitive and aversive reinforcers on visual memory formation.

MATERIALS AND METHODS

FLIES AND PREPARATION

The wild type *Drosophila melanogaster* strain Canton S was employed throughout the study. Flies were reared on standard cornmeal medium at 25°C and 60% relative humidity under a 14-h light/10-h dark cycle. All flies were handled without anesthesia until experiments and used 2–6 days after eclosion.

Before starvation, flies were collected within 1 day after eclosion and kept in new food vials for at least 1 day to control their feeding status. At the beginning of experiments, they were transferred to moistened empty vials and starved for either 24–28 or 48–52 h, roughly calibrated by mortality (see Section "Results" for further details). Water was provided by means of tissue paper on the vial bottom and folded filter paper (∅ 90 mm) clamped by a slit of a plug.

APPARATUS

Flies in a Petri dish arena were illuminated from below through filter paper that contained a chemical substance. The experimental setup consisted of three major parts: (1) an LCD monitor used to generate visual stimuli; (2) a cylindrical arena, where flies were trained and tested; (3) a video device that recorded the back-lit arena (**Figure 1**).

The LCD monitor (MM19SE, ASUS Computer GmbH, Ratingen, Germany) was horizontally laid and was used to generate visual stimuli that were fitted to the Petri dish diameter (**Figure 1**). For conditioning, green (0:255:0 in R:G:B) or blue (0:0:255) stimuli were presented on a black background (0:0:0). For preference analyses, the arena was illuminated with red light (200:0:0). **Figure 2** shows the spectral and intensity characteristics of these stimuli. Filter paper soaked with sucrose (dried), water or acid solution increased light transmission by 40 or 100%, respectively. Scheduled Slide Show function in PowerPoint 2007 (Microsoft Corporation, Redmond, WA, USA) was used to automatically change the visual stimuli. Routinely, four Petri dishes were placed on the monitor in fixed positions using a plastic frame (**Figure 1B**).

The cylindrical arena consisted of a Petri dish (∅ 92 mm, Sarstedt, Nümbrecht, Germany) on which flies could freely move, a pipe wall, and a second Petri dish used for a lid (**Figure 1A**). A circular piece of filter paper [round filter, ∅ 90 mm (trimmed to 84 mm when used), Hartenstein, Würzburg, Germany) was put on the floor of the Petri dish, depolarizing the light generated by the LCD screen (**Figure 1A**). Chemical substances were applied with the filter paper. A plastic ring that fitted to the inner diameter of a Petri dish (outer ∅ 84 mm, inner ∅ 79 mm, height 5 mm) was used to clamp the filter paper to the dish and to fix the cylindrical pipe (**Figure 1A**).

The pipe had a black surface (∅ 79 mm, height 139 mm) and served both as a wall to prevent flies from flying away and as a space to keep flies when exchanging Petri dish arenas (**Figure 1A**). Its smooth inner surface was coated with Fluon (Fluon® GP1, Whitford Plastics Ltd., UK) to prevent flies from hanging on the wall. Consequently, flies were forced to stay on the filter paper at the bottom of the arena (**Figure 1C**).

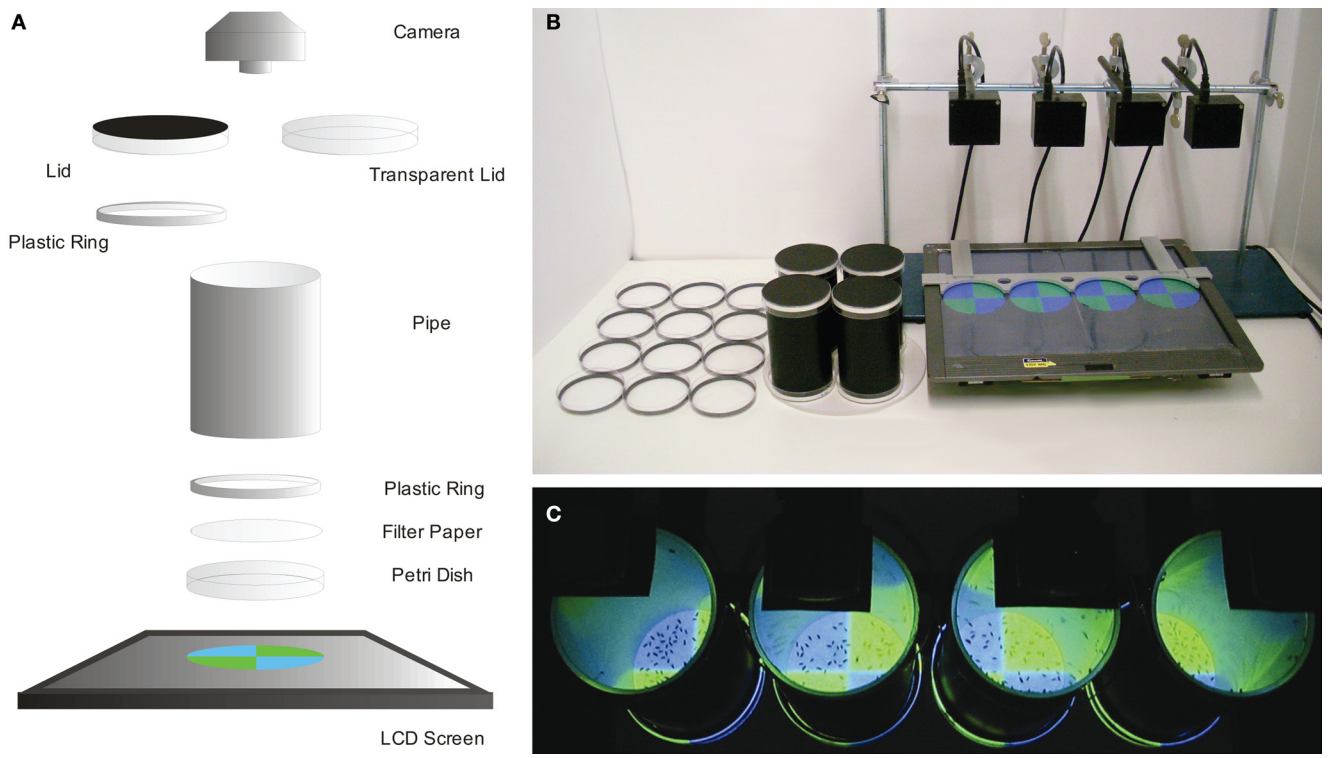
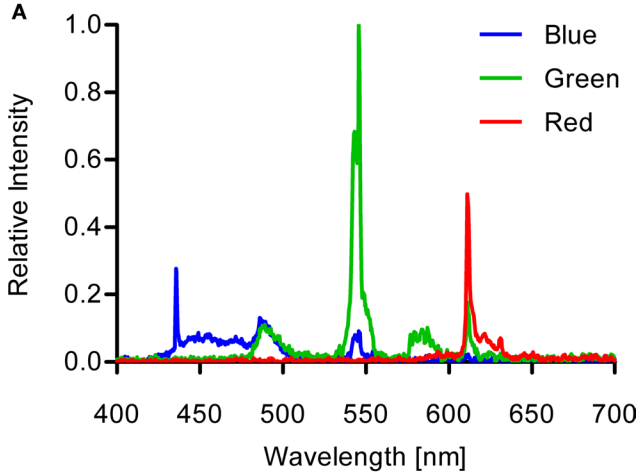


FIGURE 1 | Conditioning setup. (A) Scheme showing the principal components of the experimental setup. A Petri dish is illuminated from below using an LCD screen. Chemical solutions are presented on filter paper which is clamped on the dish by a plastic ring. A plastic pipe (inside coated with Fluon) connects the bottom dish and a lid (Petri dish). During training the cylinder is closed by an opaque lid (coated with Fluon), while during a test phase, a transparent lid enables recording flies from above. **(B)** The setup. From left to right: Petri dishes with filter papers (US-soaked, water-soaked and neutral from the left), cylinders closed with the opaque lids (top and bottom), the LCD screen with a plastic frame presenting test visual stimuli, cameras fixed by a stand. **(C)** Top view during the test phase. The flies are recorded with cameras from above (black rectangles).



B

Stimulus	Intensity [cd m^{-2}]
Blue	3.1
Green	24.5
Red	5.0

FIGURE 2 | Visual stimulus properties. (A) Spectra of blue, green and red stimuli generated by the LCD screen. **(B)** Intensities of the different light stimuli.

A Petri dish, transparent or opaque (laminated with black foil), was used for a lid (**Figure 1A**) at the top. During training, we used the opaque lid coated with Fluon and containing a plastic ring (see above). During the test phase, we used the transparent Petri dish (**Figure 1A**).

During the test phase, the arena (Petri dish floor) was video recorded from above with a CMOS camera (Sansun Webcam SN-509A, SANSUN, Deutschland, Germany; Easy cam, Typhoon, Germany, or Firefly MV, Point Grey, Richmond, Canada) (**Figure 1C**). We developed software that allows setting of four cameras independently (e.g., brightness, exposure time, frame rate, recording delay, compression, etc.). Typically, each arena was recorded for 90 s of the test at one frame per second.

CONDITIONING

Conditioned and unconditioned stimuli

All experiments were performed in darkness. During the training phase, the whole arena was illuminated from the bottom either with green or blue light, which were used as CS in a differential conditioning procedure (CS; **Figure 3A**; see **Figure 2** for visual stimulus properties). In the test phase, the four quadrants of the arena were separately illuminated with green or blue light so that flies had to choose between both stimuli. Diagonal quadrants were illuminated with the same stimulus (**Figures 1C and 4A**).

The spectra of the visual stimuli generated by the subpixels of the LCD monitor were measured with a CCD spectrometer (Tristan USB, m-u-t AG, Wedel, Germany). A Luminance meter (BM-9, TOPCON TECHNOHOUSE CORPORATION, Tokyo, Japan) was used to measure the intensities of the stimuli (**Figure 2**).

Filter paper was used to present the unconditioned stimuli (US). In *appetitive conditioning*, sucrose was used as a US as it proved to be the most efficient reward among five different sugars in olfactory learning (data not shown). The filter paper was soaked with 1.5 M sucrose solution and subsequently dried. Filter paper that was presented with the non-reinforced stimulus was soaked with water and subsequently dried. In the test phase following appetitive conditioning, no US was presented; only visual stimuli and untreated filter papers were presented.

In *aversive conditioning*, either 1 M acetic acid or 1 M formic acid solution was used as a US. Contrarily to appetitive conditioning, the filter paper on which these solutions were applied was not dried. Accordingly, the filter paper that was presented with the non-reinforced light was soaked with the same amount of water. The test arena contained either a dry filter paper previously soaked with water or a filter paper soaked with the same acid solution as the US (see Section "Results" for further details).

Appetitive conditioning

A group of flies was trained following a differential conditioning procedure, i.e., only one of two consecutively presented visual stimuli (i.e., green and blue light) was paired with a sucrose reward (**Figure 3**). Two groups of flies were reciprocally trained regarding CS–US contiguity: Green+/Blue– and Blue+/Green– (**Figure 3A**). Typically, four groups of animals were simultaneously trained and tested in parallel (**Figures 1B,C**).

Using an aspirator, 50–100 flies were introduced into the cylinder from the lid. A switch of US presentation was carried out by exchanging Petri dishes at the bottom. First, the cylinder was inverted and after a gentle tap, the Petri dish now being on the top was quickly replaced by a new Petri dish (with or without US). Subsequently, flies were transferred to the new dish by inverting the cylinder again and by delivering a further gentle tap. This "inversion & tap" procedure was done using a soft mouse pad and intended to transfer the flies by detaching them from a Petri dish. It cannot therefore be assimilated with the aversive shaking used for reinforcement in other conditioning protocols of *Drosophila* (Menne and Spatz, 1977; Mery and Kawecki, 2005; van Swinderen et al., 2009). Finally, the cylinder was immediately put in a fixed position on the LCD screen (**Figure 1B**).

Training (first CS presentation) started approximately 60 s after the introduction of the flies. The duration of CS/US presentation was 60 s (**Figure 3B**). An inter-CS interval (ICSI) caused by a fly transfer lasted typically 10–12 s (**Figure 3B**). In the experiment with a longer ICSI, the bottom dish was removed and replaced with a Fluon-coated lid, and flies were kept in this double-lid arena during the interval. Prior to the next CS, one of the lids was replaced with another Petri dish. Such a training trial was repeated four times unless otherwise stated.

After the last training trial, flies were placed on a dish without US. During the test phase, a transparent lid was used for video recording from top (**Figure 1A**). The test of immediate memory started ~60 s after the offset of the last CS (**Figure 3B**), and the preference of trained flies for the two visual stimuli was recorded for 90 s (**Figure 1C**). For testing longer retention performances (**Figure 8**), flies were transferred into moistened empty vials using

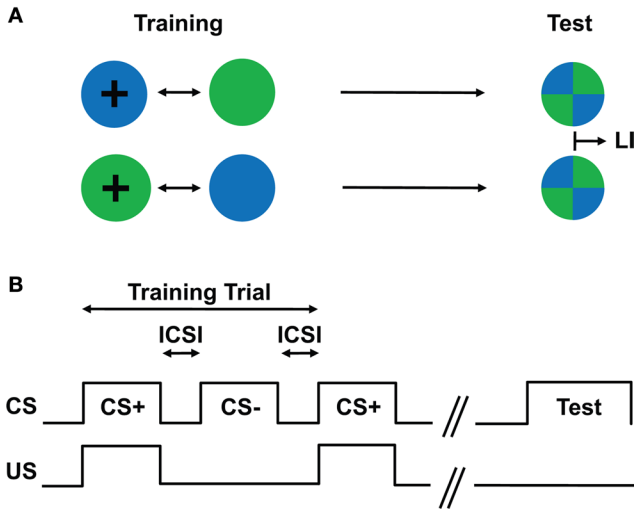


FIGURE 3 | Conditioning design. (A) Schematic drawing of the training and test situations. Two groups of flies are trained with different CS/US contingency: one group of flies is trained such that green light is paired with a US, whereas blue light is presented without any reinforcing stimulus (i.e., Green+/Blue–; first row); another group of flies is trained with the reversed contingency, i.e., Blue+/Green– (second row). After such training, flies are allowed to choose between the previously reinforced (CS+) and the non-reinforced stimulus (CS–). The difference of the stimulus preferences of the two groups in the test provides a measure of their memory (LI). **(B)** Conditioning protocol. After a pre-training period of 60 s, two differential visual stimuli (CS) were sequentially presented for 60 s with an inter-CS interval (ICSI) of typically 10–12 s. Only one of the two CSs was paired with the US (CS+) for 60 s. One training trial consisted of a CS+ and a CS– presentation.

a funnel after training. The flies were kept in darkness until the test. The test started approximately 60 s after reintroduction of the flies.

The reciprocal experiment was performed immediately afterwards using the identical apparatus. In the reciprocal experiment, the non-reinforced stimulus of the first experiment was now paired with the US; i.e., if the green light was rewarded in the first experiment (Green+/Blue–), the reciprocal group experienced blue with the reward (Blue+/Green–; **Figure 3A**). The difference of these reciprocally trained groups in the visual stimulus preference was used to calculate a learning index (LI; see below).

To exclude a non-associative effect that stems from the order of reinforcement, approximately one half of the experimental groups received the US together with the first visual stimulus (CS+/CS–), and the other half received the US with the second stimulus (CS–/CS+).

Aversive conditioning

The training protocol was identical to that of appetitive conditioning with the difference that aversive chemical substances were used as US. The interval between the training and the test phases was 90 s, during which the cylinder was closed by lids at the bottom and top. Shortly before the test phase started, one lid was replaced with a Petri dish for a respective test (see Section "Results" for details), and the other was replaced with a transparent lid. Trained flies were tested both in the presence or absence of the US.

STIMULUS PREFERENCE

To test the attractive/aversive nature of a chemical substance, the flies' preference was also measured using the cylindrical arena. To provide a choice situation, a piece of circular filter paper was cut into two halves. These halves were laid next to each other in a Petri dish and clamped with a plastic ring (**Figure 1A**). Each half presented water or the respective test substance. Papers were freshly prepared prior to an experiment to avoid desiccation. The arena was back-lit in red for video recording (see **Figure 2** for visual stimulus properties). Except for these adaptations, the setup was identical to that used for conditioning.

Approximately 50 flies were directly introduced into the choice arena after recording was started. The arena was recorded for 240 s. To avoid potential positional biases, the sides of the test substance and the control were alternated.

VIDEO RECORDING AND DATA ANALYSIS

Stimulus preference was determined by the flies' distribution. To this end, we counted in every frame of our video recordings the number of flies in each region of interest of the arena (i.e., diagonal quadrants in the case of conditioning experiments and a half arena in the case of preference experiments). All videos in this study were recorded at one frame per second.

The number of flies was scored semi-automatically using a preset macro for ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA; **Figure 4**). First, the video was gray-scaled, the circular arena in the video frames was cropped, and the surrounding was cleared (**Figure 4A**). Each region of interest (see above) was outlined (**Figure 4A**) and flies located therein were counted using the "Analyze Particles" function after manually setting a threshold that separated the flies from the background and a size range that excluded non-fly particles (**Figures 4A,B**). The threshold was determined by eye while the size range was set according to a histogram of the particle area in the respective quadrant (**Figure 4B**). Flies touching a border of two compartments were excluded. The error rate of this automated counting procedure was calculated by comparing manual and machine based counting in 20 randomly selected quadrants. It ranged from two to nine percent depending on the camera used.

PREFERENCE AND LEARNING INDICES

Conditioned behavior was quantified based on the flies' preference for the CS in both reciprocal experiments (Tully and Quinn, 1985; Rescorla, 1988; Scherer et al., 2003). We first calculated a preference index for green (PI_G) for each time point as the number of flies on the Green quadrants (#Green) minus the number on the blue quadrants (#Blue) divided by the total number of flies counted. PI_G was calculated in both reciprocal experiments [i.e., Green+/Blue– (G+ B–) and Blue+/Green– (G– B+)]:

$$PI_G\ (G+\ B-) = (\#Green - \#Blue)/\#Total$$

$$PI_G\ (G-\ B+) = (\#Green - \#Blue)/\#Total$$

PI_G values can thus range from –1 to 1. Positive values indicate that more flies prefer the "green" quadrants whereas negative values indicate that more flies prefer the "blue" quadrants. To quantify the flies' preference/avoidance for a chemical substance, a PI was calculated and analyzed in the same way: the difference of the number of flies in the two halves was divided by the total number of flies.

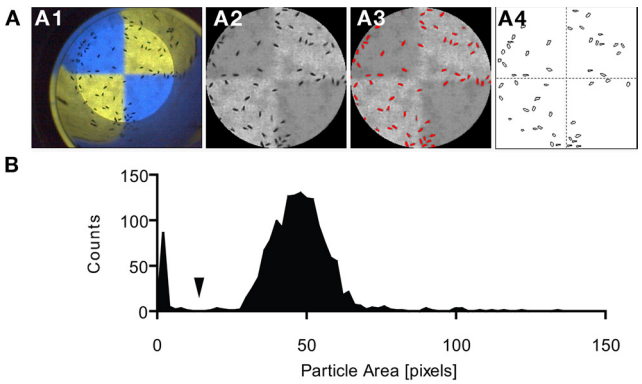


FIGURE 4 | Semi-automatic fly counting. Using ImageJ, flies in every region of interest (e.g., quadrant) were counted in every frame (automated using a macro). **(A)** The raw image **(A1)** was trimmed by manually selecting the test arena, and the image was gray-scaled **(A2)**. A threshold was set to separate the flies from the background (**A3**; recognized objects marked in red). The "Analyze Particles" function was used to count the objects in each quadrant and frame **(A4)**. **(B)** Histogram of the particle area distribution on a quadrant of one test video. Particles smaller than a certain size (arrow) were regarded as non-fly particles and excluded from counting.

For single-fly conditioning, the difference between the number of frames where a given fly was on the Green and Blue quadrants was taken as a preference.

An LI was calculated by subtracting PI_G values of the two reciprocally trained groups and by dividing the resulting value by 2:

$$LI = [PI_G\ (G+\ B-) - PI_G\ (G-\ B+)]/2$$

Like a preference index, LI can range from −1 to 1. A positive LI indicates conditioned approach, whereas a negative LI indicates conditioned avoidance. If flies do not show associative memory in the test, the LI would become 0.

The LI was calculated in each frame of a recorded video. For bar graph presentation and statistical comparisons of different groups, all single LIs in the entire test phase (1–90 s) were averaged.

STATISTICS

The significance level of all statistical tests was set to 5%. All groups were first tested for normal distribution with the Shapiro–Wilk test with Bonferroni correction and for homogeneity of variance with Bartlett's test or *F*-test. As in no case our data significantly violated the assumption of the normal distribution, parametric comparisons [i.e., one-sample *t*-test with Bonferroni correction, Student's and Welch's *t*-tests, one-way ANOVA followed by planned pair-wise comparisons (Bonferroni)] were applied as specified in the figure legends. All statistical calculations were performed using the software Prism 5 (GraphPad, San Diego, CA, USA).

RESULTS

APPETITIVE VISUAL LEARNING

Repetition of training and order of reinforcement

First, we examined the effect of training repetition. Twenty-four hour starved flies were subjected to 0, 1, 2, 4 or 8 cycles of a training trial. For the group with no training trial, flies were randomly assigned to the groups "green rewarded" or "blue rewarded."

In the test phase, flies having undergone four trials of a sucrose reward in the presence of green light showed a higher preference for the green stimulus than the preference for blue exhibited by the reciprocal group rewarded on blue light (**Figure 5A**). Flies without training (naïve flies) showed a significant preference for the green light ($P < 0.001$). This might explain the shift of preference toward green after training (**Figure 5A**). By analyzing the time course of memory performance (i.e., differences in the preferences), we found that the choice of all trained groups reached an asymptote within 20 s (**Figure 5B**).

All the trained groups showed a significant conditioned approach to the rewarded stimulus ($P < 0.01$) while no significant associative memory was detected in the group without training ($P > 0.05$; **Figures 5B,C**). The performance of the trained groups was significantly better than that of the group without training ($P < 0.01$; **Figure 5C**). Comparison among the groups with training (one to eight trials) revealed a significant difference ($P < 0.05$). The memory tended to increase with training repetition ($P < 0.05$; comparison of groups trained with two and four trials; **Figure 5C**).

In order to analyze potential non-associative effects of reinforcement order (Tully and Quinn, 1985; Kim et al., 2007a), we discriminated LIs according to whether sugar was delivered with the first or second CS in each trial (i.e., CS+/CS− vs. CS−/CS+, respectively; **Figure 5D**). We compared the performance of these two groups and found that the order of reinforcement did not significantly affect memory ($P > 0.05$, **Figure 5D** for the groups with four-cycle training trial; data not shown for the groups with one-, two-, and eight-cycle trials). As the intervals between the last US presentation and the test are different in these two groups, the periodical stimulus presentation protocol did not significantly modulate visual memory. Since such a non-associative effect might however become evident only in some mutants or under certain experimental conditions (Acevedo et al., 2007), we randomized the order of reinforcement in all groups of this study. In the following experiments four-cycle training was used since immediate memory reached an asymptote (**Figure 5C**).

Different inter-CS intervals

In our associative training, we alternately presented two color stimuli. To examine the role of the interval between the two colored cues, we trained flies with different ICSIs (10, 30 or 90 s, **Figure 6A**). All other training and test conditions were kept constant, while the total length of each training protocol differed accordingly. All groups displayed significant conditioned behavior (**Figure 6A**) and their performances did not significantly differ from each other ($P > 0.05$). Therefore, the ICSI of 10 s was adopted for further experiments.

Starvation period

Appetitive learning requires appropriate appetitive motivation, which can be varied through starvation of flies (Tempel et al., 1983; Krashes and Waddell, 2008; Colomb et al., 2009). The resistance to starvation is controlled by many physiological factors and appears to fluctuate significantly. All results described above were obtained from flies starved for 24 h. This starvation period yielded a level of mortality around 20%. In order to measure the effect of the starvation period on visual learning, three groups of flies were starved for

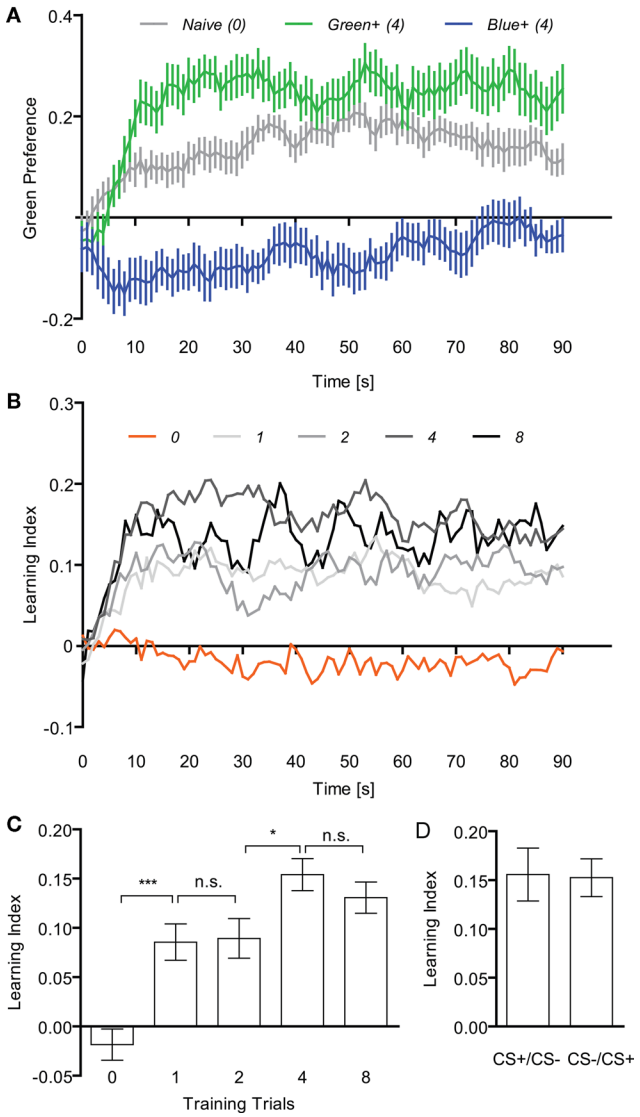


FIGURE 5 | Appetitive visual associative memory. (A) Time course of the mean preference for the green stimulus of naïve flies (no training) and flies that received four training trials [two reciprocal groups: Green+/Blue– (green) and Blue+/Green– (blue). Flies that received a sugar reward with green light showed a higher preference for the green stimulus during the test phase than the reciprocal group having received the same reward with blue light. **(B)** Time course of the mean LIs of flies with (1, 2, 4 or 8) or without (0) training trials. **(C)** Effect of training repetition. Without training no significant memory could be found [one-sample *t*-test, $t_{(13)} = 1.159$, $P > 0.05$], whereas all trained groups showed significant memory [one-sample *t*-test, one trial: $t_{(17)} = 4.632$, $P < 0.01$; two trials: $t_{(19)} = 4.446$, $P < 0.01$; four trials: $t_{(19)} = 9.490$, $P < 0.001$; eight trials: $t_{(17)} = 8.242$, $P < 0.001$]. Comparison among the groups with training (one to eight trials) revealed significant difference [one-way ANOVA, $F_{(3, 72)} = 3.512$, $P < 0.05$]. The memory tended to increase with training repetition [e.g., comparison of groups trained with two and four trials: *t*-test, $t_{(38)} = 2.632$, $P < 0.05$]. $n = 18$–20. **(D)** Effect of the order of CS presentation (four trials). The same data as in C were sorted to CS+/CS– and CS–/CS+ and reanalyzed. Either the first or second visual stimulus of each training trial was paired with the US. No significant difference was found between both groups [*t*-test, $t_{(18)} = 0.096$, $P > 0.05$, $n = 10$]. In all the diagrams, bars (points) and error bars indicate means and the standard error of the mean, respectively. Asterisks indicate statistical significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

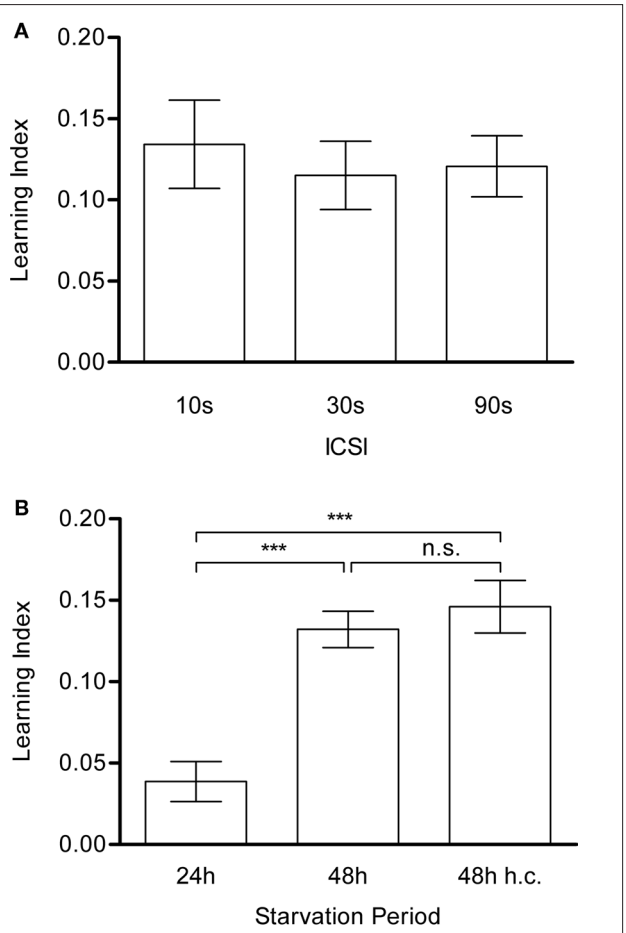


FIGURE 6 | Inter-CS interval and starvation-dependency of visual appetitive memory. (A) The effect of duration of the inter-CS interval (ICSI). The interval lasted 10, 30 or 90 s. All groups showed significant memory [one-sample *t*-test, 10 s: $t_{(19)} = 4.925$, $P < 0.001$; 30 s: $t_{(19)} = 5.476$, $P < 0.001$; 90 s: $t_{(17)} = 6.394$, $P < 0.001$] while no significant difference could be found among all groups [one-way ANOVA, $F_{(2,55)} = 0.1897$, $P > 0.05$]. $n = 18$–20. **(B)** The effect of starvation periods. Flies were starved for 24, 48 or 48 h at high humidity conditions (h.c.). All groups showed significant memory [one-sample *t*-test, 24 h: $t_{(17)} = 3.154$, $P < 0.05$; 48 h: $t_{(15)} = 11.87$, $P < 0.001$; 48 h h.c.: $t_{(12)} = 9.022$, $P < 0.001$], while longer starvation resulted in a significantly higher performance than short starvation [*t*-test, $t_{(32)} = 5.288$, $P < 0.001$]. Different humidity conditions had no significant effect on the conditioned approach [*t*-test, $t_{(27)} = 0.7242$, $P > 0.05$]. $n = 13$–16.

two different periods. The first group was starved for 24 h as in the previous experiments. However, almost no dead flies were found in the starvation vials at the time point of experiments, suggesting that resistance to starvation may change over time. The second and third group were starved for 48 h and yielded a mortality rate that was similar to that of the previous experiments (see **Figures 5 and 6**). To prevent potential water deprivation caused by long starvation, the third group was kept in a humidified box.

Although all three groups acquired a significant memory ($P < 0.05$; **Figure 6B**), the performance of the 24h-starved flies was significantly lower than that of the flies starved for 48 h ($P < 0.001$; **Figure 6B**). The high humidity during the starvation did not cause a significant difference ($P > 0.05$; **Figure 6B**). Thus, these results indicate that the expression of visual appetitive memory varies

with the amount of starvation, and that this variation is not due to water deprivation. As the performance of 48 h-starved flies was comparable to that of flies in the previous experiments with 24 h-starvation (see **Figures 5 and 6A**), the calibration of a starvation period might be necessary to stably assess appetitive memory.

Single-fly conditioning

Typically, 50–100 flies were collectively trained and tested. However, this *en masse* conditioning might not reflect single fly behavior, as the choice of individual flies could be influenced by other flies (Chabaud et al., 2009). Therefore, we examined whether the performance of flies that were trained and tested in a group was different from that of flies that were trained and tested individually. We compared the LIs obtained after single-fly conditioning and after *en masse* conditioning. The former is based on the time spent on the CS+ and CS–, whereas the latter results from the differential distribution of flies. LIs of flies that underwent training and test in a group (Group) or individually (Individual) did not differ significantly from each other ($P > 0.05$) despite presenting significantly different variances ($P < 0.001$; **Figure 7**). This suggests that the choice of individual flies in our previous tests was not affected by the *en masse* protocol.

Memory retention

To address the stability of appetitive visual memories, flies were tested 5 min, 1, 3 or 6 h after receiving four training trials. After the training, flies were removed from the arena and kept in a vial during the respective retention interval. The memory decayed to ca. 50% within 3 h (**Figure 8**). It diminished after 6 h (**Figure 8**).

AVERSIVE VISUAL LEARNING

Aversive chemical substances

To find a chemical that might function as an aversive reinforcer for visual conditioning, we examined the flies' avoidance when confronted with different substances. We chose three different acids, one inorganic (phosphoric acid) and two organic ones (formic acid and acetic acid). Acetic acid was previously shown to be aversive to flies (Joseph et al., 2009). Additionally, we measured avoidance of sodium chloride and quinine as they were previously shown to be potent aversive reinforcers in fly conditioning (Quinn et al., 1974; Gerber and Hendel, 2006).

For the measurement of avoidance, the apparatus used for appetitive conditioning was slightly modified. The filter paper on the arena ground was split in two halves to create a choice situation: one half contained the substance to be tested and the other half the solvent used to dilute the substance (i.e., water). As in the previous quantification of aversive memory, a preference index was determined based on the distribution of the flies on the two halves.

Both organic acids, formic acid and acetic acid, strongly repelled flies at a concentration of 1 M ($P < 0.001$, **Figure 9A**). Lower concentrations barely evoked avoidance (**Figure 9A**). Interestingly, we found that phosphoric acid did not repel flies at any tested concentration although it was more acidic than acetic acid or formic acid at 1 M ($P > 0.05$, **Figure 9A**). Similarly, sodium chloride and quinine (both at the maximum soluble concentrations; 6 and 0.1 M, respectively) did not evoke significant avoidance in our setup ($P > 0.05$, **Figure 9B**). Thus, we used acetic acid and formic acid as potential aversive reinforcers in our conditioning experiments.

Aversive visual learning

To allow comparison between aversive and appetitive conditioning, we applied the same training protocol as for appetitive learning except for the use of different reinforcing substances. Flies were starved and trained with four conditioning trials in the same apparatus. No significant visual memory could be detected with 1 M formic acid or 1 M acetic acid when the test arena did not contain the corresponding acid ($P > 0.05$, **Figure 10**). In *Drosophila* larvae, aversive olfactory memory can be expressed only in the presence of an aversive stimulus presented during the test situation (Gerber and Hendel, 2006). Thus, flies could have established an aversive visual memory but the absence of aversive reinforcer during the test might have prevented revealing such a memory. Therefore,

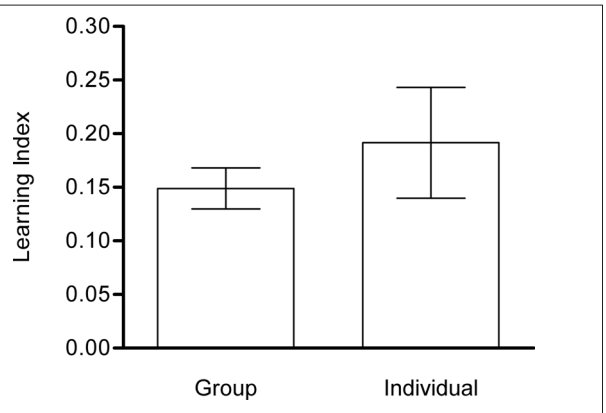


FIGURE 7 | Single-fly vs. *en masse* conditioning. Learning indices of flies that undergo training and test in a group (Group) or individually (Individual) did not differ significantly from each other [Welch's *t*-test, $t_{(27)} = 0.7742$, $P > 0.05$] despite significantly different variances [*F*-test, $F_{(22,16)} = 0.859$, $P < 0.001$). Both groups showed significant memory [one-sample *t*-test, Group: $t_{(16)} = 7.802$, $P < 0.001$; Individual: $t_{(22)} = 3.716$, $P < 0.01$]. Note that the learning index of single-fly conditioning is based on the time spent on CS+ and CS–, whereas memory of *en masse* conditioning is measured with the differential distribution of flies. $n = 17$ and 23.

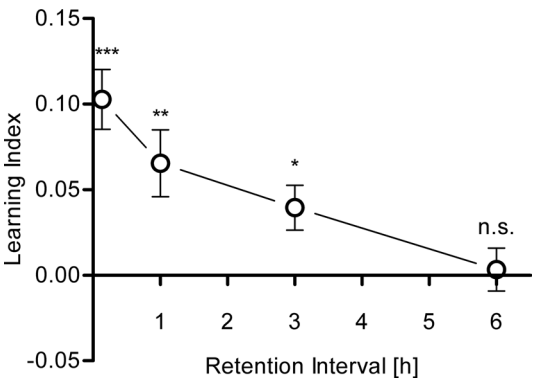


FIGURE 8 | Memory retention. Learning indices of flies that were trained with four training trials and tested after 5 min., 1, 3 and 6 h. Significant memory was found up to 3 h after training [one-sample *t*-test, 5 min: $t_{(34)} = 5.891$, $P < 0.001$; 1 h: $t_{(34)} = 3.358$, $P < 0.01$; 3 h: $t_{(29)} = 3.008$, $P < 0.05$]. After 6 h, no significant memory was detected anymore [one-sample *t*-test, $t_{(15)} = 0.2622$, $P > 0.05$]. $n = 16$–35.

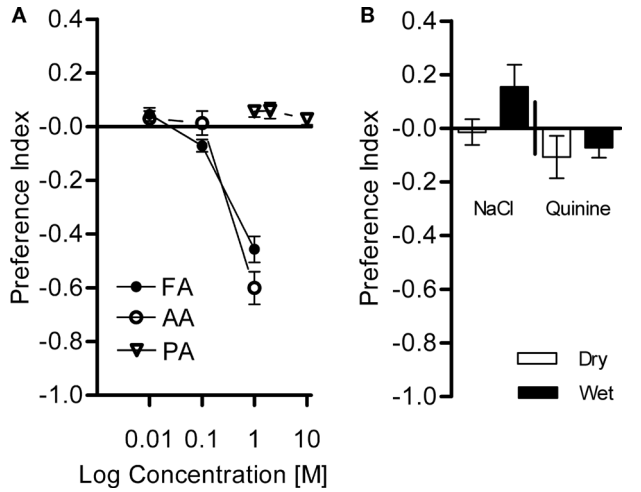


FIGURE 9 | Avoidance of diverse chemical substances. Choices between different chemical solutions and the control (water) were given to naïve flies. **(A)** Acid avoidance. Flies were tested with formic acid (FA), acetic acid (AA) at 0.01–1 M, and phosphoric acid (PA) at 1–10 M. Strong avoidance was found for FA and AA at 1 M [one-sample *t*-test, AA 1 M: $t_{(7)} = 16.73$, $P < 0.001$; FA 1 M: $t_{(7)} = 15.13$, $P < 0.001$], whereas moderate avoidance, if at all, was observed at lower concentrations [one-sample *t*-test, AA 0.01 M: $t_{(7)} = 1.134$, $P > 0.05$; AA 0.1 M: $t_{(7)} = 4.787$, $P < 0.05$; FA 0.01 M: $t_{(7)} = 0.2669$, $P > 0.05$; FA 0.1 M: $t_{(7)} = 1.358$, $P > 0.05$]. PA did not evoke a significant avoidance at any of the tested concentrations [one-sample *t*-test, PA 1 M: $t_{(7)} = 1.356$, $P > 0.05$; PA 2 M: $t_{(7)} = 0.2009$, $P > 0.05$; PA 10 M: $t_{(7)} = 0.5641$, $P > 0.05$]. $n = 8$. **(B)** Avoidance of NaCl (6 M) and quinine (0.1 M). Both substances were assayed with dry or wet filter paper. No avoidance of the flies to these substances at any condition was found [one-sample *t*-test, NaCl dry: $t_{(9)} = 0.3021$, $P > 0.05$; NaCl wet: $t_{(9)} = 1.872$, $P > 0.05$; quinine dry: $t_{(9)} = 1.353$, $P > 0.05$; quinine wet: $t_{(7)} = 1.959$, $P > 0.05$]. $n = 10$.

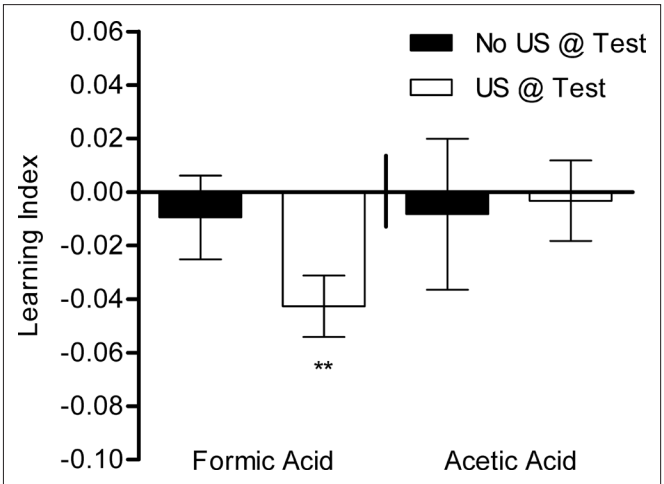


FIGURE 10 | Aversive visual associative memory. Memories of flies that were trained with formic acid or acetic acid instead of sugar. The same training protocol was applied as for appetitive conditioning. Conditioned avoidance was tested in the presence or absence of the respective reinforcer (white or black bars, respectively). Significant memory was only found with formic acid when formic acid was present during the test [one-sample *t*-test, $t_{(39)} = 3.714$, $P < 0.01$]. No significant memory could be detected without a reinforcer in the test or using acetic acid as reinforcer [one-sample *t*-test, FA No US @Test: $t_{(39)} = 0.6058$, $P > 0.05$; AA US @Test: $t_{(19)} = 0.2170$, $P > 0.05$; AA No US @ Test: $t_{(19)} = 0.2937$, $P > 0.05$]. $n = 20$–40.

we additionally measured the response of flies in a test situation in which the corresponding US was made available. Flies showed a small, yet significant, memory in the case of formic acid ($P < 0.01$), but not acetic acid (**Figure 10**). These results suggest that formic acid can act as an aversive reinforcer and that visual memories can be expressed if formic acid is presented in the test. Although acetic acid induced avoidance in a similar way as formic acid, it did not act as a reinforcer under the examined conditions.

DISCUSSION

A NEW VERSATILE VISUAL CONDITIONING PARADIGM

We successfully established a new behavioral paradigm for visual classical conditioning in adult *Drosophila*. Paired presentation of an appetitive or aversive chemical (US) and a visual stimulus (green or blue light; CS) significantly increased flies' preference/avoidance for the conditioned visual stimulus (**Figures 5A and 10**). All groups of flies were exposed to the same amount of CS and US; the only difference between two reciprocally trained groups was the CS–US contiguity. Thus, this conditioning design excludes the contribution of non-associative effects to the LI (Rescorla, 1988). We demonstrated that appetitive and aversive visual memories of the fly can be measured in the same setup (**Figures 1, 5, and 10**). This assay may therefore be applied to explore molecular and cellular mechanisms that dissociate appetitive and aversive visual memories using *Drosophila* genetics. Such comparison has been previously done by combining a similarly versatile behavioral protocol and pharmacology in the cricket (Unoki et al., 2006; Nakatani et al., 2009). We also showed that memories of single-fly and *en masse* conditioning are not significantly different (**Figure 7**), so that we suppose that the behavior analyzed in the *en masse* assay reflects individual behavior, at least in the case of immediate appetitive memory (**Figure 7**). Collective behavior might become influential under some experimental condition, because a recent report on aversive olfactory conditioning revealed that a particular memory component is selectively affected in adult flies (Chabaud et al., 2009).

With a fixed time period of starvation, scores of appetitive memory seemed to fluctuate over a long time range (e.g., season). This fluctuation can be calibrated by varying the starvation period according to the mortality rate, implying that feeding motivation is correlated with resistance to starvation (Shiraiwa and Carlson, 2007) (**Figure 6B**). Thus, visual memory in our setup is independent from starvation resistance. This also suggests that a fixed time period of starvation may lead to different levels of feeding motivation, thus implying that starvation has to be calibrated in order to compare appetitive memories of different genotypes (Thum et al., 2007; Shiraiwa and Carlson, 2007).

Appetitive visual memories in our assay were retained for several hours (**Figure 8**). Although this performance is rather short compared to memory duration found in appetitive olfactory memory lasting more than 24 h (Krashes and Waddell, 2008; Colomb et al., 2009), it might still allow the analysis of different memory components (see Quinn and Dudai, 1976; Honjo and Furukubo-Tokunaga, 2009). Furthermore, by optimizing

training conditions (e.g., application of spaced training or more repetitions), our protocol might be improved to allow the study of longer-lasting memories.

Furthermore, we demonstrated that this setup can be adapted to measure preferences for chemical and visual stimuli (**Figure 9**). It is also applicable to the analysis of many different kinds of behaviors that involve freely moving flies, such as circadian rhythm. Altogether, this new behavioral paradigm can become a significant alternative to hitherto established visual learning assays, as it is simple to set up and enables rapid and reproducible data acquisition.

In contrast to appetitive memory with sucrose, aversive memory was marginal with formic acid and not detectable with acetic acid, although they both induced similarly robust avoidance (**Figures 9 and 10**). Such differential processing of acetic acid in reflexive (unconditioned) and conditioned avoidance is similar to findings in *Drosophila* larvae (Schipanski et al., 2008). Aversive memory of formic acid was expressed only if it was presented during the test (**Figure 10**). This result corroborates conclusions and the rationale of a previous study using *Drosophila* larvae (Gerber and Hendel, 2006). Larvae only exhibited aversive memory if an aversive US was present in the memory test. It can be argued that in our case, the presentation of formic acid together with a previously not reinforced CS may induce a new aversive association that counteracts conditioned avoidance of the previous CS+. This is, however, an unlikely scenario in our experiments, because initial conditioned avoidance was maintained in the entire test period (90 s) without significant decrease (data not shown).

Since chemical stimulation of the tarsi or antennae was shown to signal less potent sugar reinforcement than the proboscis stimulation (Wright et al., 2007; de Brito Sanchez et al., 2008) and formic acid is unlikely to induce proboscis extension, weak aversive memory might be due to little contribution of the proboscis. Presentation of formic acid as an olfactory stimulus or testing flies with the defective olfactory system (e.g., *Or83b*$^{-}$) may clarify the contribution of olfaction to formic acid perception. In any event, improvements of the protocol and/or US are therefore important for examining aversive memory in future.

COMPARISON WITH OTHER VISUAL LEARNING ASSAYS

Our assay has many similarities with the visual aversive learning paradigm developed by Menne and Spatz, where colored illumination of a vial was paired with vigorous shaking as aversive reinforcement (Menne and Spatz, 1977). Similar to our setup, the entire arena was illuminated, and freely moving flies were handled as a group (Menne and Spatz, 1977). Since they also applied differential conditioning and a discrimination task, this paradigm was successfully used in studies of color vision in *Drosophila* (Menne and Spatz, 1977; Bicker and Reichert, 1978; Hernández de Salomon and Spatz, 1983). Later, Gerber et al. (2004) established a behavioral paradigm for appetitive visual memory in larvae by illuminating an entire arena from the bottom. Encouraged by these successful precedents, we employed illumination of the entire arena for CS presentation (**Figure 1**). The application of a computer screen and a mechanism to keep flies on the bottom of the arena added versatility in CS and US presentation (**Figure 1**).

Visual learning in adult flies has been extensively studied using the so-called flight simulator (Wolf and Heisenberg, 1991). In this computer-controlled setup, a fruit fly flying stationary in the middle of a cylindrical arena learns that flying toward certain directions (i.e., quadrants) of the arena is permitted while flying toward other directions is punished by means of a heat beam. Different landmarks displayed on the wall of the arena signalize the safe and dangerous quadrants. Visual memory formed in the flight simulator is reproducibly robust (Heisenberg et al., 2001). In particular, presentation of complex visual objects as landmarks is possible (Liu et al., 2006). Our assay has two major differences compared to the flight simulator. First, the behavioral apparatus is much easier to set up and maintain, and involves less customized mechanics and electronics. Second, the demands for flies are less in our assay. In the flight simulator, the tethered fly needs to keep flying at least for several minutes in order to be trained and tested. Consequently, mutants with defective wings or flight cannot be examined in this apparatus (Brembs et al., 2007). Since the threshold of walking appears to be lower than that of flight in *Drosophila*, a broader range of mutants may be examined using our novel paradigm.

FURTHER POSSIBILITIES

We constructed a setup where various behaviors of unconstrained flies can be recorded (**Figure 1**). We designed our conditioning paradigm in order to make it comparable to that commonly used in olfactory learning in many respects: the conditioning protocol, the appetitive US (sucrose) and its presentation with filter paper (Schwaerzel et al., 2003). Thus, appetitive memories of different sensory modalities can be directly compared. In several cases including ours, the same US application seems to be more effective on olfactory memory than visual memory (Hori et al., 2006; Krashes and Waddell, 2008; Colomb et al., 2009) (**Figure 8**).

The application of an LCD monitor allows generating a variety of visual stimuli. This opens a possibility for studying visual perception beyond associative memory *per se*. For example, the setup might be useful for studying color discrimination (intensity-independent spectral discrimination of light). Indeed, several seminal reports exploited visual associative learning to show the existence of such color vision in *Drosophila* and its psychophysical characteristics (Menne and Spatz, 1977; Bicker and Reichert, 1978; Hernández de Salomon and Spatz, 1983). Combined with a wide range of genetic techniques and resources, our setup may serve as a model to study the molecular and cellular mechanisms of insect vision.

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Learning at old age: a study on winter bees

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Ageing is often accompanied by a decline in learning and memory abilities across the animal kingdom. Understanding age-related changes in cognitive abilities is therefore a major goal of current research. The honey bee is emerging as a novel model organism for age-related changes in brain function, because learning and memory can easily be studied in bees under controlled laboratory conditions. In addition, genetically similar workers naturally display life expectancies from 6 weeks (summer bees) to 6 months (winter bees). We studied whether in honey bees, extreme longevity leads to a decline in cognitive functions. Six-month-old winter bees were conditioned either to odours or to tactile stimuli. Afterwards, long-term memory and discrimination abilities were analysed. Winter bees were kept under different conditions (flight/no flight opportunity) to test for effects of foraging activity on learning performance. Despite their extreme age, winter bees did not display an age-related decline in learning or discrimination abilities, but had a slightly impaired olfactory long-term memory. The opportunity to forage indoors led to a slight decrease in learning performance. This suggests that in honey bees, unlike in most other animals, age *per se* does not impair associative learning. Future research will show which mechanisms protect winter bees from age-related deficits in learning.

Keywords: olfactory learning, tactile learning, honey bee, longevity, ageing

INTRODUCTION

In the Western world, the human population grows continually older. A side-effect of the aging population is an increase in age-related diseases such as Alzheimer's disease and Parkinson's disease, which ultimately result in a decline of cognitive functions. This makes the study of age-related changes in cognitive functions a major goal of this century.

Over the last years, we have gained important insight into ageing processes of the brain through a number of vertebrate and invertebrate models. Most experiments on learning at old age have been conducted in organisms like the fruit fly *Drosophila melanogaster* (Tamura et al., 2003; Grotewiel et al., 2005), the nematode *Caenorhabditis elegans* (Murakami and Murakami, 2005) and the mouse *Mus musculus* (Gower and Lamberty, 1993; Unno et al., 2007). In these animals, ageing of the central nervous system is mostly related to the age of the individuals. Although the plasticity of ageing and cognitive decline is of major interest, we lack models in which ageing processes in the brain are decoupled from chronological age.

The honey bee is beginning to be recognized as a new model for studying the biology of aging, because it offers a number of advantages. Life span of honey bee workers can range between 6 weeks and more than 6 months. Summer workers usually live up to 6 weeks, whereas winter bees become 6 months old and older (Omholt and Amdam, 2004; Remolina and Hughes, 2008). A fascinating aspect of this plasticity is that all of these workers could have similar genotypes, because life span in honeybee workers is mostly regulated by environmental factors (Winston, 1987; Seeley, 1995). These characteristics make honey bees an excellent tool for testing candidate genes for ageing and regulation of lifespan (for review see Rueppell et al., 2004) as identified in other model organisms (Keller and Jemielity, 2006).

Honey bee ageing is very plastic. After nursing the brood and the queen in the hive, a task which is accompanied by slow senescence, they forage outside the hive for pollen, nectar, water and propolis, which leads to rapid senescence (for review see Amdam and Page, 2005). But foragers are capable of reverting to nursing tasks if the nurse bees have been removed from a colony, and nurse bees can be induced to forage precociously (Robinson et al., 1989, 1992; Huang and Robinson, 1996; Behrends et al., 2007). Whereas the former procedure increases life span, the later reduces life span, because the foraging duration of a honey bee is more or less fixed. Therefore, bees initiating foraging early in life will live shorter, while individuals starting to forage late will live longer (Guzmán-Novoa et al., 1994; Tofilski, 2000; Page and Peng, 2001; Rueppell et al., 2008, 2009).

Another advantage of honey bees is their unique behavioural repertoire including tactile, olfactory and visual learning which can be studied under controlled laboratory conditions and in the field (for review see Menzel and Müller, 1996; Giurfa, 2007; Srinivasan, 2010). This allows a detailed analysis of brain functions at all ages. Taken together, these characteristics make the bee particularly interesting for the study of age-related changes in brain functions and their underlying mechanisms.

Winter bees are workers that emerge shortly before the end of the foraging period and survive up to 6 months and longer (for review see Remolina and Hughes, 2008). They stay in a cluster inside the hive and engage in thermoregulation and queen-care until the start of the next foraging period. Winter bees do not hibernate but stay active in the hive throughout the winter time. Therefore, winter bees or *diutinus* bees provide a unique life form, since their physiological properties closely resemble those of young summer hive bees, although their age and experience is extremely

different. Thus winter bees have well-developed hypopharyngeal glands and low titres of juvenile hormone (Fluri et al., 1982), which is a releaser of foraging behaviour at high concentrations (Schulz et al., 2002). In addition, winter bees have high titres of vitellogenin. This protein is an egg yolk precursor which is used for producing brood food and, at the same time, protects bees from oxidative cellular damage (Seehuus et al., 2006a). Finally, the transition from in-nest behaviour to foraging is delayed by several months in winter bees.

To analyse associative learning, different paradigms for conditioning under controlled laboratory conditions have been developed (Kuwabara, 1957; Bitterman et al., 1983; Menzel and Müller, 1996; Erber et al., 1997; Giurfa and Malun, 2004; Giurfa et al., 2009). We decided to study classical olfactory conditioning of the proboscis extension response (PER) and operant tactile conditioning (Bitterman et al., 1983; Erber et al., 1997). Classical olfactory conditioning might reveal deficits in the olfactory system commonly associated with old-age diseases like Alzheimer's disease (Wilson et al., 2009). Tactile learning, which requires more activity on part of the bee, was chosen to test for age-dependent deficits in the motor system, such as frequently found in patients with Parkinson's disease (Chung et al., 2003).

A number of tactile and olfactory conditioning experiments using summer bees maintained under different hive conditions have shown that chronological age has no effect on associative learning in older worker bees (Behrends et al., 2007; Rueppell et al., 2007; Scheiner and Amdam, 2009). In those experiments, social role determined learning performance rather than chronological age. However, bees in those experiments were much younger than winter bees, on average no older than 50 days.

Associative learning of the proboscis extension response is strongly determined by individual evaluation of the sucrose reward. Bees showing proboscis extension to stimulation of the antennae with low sucrose concentrations learn tactile and olfactory cues faster than bees with low responsiveness to sucrose (Scheiner et al., 1999, 2001a,b, 2003, 2005). To determine whether learning differences were solely a result of differences in responsiveness to sucrose, we separately tested for this parameter.

In our study, we analysed the associative learning, memory and discrimination abilities of winter bees aged between 160 and 180 days. We compared their learning performance to that of much younger summer bees performing different social tasks and to that of winter bees of an indoor flight room. The latter had the opportunity to forage for pollen, sucrose solution and water. We thus wanted to answer the question if the learning performance of outdoor winter bees is related to their inability to perform flights over the cold winter period.

MATERIALS AND METHODS

OUTDOOR WINTER BEES

Experiments were performed between winter 2006 and summer 2009 in Berlin. Our outdoor overwintering colony contained approximately 7,000 bees (*Apis mellifera carnica*). At the end of the foraging period, we marked 1,000 newly emerged bees at the thorax with paint (shellack mixed with colour pigments) and introduced them to this colony. This allowed us to identify their age the next spring. Bees were 160–180 days old when tested in the behavioural assays and presumably had never foraged, because we introduced the bees just before the temperatures dropped to below 8°C and collected the bees from the combs before temperatures allowed foraging activity the next spring. Most of the bees tested for learning abilities had full guts when we mounted them. Because bees can take cleansing flights in the winter period to defecate, this is a further indicator that the climatic conditions did not allow these bees to leave the hive in the winter before we collected them. The bees introduced into the overwintering colony stemmed from a naturally mated queen.

INDOOR WINTER BEES

At the end of the foraging period, we placed a small colony of about 3,000 bees in a flight room (5 m × 4 m) maintained at a 12:12 light/dark cycle at 28°C and 45% humidity. Bees could daily engage in flight and foraging activities on artificial pollen, water and sucrose feeders. For the behavioural assays we collected bees that were engaged in flight after presenting a pollen source near the hive entrance.

The indoor winter bees did not have the same genetic origin as the outdoor winter bees but also came from a naturally mated queen. Because we measured individual gustatory responsiveness prior to conditioning, we could nevertheless compare the learning performance of the two groups of bees. Gustatory responsiveness is the most important determinant of associative learning performance (for review see Scheiner et al., 2004) and bees of different patrilines (Scheiner and Arnold, 2010) and bees of highly different genetic strains (Scheiner et al., 2001a,b) only differed in their learning performance when the gustatory responsiveness of the patrilines or strains differed. A joint analysis of gustatory responsiveness and associative learning performance can thus explain whether possible learning differences are solely a result of differences in sensory responsiveness or whether they are "real" differences in the learning/memory system.

SUMMER BEES

To compare the learning performance of winter bees with that of summer bees, we collected nurse bees and foragers from a garden colony containing about 7,000 bees. Because bees have a high probability of being nurse bees when they are 6 days old, we only collected bees of this age group which were putting their heads in a cell with brood. Only bees with intact wings and furry coat on their thorax were regarded as nurse bees. Foragers were collected from the hive entrance after returning to the colony. We only used nectar foragers for this experiment. Foragers were not collected according to age, because (1) they were to represent a mixed sample of bees which is typically used in conditioning experiments and because (2) age has no effect on learning performance in foragers (Behrends et al., 2007; Scheiner and Amdam, 2009). The bees tested in this experiment stemmed from a naturally mated queen, which however, was different from that used for producing the winter bees.

PREPARATION OF BEES FOR CONDITIONING

After collecting the bees individually in glass vials, they were cooled in a refrigerator until they showed first signs of immobilization. Then they were mounted on metal tubes with a tape between

head and thorax and one tape on the abdomen to prevent stinging. Afterwards the bees rested for 1 h in a humidified chamber (Bitterman et al., 1983; Scheiner et al., 1999). For tactile learning, their complex eyes were occluded with black acrylic paint prior to resting to block visual input (Erber et al., 1997).

GUSTATORY RESPONSIVENESS

Before conditioning, we tested all bees for gustatory responsiveness using the proboscis extension response (PER). In time intervals of 2 min the bees were tested for PERs to a series of water and increasing sucrose concentrations presented to both antennae. We used the following concentrations of 0; 0.1; 0.3; 1.0; 3.0; 10 and 30% sucrose (weight/volume). During each stimulation we applied a droplet of 1 µl sucrose solution to the tips of the antennae and recorded if the bee displayed the PER. Afterwards, a gustatory response score (GRS) was calculated for each bee. It comprises the sum of all PERs during the assay. The GRS ranged between 0 and 7. The score of 7 indicates a highly responsive bee, a score of 0 implies no responsiveness to sucrose (Scheiner et al., 2004).

OLFACTORY CONDITIONING

After measuring gustatory responsiveness, bees with a GRS > 0 were tested for olfactory acquisition performance by conditioning the PER to carnation odour. Prior to the conditioning procedure the bees were tested for their spontaneous response to the conditioned odour carnation and the new odour stimulus cineole with an inter-trial interval of 5 min. Bees displaying spontaneous proboscis extension to either odour were discarded. For conditioning, a bee was placed in a constant air stream for 8 s and was stimulated with 5 ml of an odour/air mixture delivered by a 20-ml syringe (2 µl odour on a small piece of filter paper) in front of the bee.

The first conditioning trial started 5 min after the spontaneous response tests. This was done by eliciting the PER with a droplet of 30% sucrose solution while the bee experienced the carnation odour. The bee was allowed to drink about 1 µl of the sucrose solution as a reward. If the bee did not respond to the sucrose stimulus, it was discarded from further conditioning. If the bee responded with spontaneous PER to the odour stimulus in the first trial it was also discarded. At each of the six conditioning trials it was recorded whether the bee displayed a conditioned PER. An acquisition score was calculated which ranged from 0 to 5. It comprises the sum of all conditioned PERs. Five minutes after the conditioning procedure we tested the bees' responses to the new odour cineole and then the response to the conditioned odour carnation. The inter-trial interval was again 5 min. Tests for the new odour and for the conditioned odour were repeated 24 and 48 h after conditioning.

TACTILE CONDITIONING

Tactile conditioning was performed similarly to olfactory conditioning. Only bees with a GRS > 0 were used for conditioning. The tactile stimulus consisted of a 3 × 4 mm copper plate. The tactile pattern which was presented as the conditioned stimulus was engraved vertically and the pattern which was used as an new stimulus was engraved horizontally. Prior to the conditioning procedure, bees were tested for their spontaneous responses to the plate when it was placed in the scanning range of their antennae. The inter-trial interval was 5 min throughout the experiment. Six conditioning trials were performed. At each conditioning trial, the plate with the vertical pattern was brought into the scanning range of the bees' antennae with the help of a micromanipulator. While the bee scanned the surface of the plate we elicited the PER by applying a droplet of sucrose solution to the antennae of the bee. Once the bee extended its proboscis it was allowed to drink approximately 1 µl of sucrose solution. If the bee touched the plate with its proboscis, the plate was cleaned with 70% ethanol and subsequently with water. Testing conditioned responses without reward was performed 5 min after the last of the six conditioning trials. First, we tested the response to the new horizontal pattern and 5 min later the response to the conditioned vertical pattern. This test was repeated 24 and 48 h later to test for short-term, mid-term and long-term memory.

STATISTICS

For sucrose-concentration response curves, learning curves of olfactory and tactile conditioning and graphic displays of memory tests, percentages of conditioned PERs were calculated (PASW 18.0). Numbers of bees' responses in the discrimination and memory tests were compared using two-tailed Fisher Exact Probability Tests (GraphPad Instat 3). Acquisition scores are a measure of overall learning success. They represent the total number of conditioned responses during the acquisition phase (Scheiner et al., 1999). Gustatory response scores are a measure for gustatory responsiveness (Scheiner et al., 2004). Both types of scores were not distributed normally as revealed by Kolmogorov–Smirnov Tests. Therefore, we compared the respective scores between two groups using two-tailed Mann–Whitney *U* Tests. When more than two groups were compared, we performed nonparametric ANOVA (Kruskal–Wallis *H* Tests) and used Dunn's Test as *post hoc* test to compare pairs. All tests were two-tailed.

RESULTS

OLFACTORY ACQUISITION AND GUSTATORY RESPONSIVENESS OF OUTDOOR WINTER BEES AND SUMMER BEES

Despite their high chronological age, outdoor winter bees showed no impairment of associative olfactory learning and displayed a typical olfactory PER learning curve, similar to that of summer foragers or nurse bees (**Figure 1B**). To test whether outdoor winter bees differed significantly in their classical olfactory learning performance from summer bees, we compared the acquisition scores of winter bees with those of nurse bees and of summer foragers. Acquisition scores comprise the total number of conditioned responses during conditioning. Winter bees did not differ in their acquisition scores from summer nurse bees or from summer foragers (**Figure 1D**; $H = 3.90$, $p = 0.15$, $n_{\text{summer nurse bees}} = 35$, $n_{\text{summer foragers}} = 20$, $n_{\text{outdoor winter bees}} = 30$, Kruskal–Wallis *H* Test).

Because individual olfactory learning performance is strongly dependent on gustatory responsiveness (for review see Scheiner et al., 2004), we also compared this factor between the different groups. The sucrose-concentration response curve of winter bees was intermediate between that of summer nurse bees and that of summer foragers (**Figure 1A**). Gustatory response scores (GRS), which are a measure of gustatory responsiveness and were used for statistical comparison, differed significantly between the three

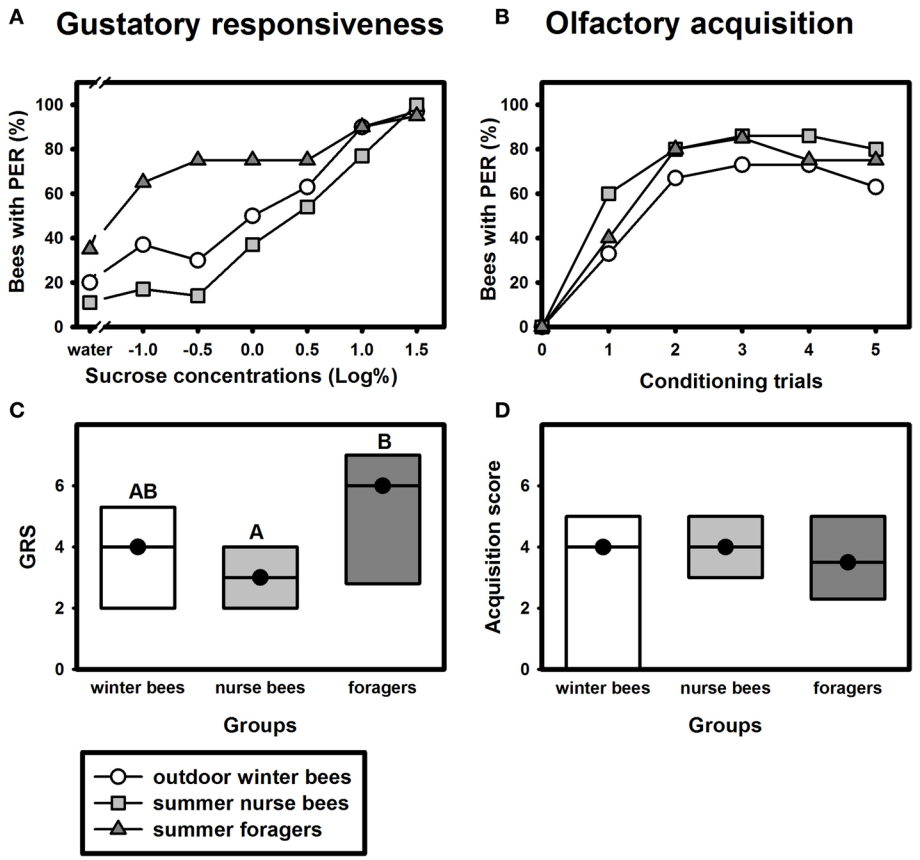

FIGURE 1 | Gustatory responsiveness and olfactory acquisition of outdoor winter bees, summer nurse bees and summer foragers. (A) Sucrose-concentration response curves of outdoor winter bees, summers nurse bees and summer foragers. The *x*-axis displays the sucrose concentration offered to the antennae of each bee. The *y*-axis shows the percentage of bees showing the proboscis extension response (PER). **(B)** Olfactory acquisition curves of outdoor winter bees, summer nurse bees and summer foragers. The *x*-axis displays the conditioning trials. The *y*-axis shows the percentage of bees showing conditioned PER. **(C)** Gustatory response scores (GRS) of outdoor winter bees, summer nurse bees and summer foragers. The *x*-axis displays the different groups. The *y*-axis shows median GRS (dots) and 25% (lower line) and 75% (upper line) quartiles. Groups with different letters differ significantly from each other. For statistics see text. **(D)** Olfactory acquisition scores of outdoor winter bees, summer nurse bees and summer foragers. The *x*-axis displays the different groups. The *y*-axis shows median acquisition scores (dots) and 25% (lower line) and 75% (upper line) quartiles. The groups did not differ from each other.

groups (**Figure 1C**; $H = 9.61$, $p \leq 0.01$, Kruskal–Wallis H Test). However, outdoor winter bees did not differ in their GRS from summer nurse bees ($p > 0.05$) or from summer foragers ($p > 0.05$). But foragers were significantly more responsive than nurse bees ($p \leq 0.01$, Dunn's Test).

OLFACTORY MEMORY AND DISCRIMINATION OF OUTDOOR WINTER BEES AND SUMMER BEES

After training, we tested how well the bees discriminated between the conditioned odour carnation and the new odour cineole at 5 min, 24 and 48 h after conditioning. Of the outdoor winter bees surviving the 48-h test, individuals responded significantly more often to the conditioned odour carnation than to the new odour cineole at all time points after conditioning and thus demonstrated significant discrimination (**Figure 2**; 5 min: $p \leq 0.001$, 24 h: $p \leq 0.05$, 48 h: $p \leq 0.01$, $n_{\text{outdoor winter bees}} = 27$, Fisher Exact Probability Test).

Of the nurse bees surviving the 48-h test, individuals responded significantly more often to the conditioned odour carnation than to the new odour cineole 5 min and 48 h after conditioning and thus displayed significant discrimination, but not 24 h after conditioning (**Figure 2**; 5 min: $p \leq 0.001$, 24 h: $p = 0.06$, 48 h: $p \leq 0.001$, $n_{\text{summer nurse bees}} = 29$, Fisher Exact Probability Test).

Five minutes and 24 h after conditioning, nurse bees did not differ from winter bees in their responses to the conditioned odour or to the new odour (5 min CS: $p = 0.19$, new odour: $p = 0.72$, 24 h CS: $p = 0.58$, new odour: $p = 0.16$). Interestingly, nurse bees responded significantly more often to the conditioned odour than winter bees 48 h after conditioning ($p \leq 0.05$), while not differing in their responses to the new odour ($p = 0.73$, Fisher Exact Probability Test). They apparently developed a better olfactory long-term memory than winter bees.

Foragers surviving the 48-h test displayed significant discrimination between the two odours only 48 h after conditioning (**Figure 2**; $p \leq 0.01$) but not 5 min ($p = 0.66$) or 24 h ($p = 0.37$) after conditioning. They did not differ from winter bees in their responses to the conditioned odour or to the new odour 5 min or 24 h after conditioning (5 min CS: $p = 1.00$, 5 min new odour: $p = 0.17$, 24 h CS: $p = 1.0$, 24 h new odour: $p = 0.68$, $n_{\text{summer foragers}} = 10$). But similar

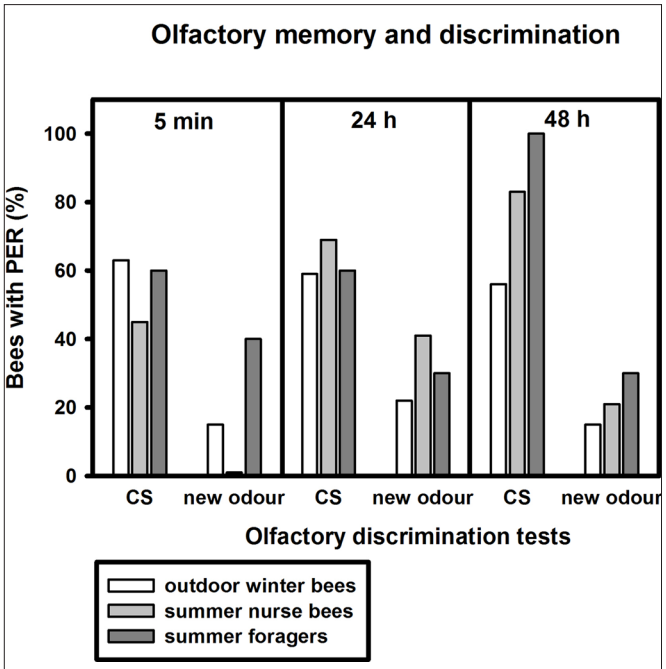

FIGURE 2 | Olfactory memory and discrimination of outdoor winter bees, summer nurse bees and summer foragers. The *x*-axis displays the tested stimuli (conditioned odour carnation (CS) or new odour cineole) at 5 min, 24 h or 48 h after conditioning. The *y*-axis shows the percentage of bees displaying the proboscis extension response (PER). For statistics see text. The same individuals as in **Figure 1** are displayed minus individuals that did not survive the 48-h test.

to nurse bees, foragers displayed a significantly stronger long-term memory 48 h after conditioning than winter bees ($p \leq 0.05$), while not differing in their responses to the new odour ($p = 0.36$, Fisher Exact Probability Test).

TACTILE ACQUISITION AND GUSTATORY RESPONSIVENESS OF OUTDOOR WINTER BEES AND SUMMER BEES

Similar to olfactory PER conditioning, winter bees showed a tactile learning curve which was comparable to that of summer nurse bees or summer foragers (**Figure 3B**). Tactile acquisition scores did not differ between outdoor winter bees and nurse bees or foragers which were tested in the summer (**Figure 3D**; $H = 3.01$, $p = 0.26$, $n_{\text{summer nurse bees}} = 34$, $n_{\text{summer foragers}} = 48$, $n_{\text{outdoor winter bees}} = 44$, Kruskal–Wallis *H* Test). These data show that winter bees in principle did not differ in their tactile acquisition performance from summer bees with different social roles.

Because individual tactile learning performance is strongly dependent on gustatory responsiveness (for review see Scheiner et al., 2004), we also compared this factor between the groups. The sucrose-concentration response curve of winter bees was very similar to that of summer foragers (**Figure 3A**). As before, gustatory response scores differed overall between the three groups (**Figure 3C**; $H = 6.15$, $p \leq 0.05$, Kruskal–Wallis *H* Test). However, winter bees did not differ significantly in their GRS from summer nurse bees ($p > 0.05$) or from summer foragers ($p > 0.05$). But summer foragers were significantly more responsive than summer nurse bees ($p \leq 0.05$, Dunn's Test).

TACTILE MEMORY AND DISCRIMINATION OF OUTDOOR WINTER BEES AND SUMMER BEES

After training, conditioned winter bees showed significant discrimination between the conditioned vertical pattern and a new horizontal pattern 5 min and 48 h after training, but not 24 h after conditioning (**Figure 4**; 5 min: $p \leq 0.05$, 24 h: $p = 0.13$, 48 h: $p \leq 0.01$, $n_{\text{winter bees}} = 44$, Fisher Exact Probability Test). In contrast to olfactory conditioning, the bees showed a significant decline in conditioned responses to the vertical pattern between 5 min and 24 h after training ($p \leq 0.05$, Fisher Exact Probability Test).

Of the bees surviving the 48-h memory tests, nurse bees displayed significant tactile discrimination 5 min and 24 h after conditioning (5 min: $p \leq 0.05$, 24 h: $p \leq 0.05$) but not 48 h after conditioning ($p = 1.00$). Winter bees did not differ from summer nurse bees in their responses to the conditioned pattern or to the new pattern at all test points after conditioning (**Figure 4**; 5 min CS: $p = 0.16$, new pattern: $p = 0.06$, 24 h CS: $p = 0.32$, new pattern: $p = 1.00$, 48 h CS: $p = 0.31$, new pattern: $p = 0.54$, $n_{\text{outdoor winter bees}} = 44$, $n_{\text{summer nurse bees}} = 29$, Fisher Exact Probability Test).

Similar to the nurse bees, surviving foragers displayed significant tactile discrimination 5 min and 24 h after conditioning (5 min: $p \leq 0.05$, 24 h: $p \leq 0.05$) but not 48 h after conditioning ($p = 0.49$). Foragers did not differ significantly from winter bees in their responses to the conditioned pattern at all time points after conditioning. But they showed the trend to respond more often to the conditioned pattern in the mid-term and long-term memory tests 24 h 48 h after conditioning than winter bees (**Figure 4**; 5 min: $p = 0.78$, 24 h: $p = 0.08$, 48 h: $p = 0.08$, $n_{\text{summer foragers}} = 17$). In addition, foragers responded less often to the new pattern 48 h after conditioning, while not differing from winter bees in their responses to the new pattern in the earlier tests (5 min: $p = 0.35$, 24 h: $p = 1.00$, 48 h: $p \leq 0.05$, Fisher Exact Probability Test).

OLFACTORY ACQUISITION AND GUSTATORY RESPONSIVENESS OF OUTDOOR WINTER BEES AND INDOOR WINTER BEES

Outdoor winter bees normally do not have the chance to perform flights during the winter time, because of cold temperatures outside. The learning performance of foragers, however, generally decreases with increasing foraging duration (Behrends et al., 2007; Scheiner and Amdam, 2009). In addition, foraging activity leads to decreased stress resistance and accumulation of brain damage (Seehuus et al., 2006b). We therefore wanted to test if the opportunity to fly in the winter time reduced the learning performance of winter bees.

Indoor winter bees had the opportunity to fly at room temperature and to collect pollen, sucrose solution or water during the winter period. They were placed in a room maintained at constant 22°C and a light/dark cycle of 12 h/12 h. Despite these conditions, we hardly observed any breeding activity in this colony or in similar colonies maintained over different winter periods.

Indoor winter bees displayed a similar olfactory acquisition curve to that of outdoor winter bees (**Figure 5B**). Their olfactory acquisition scores did not differ significantly from that of outdoor winter bees (**Figure 5D**; $Z = 1.18$, $p = 0.24$,

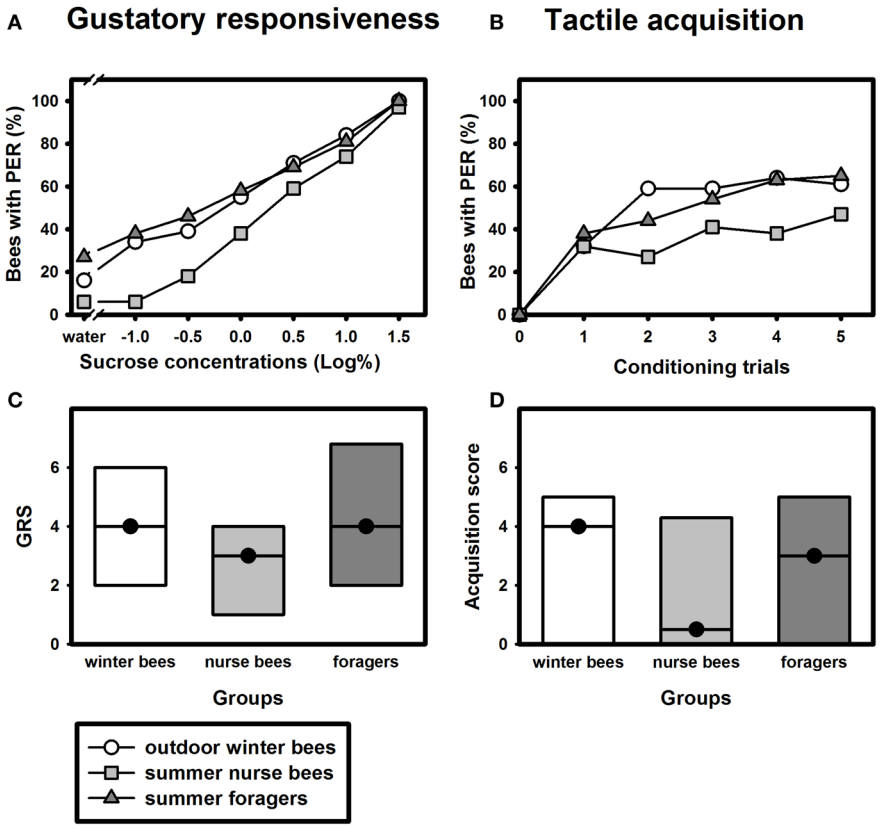

FIGURE 3 | Gustatory responsiveness and tactile acquisition of outdoor winter bees, summer nurse bees and summer foragers.
(A) Sucrose-concentration response curves of outdoor winter bees, summers nurse bees and summer foragers. The *x*-axis displays the sucrose concentration offered to the antennae of each bee. The *y*-axis shows the percentage of bees showing the proboscis extension response (PER). **(B)** Tactile acquisition curves of outdoor winter bees, summers nurse bees and summer foragers. The *x*-axis displays the conditioning trials. The *y*-axis shows the percentage of bees showing conditioned PER. **(C)** Gustatory response scores (GRS) of outdoor winter bees, summers nurse bees and summer foragers. The *x*-axis displays the different groups. The *y*-axis shows median GRS (dots) and 25% (lower line) and 75% (upper line) quartiles. **(D)** Tactile acquisition scores of outdoor winter bees, summers nurse bees and summer foragers. The *x*-axis displays the different groups. The *y*-axis shows median acquisition scores (dots) and 25% (lower line) and 75% (upper line) quartiles.

$n_{\text{indoor winter bees}} = 40$; $n_{\text{outdoor inter bees}} = 30$, Mann–Whitney *U* Test). However, indoor winter bees were more responsive to sucrose (**Figure 5A**), which was used as reward during conditioning. They had significantly higher gustatory response scores than respective outdoor winter bees (**Figure 5C**; $Z = 2.69$, $p \leq 0.01$, Mann–Whitney *U* Test).

As bees with higher GRS usually reach higher acquisition scores than bees with low GRS (Scheiner et al., 1999, 2001a,b, 2003), our data suggest that indoor winter bees displayed poorer acquisition than outdoor winter bees. This phenomenon becomes particularly apparent in bees with low gustatory responsiveness (GRS 1–2) or intermediate responsiveness (GRS 3–4). Despite equal responsiveness, outdoor winter bees in these GRS classes appeared to reach higher acquisition scores than respective indoor bees, although these differences were not statistically significant, probably due to the comparatively small sample sizes (data not shown). In individuals with high gustatory responsiveness (GRS classes 5–7), there was no such apparent difference in acquisition scores between indoor and outdoor winter bees (data not shown).

OLFACTORY MEMORY AND DISCRIMINATION OF OUTDOOR WINTER BEES AND INDOOR WINTER BEES

Of bees surviving the 48-h test, indoor winter bees demonstrated significant olfactory discrimination and long-term memory (**Figure 6**). Bees responded significantly more often to the conditioned odour carnation than to the new odour cineole 5 min ($p \leq 0.05$), 24 h ($p \leq 0.01$) and 48 h ($p \leq 0.01$, Fisher Exact Probability Test) after conditioning.

Outdoor winter bees did not differ from indoor winter bees in their responses to the conditioned odour or to the new odour at all test points after conditioning (**Figure 6**; 5 min CS: $p = 0.58$, new pattern: $p = 0.72$, 24 h CS: $p = 1.00$, new pattern: $p = 0.73$, 48 h CS: $p = 0.78$, new pattern: $p = 1.00$, $n_{\text{indoor winter bees}} = 24$, $n_{\text{outdoor winter bees}} = 24$; Fisher Exact Probability Test).

DISCUSSION

LEARNING OF OUTDOOR WINTER BEES

Our results demonstrate that winter bees show an average associative learning performance in classical olfactory conditioning (**Figure 1**) and operant tactile conditioning (**Figure 3**), despite their

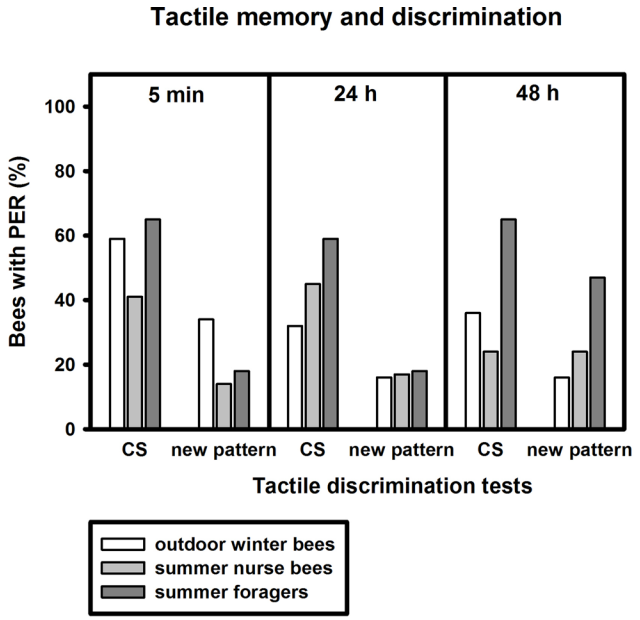

FIGURE 4 | Tactile memory and discrimination of outdoor winter bees, summer nurse bees and summer foragers. The *x*-axis displays the tested stimuli (conditioned vertical pattern (CS) or new horizontal pattern) at 5 min, 24 h or 48 h after conditioning. The *y*-axis shows the percentage of bees displaying the proboscis extension response (PER). For statistics see text. The same individuals as in **Figure 2** are displayed minus individuals that did not survive the 48-h test.

high chronological age. Tactile and olfactory acquisition scores of winter bees were very similar to those of summer foragers or summer nurse bees (**Figure 2** for olfactory learning and **Figure 4** for tactile learning), although the latter were much younger.

Naturally, learning performance of summer bees depends on a number of variables, like changes in nectar source profitability, in-hive conditions or changes in season (Scheiner et al., 2003). Therefore, the summer bees tested in our experiments might have differed from summer bees tested in another year or in another week of the same season. However, all of our earlier experiments demonstrate that individual gustatory responsiveness is *the* decisive determinant of associative learning performance in honey bees. Summer bees tested at different weeks of the foraging season, for example, do not differ in their learning performance, when they are equally responsive to gustatory stimuli tested with the proboscis extension response (Scheiner et al., 2003).

Our summer foragers and nurse bees did not differ from the tested winter bees in their gustatory response scores or in their associative learning performance. Therefore, our data imply that there are no fundamental differences in the relationship between sensory responsiveness and associative learning between winter and summer bees.

The learning abilities of outdoor winter honey bees are thus very different from a number of other insect species and vertebrates. In *Drosophila melanogaster*, for example, artificial selection for high life span led to low olfactory learning performance, implying a negative correlation between longevity and learning performance in this insect (Burger et al., 2008). In the American cockroach (*Periplaneta americana*), experiments on a vision-based learning paradigm showed a negative relationship between age and learning performance (Brown and Strausfeld, 2009). Old mice were deficient in learning a discrimination reversal and different complex maze tasks, while performing normally in a simple spatial discrimination task (Warren, 1986).

The excellent learning and memory performance of outdoor winter bees suggests that their brains do not develop behavioural signs of cognitive senescence as active foragers do in the summer time. The latter typically display reduced acquisition in tactile and olfactory learning after foraging for more than 2 weeks (Behrends et al., 2007; Scheiner and Amdam, 2009). This is further evidence that in honey bees, chronological age does not affect cognitive functions in the same way as in most other species. The function of the individual in the hive (reduced-activity state in the hive or active foraging activity outside the hive) appears to be the decisive factor determining how fast a bee shows signs of senescence.

By which mechanisms winter bees are protected from cognitive senescence, however, remains unclear. According to the oxidative stress hypothesis of Harman (1956), cumulative oxidative damage causes ageing and a reduction of lifespan, whereas protection from oxidative damage increases life span. It is therefore conceivable that winter bees are protected from oxidative damage in the brain, leading to longer life span and protection from cognitive senescence. In some organisms, cognitive senescence has been attributed to signs of oxidative stress in the brain. In *Caenorhabditis elegans*, mutants with elevated oxidative stress levels displayed an impaired learning performance (Murakami and Murakami, 2005). In mice, Forster et al. (1996) showed that loss of cognitive function at high age is associated with oxidative protein damage.

Therefore, winter bees should have a lower rate of reactive oxygen species production than summer bees. Alternatively, winter bees should be more able to repair oxidative damage in the brain or to eliminate reactive oxygen species or to reduce the number of reactive oxygen species than summer bees. Corona et al. (2005) measured mRNA levels for eight antioxidant genes and five genes encoding mitochondrial proteins involved in cellular respiration in short-lived summer workers and queens, which live up 5 years (for review see Remolina and Hughes, 2008). Interestingly, levels of antioxidant mRNA in workers increased with age or did not change, whereas they decreased in queens. Therefore, differences in oxidative stress resistance alone seem unlikely to explain the longevity differences observed in honey bees, because summer foragers live much shorter than winter bees and queens.

Another study on oxidative stress comes from Seehuus et al. (2006b). The authors measured levels of oxidative carbonylation in the brain. They demonstrate that foragers have higher levels of oxidative carbonylation than nurse bees and that winter bees have lower levels of oxidative carbonylation than summer foragers. Therefore, oxidative stress tolerance could be one factor leading to exquisite acquisition and memory in winter bees, but other factors are certainly also involved.

One candidate for protecting winter bees from oxidative damage in the brain is vitellogenin, because winter bees have similar levels of vitellogenin as nurse bees (Fluri et al., 1982). Vitellogenin is an egg yolk precursor which is accumulated in nurse bees, because it is normally required for producing brood food. In addition,

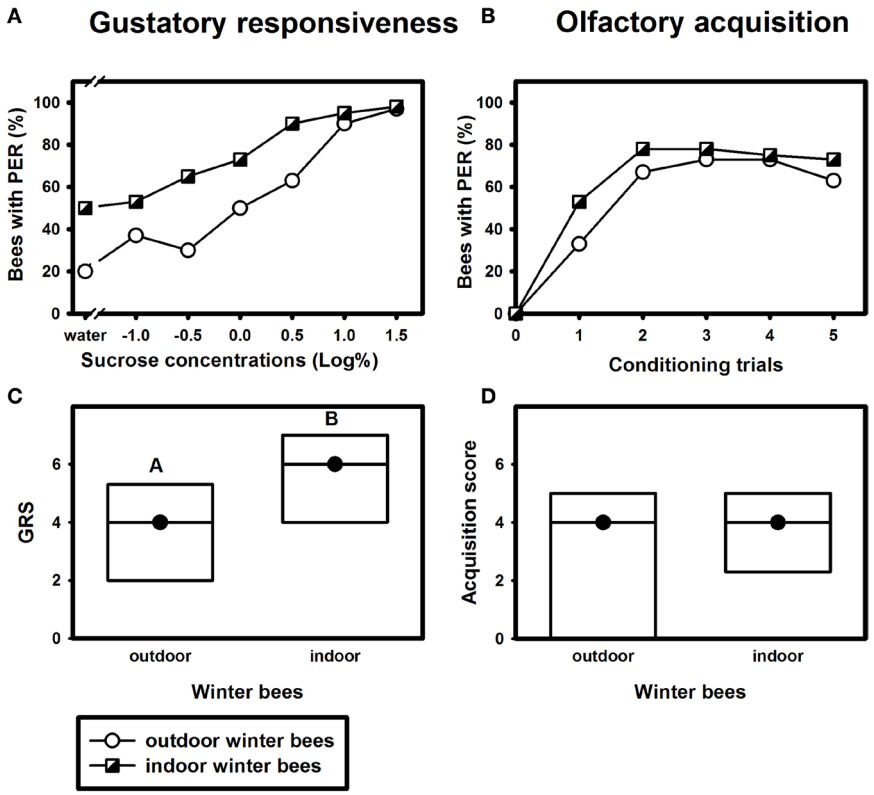

FIGURE 5 | Gustatory responsiveness and olfactory acquisition of outdoor winter bees and indoor winter bees. (A) Sucrose-concentration response curves of outdoor winter bees and indoor winter bees. The *x*-axis displays the sucrose concentration offered to the antennae of each bee. The *y*-axis shows the percentage of bees showing the proboscis extension response (PER). **(B)** Olfactory acquisition curves of outdoor winter bees and indoor winter bees. The *x*-axis displays the conditioning trials. The *y*-axis shows the percentage of bees showing conditioned PER. **(C)** Gustatory response scores (GRS) of outdoor winter bees and indoor winter bees. The *x*-axis displays the different groups. The *y*-axis shows median GRS (dots) and 25% (lower line) and 75% (upper line) quartiles. Groups with different letters differ significantly from each other. For statistics see text. **(D)** Olfactory acquisition scores of outdoor winter bees and indoor winter bees. The *x*-axis displays the different groups. The *y*-axis shows median acquisition scores (dots) and 25% (lower line) and 75% (upper line) quartiles. The outdoor winter bees were the same as in **Figure 1**.

vitellogenin is a potent antioxidant in the honey bee, protecting the brain from oxidative cell damage (Seehuus et al., 2006a). The neuroprotective properties of this protein may allow winter bees to survive the long winter period without severe brain damage. Furthermore, upstream effects of the *vitellogenin* gene, such as the maintenance of immune functions (Amdam et al., 2005) and its regulatory effects on juvenile hormone (Guidugli et al., 2005) may contribute to behavioural and physiological differences between stress-resistant winter bees and summer bees with lower stress resistance.

FLIGHT ACTIVITY AND LEARNING PERFORMANCE

Another possible explanation for the excellent learning performance and longevity of winter bees lies in their reduced flight activity. A number of experiments indicate that foragers are prompt to intrinsic mortality factors like energetic expenditure, physiological exhaust and mechanical senescence (Neukirch, 1982; Cartar, 1992; Crailsheim et al., 1996; Rueppell et al., 2007). In addition, foraging activity leads to high levels of oxidative stress in flight muscle tissue (Williams et al., 2008). The mean time used per one foraging trip increases when bees reach their 50th foraging trip (Tofilski, 2000). This decline in physical performance may also be associated with changes in learning performance.

To study whether foraging activity leads to a reduced associative learning performance we placed a small hive inside an indoor flight room at the end of the foraging season. The bees in this colony were encouraged to forage for water, sucrose solution or pollen. Their frequent flight activity apparently affected their associative learning performance and possibly their gustatory responsiveness. Although the indoor winter bees were more responsive to gustatory stimuli than the outdoor winter bees, they did not perform better in associative learning. Particularly indoor winter bees with low (GRS 1–2) or intermediate gustatory response scores (GRS 3–4) needed more trials for associating the odour with the sucrose reward than outdoor winter bees with the same gustatory responsiveness. Indoor winter bees with high gustatory scores, in contrast, did not differ in their learning performance from outdoor winter bees. This finding suggests differential effects of frequent flight activity on bees with different gustatory responsiveness. Whether increased flight activity itself led to a decrease in gustatory response scores is an

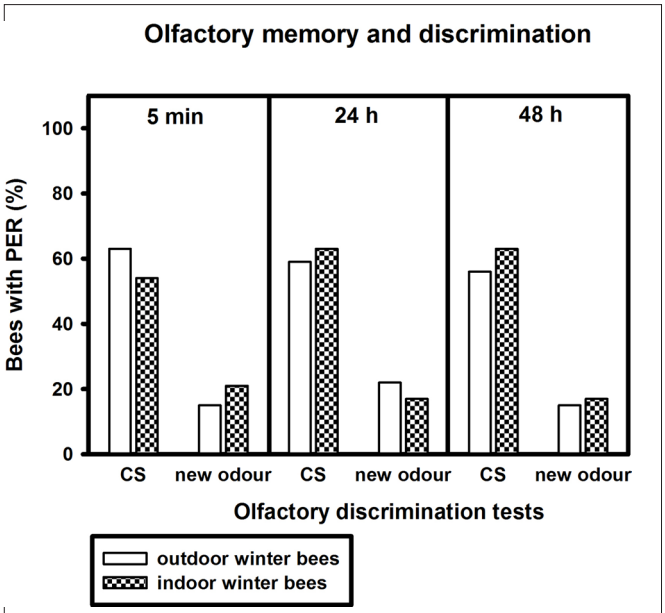

FIGURE 6 | Olfactory memory and discrimination of outdoor winter bees and indoor winter bees. The *x*-axis displays the tested stimuli (conditioned odour carnation (CS) or new odour cineole) at 5 min, 24 h or 48 h after conditioning. The *y*-axis shows the percentage of bees displaying the proboscis extension response (PER). For statistics see text. The same individuals as in **Figure 5** are displayed minus individuals that did not survive the 48-h test.

open question, because it is also conceivable that the climatic conditions in the flight room affected the gustatory responsiveness of the indoor winter bees. Another interesting question is whether individual differences in flight activity led to the observed differences in learning performance in bees with low or intermediate GRS.

LONG-TERM MEMORY OF WINTER BEES

Outdoor and indoor winter bees displayed a significant olfactory long-term memory. Forty-eight hours after conditioning, 56% of the trained outdoor winter bees and 63% of the indoor winter bees still showed conditioned proboscis extension when stimulated with the conditioned odour carnation. Nevertheless, nurse bees and foragers displayed a significantly better olfactory long-term memory than winter bees, suggesting an effect of chronological age on this form of long-term memory. But tactile long-term memory of winter bees did not differ significantly from that of summer nurse bees or foragers, although there was a similar trend observable between foragers and winter bees. These findings suggest differential effects of high chronological age on associative acquisition and memory. Long-term memory appears to be impaired, although only slightly, whereas acquisition appears normal in winter bees.

Winter bees thus behave opposite to summer foragers with long foraging duration. Although chronological age has no impact on associative learning in foragers, we found in an earlier study that foraging duration can severely impair associative acquisition in bees (Scheiner and Amdam, 2009). Although summer foragers with long foraging duration displayed an impaired acquisition, they had an extremely good long-term memory. These data are further support for the assumption that learning and memory systems are differentially affected by age and social role or foraging duration.

Although slightly impaired, winter bees can memorize the formed associations between odours, tactile patterns and a sucrose reward over days. In fact, winter bees are even capable of retaining a memory over several months, as shown by Lindauer (1963). In his experiments, winter bees remembered a food source from the previous autumn for a period of 173 days. Bees can even use olfactory experiences acquired inside the hive for later foraging decisions (Farina et al., 2007; Arenas et al., 2008; Grüter et al., 2009). An interesting question is whether winter bees trained to an odour in olfactory PER learning shortly before the winter season would remember this odour the following spring and would be able to transfer this memory to the field.

The capability of forming an extreme long-term memory over the winter period is not restricted to honey bees. A similarly long-lasting spatial form of memory of nest location was demonstrated in red wood ants which stayed underground during a hibernation period of about 210 days (Rosengren and Fortelius, 1986). In marmots (*Marmota marmota*), long-term memory was also not affected by a hibernation period of 6 months (Clemens et al., 2009). However, it needs to be stressed that winter bees do not spend the cold period in hibernation but remain active inside the hive.

DISCRIMINATION OF WINTER BEES

Winter bees showed significant discrimination between the conditioned odour carnation and the new odour cineole, regardless of whether they spent the winter confined to the hive or with the opportunity to fly about in a flight room. Discrimination between conditioned and new tactile stimuli was also apparent, although winter bees discriminated better between the odours. This finding is well in line with earlier studies demonstrating that discrimination of olfactory cues appears to be less difficult for honey bees than discrimination of tactile cues (Scheiner et al., 2003). It also shows that winter bees are capable of forming exact memories when associating odours or tactile cues with a sucrose reward. Their discrimination abilities did not differ from those of summer nurse bees or foragers. This, in turn, implies that winter bees have no deficits in their olfactory system or in their antennal motor system, despite their high chronological age. However, to answer this question in more detail, experiments analysing the odour perception and processing and the uptake of tactile information while scanning a plate are required.

CONCLUSIONS

Our study shows in insects that high chronological age does not necessarily lead to a severe decline in cognitive functions such as associative acquisition, discrimination or memory. Although extremely old, winter bees displayed normal olfactory and tactile acquisition and discrimination and slightly impaired olfactory long-term memory. This is in sharp contrast to the learning behaviour of many vertebrate and some other insect species. The honey bee thus has the potential of serving as a new model organism for studying mechanisms preventing the ageing brain from cognitive senescence.

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Balanced plasticity and stability of the electrical properties of a molluscan modulatory interneuron after classical conditioning: a computational study

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The Cerebral Giant Cells (CGCs) are a pair of identified modulatory interneurons in the Central Nervous System of the pond snail *Lymnaea stagnalis* with an important role in the expression of both unconditioned and conditioned feeding behavior. Following single-trial food-reward classical conditioning, the membrane potential of the CGCs becomes persistently depolarized. This depolarization contributes to the conditioned response by facilitating sensory cell to command neuron synapses, which results in the activation of the feeding network by the conditioned stimulus. Despite the depolarization of the membrane potential, which enables the CGGs to play a key role in learning-induced network plasticity, there is no persistent change in the tonic firing rate or shape of the action potentials, allowing these neurons to retain their normal network function in feeding. In order to understand the ionic mechanisms of this novel combination of plasticity and stability of intrinsic electrical properties, we first constructed and validated a Hodgkin-Huxley-type model of the CGCs. We then used this model to elucidate how learning-induced changes in a somal persistent sodium and a delayed rectifier potassium current lead to a persistent depolarization of the CGCs whilst maintaining their firing rate. Including in the model an additional increase in the conductance of a high-voltage-activated calcium current allowed the spike amplitude and spike duration also to be maintained after conditioning. We conclude therefore that a balanced increase in three identified conductances is sufficient to explain the electrophysiological changes found in the CGCs after classical conditioning.

Keywords: parameter estimation in Hodgkin-Huxley-type models, classical conditioning, intrinsic plasticity, persistent sodium current, delayed rectifier potassium current, high-voltage-activated calcium current, *Lymnaea stagnalis*

INTRODUCTION

The Cerebral Giant Cells (CGCs) of the snail *Lymnaea*, like their homologs in other mollusks (Weiss and Kupfermann, 1976), play an important modulatory role in the neural control of feeding (Yeoman et al., 1996; Straub and Benjamin, 2001). A recent finding is that the CGCs become persistently depolarized after single-trial food-reward classical conditioning (Kemenes et al., 2006). This depolarization is delayed by 16–24 h with respect to acquisition and early memory formation, but concomitant with the formation and duration of long-term memory. The depolarized CGCs recruit command neurons that activate the feeding motor circuit to produce the conditioned response (Kemenes et al., 2006). Membrane potential manipulation and calcium imaging experiments suggest that this recruitment occurs by calcium-dependent facilitation of sensory pathways to the feeding command neurons (Kemenes et al., 2006). Surprisingly, the depolarization of the CGCs occurs without significant changes in the firing rate or shape of action potentials (Kemenes et al., 2006). This observation has raised interesting questions about the ionic mechanisms supporting both a long-lasting depolarization and stable spike frequency in the same neuron. Frequency stabilization is necessary in the feeding network because the CGCs need to fire within a narrow frequency range (between ~ 0.6 and 1 Hz in the type of isolated CNS preparation we used in the present study) to carry out their normal (non-learning) modulatory role in supporting feeding motor output (Yeoman et al., 1994). Membrane potential depolarization already has been linked to a learning-induced increase of the persistent sodium current (I_{NaP}) of the CGCs (Nikitin et al., 2008), but the mechanism of spike frequency stabilization remained unknown.

To facilitate our understanding of the electrical mechanisms underlying this novel combination of plasticity and stability of key electrical properties in the same neuron, here we used voltage- and current-clamp data as inputs to parameter estimation in a single-compartment Hodgkin-Huxley-type model of the CGCs. The parameterized model is then validated against different data and its behavior correlated to that observed in the CGCs after conditioning.

Our main specific aim was to replicate the complex electrophysiological effects of behavioral conditioning by simulating changes in I_{NaP} and the delayed rectifier potassium current (I_D) of the CGCs (Staras et al., 2002). The inclusion of I_D in the modeling was justified by the finding that this current is also increased following conditioning (see Results). This new finding allowed us to formulate the hypothesis that a balanced increase in the conductances of I_D and

I_{NaP} underlie spike frequency stabilization during learning-induced persistent depolarization. However, while this hypothesis was being tested, the model revealed that an increase in the conductance of the also previously identified high-voltage-activated calcium current (I_{HVA}, Staras et al., 2002) was required for the stabilization of spike amplitude and duration after conditioning. Our work therefore establishes a theoretical framework for relating multiple ion channel properties to the expression of cellular correlates of conditioning.

MATERIALS AND METHODS

GENERAL DESCRIPTION OF THE MODEL

The model of the CGCs took the form of a single-compartment Hodgkin-Huxley-type neuron. We used a single- instead of a two- or multi-compartment model (e.g., Golowasch et al., 1999; Vavoulis et al., 2007), because the basic electrical properties (mean firing frequency, action potential shape, resting membrane potential, etc.) of the cell body in the CGCs after axotomy did not differ significantly from recordings made from the intact neuron in the absence of axotomy (Staras et al., 2002; Nikitin et al., 2006). Moreover, molluscan neurons have no dendritic processes on their cell bodies, so compartments modeling these processes did not need to be included. The model includes all the ionic currents previously identified in the CGCs from two-electrode voltage-clamp recordings (Staras et al., 2002; Nikitin et al., 2006), namely: (1) a transient TTX-sensitive sodium current (I_{NaT}), (2) a persistent TTX-resistant sodium current (I_{NaP}), (3) a persistent TEA-sensitive potassium current (I_D), (4) a transient 4-AP-sensitive potassium current (I_A), (5) a transient low-voltage-activated T-type calcium current (I_{LVA}) and (6) a transient high-voltage-activated calcium current (I_{HVA}). An S_K-type Ca^{2+}-dependent potassium current, rarely found in the CGCs and not characterized in detail, was not included in the model.

A detailed mathematical description of the model and the parameter estimation methods we used is given as Materials and Methods in Supplementary Material. The model for each current follows the Hodgkin-Huxley formalism, as described in Willms et al. (1999). It includes a number of activation and, in the case of transient currents, inactivation gates, which exist in either an open or close state. The opening and closing of the gates in each channel type in response to changes in the membrane potential was modeled by a set of differential equations obeying first-order relaxation kinetics. The equilibrium states and associated relaxation times of this kinetics were respectively described as sigmoid and Gaussian functions of the membrane potential (Eq. S9 and S10 in Section Materials and Methods in Supplementary Material).

For the estimation of the maximal conductances, reversal potentials and the parameters governing the response of the currents to voltage (a total of 43 free parameters), we fitted the model against a combination of voltage- and current-clamp data (Staras et al., 2002; Nikitin et al., 2006) using an efficient optimization method, which permitted the fitted model to mimic the spike shape, spontaneous tonic firing activity and current-frequency response of the biological CGCs with high accuracy (for a more detailed description, see Materials and Methods in Supplementary Material). The fitting process was repeated for a large number of randomly selected initial values of the unknown parameters providing information on the uniqueness of the estimated values and the error associated with their estimation. Furthermore, the contribution of identified ionic currents to the electrical properties of the fitted CGCs model was estimated by simulating the application of specific pharmacological agents in the biological cells.

SIMULATIONS

The fitted CGC model took the form of an 8-dimensional system of Ordinary Differential equations (ODEs; see Materials and Methods in Supplementary Material), which were encoded using custom code in the programming language ANSI C and solved in MATLAB®. All simulations were realized on a number of Intel Core 2 Duo desktop computers with 2 or 4 GB of memory and the Linux operating system. The coupled ODEs were solved using an adaptive Runge-Kutta-Fehlberg (4,5) algorithm with absolute error tolerance 10^{-6} and relative error tolerance 10^{-3}.

ELECTROPHYSIOLOGY

All the data on ionic currents used in the present study were obtained in previous experiments using standard two-electrode voltage-clamp methods. A variety of commonly used modified salines (containing TEA, 4-AP, nickel, etc.) were used to isolate specific ionic currents, as described in the same previous papers (Staras et al., 2002; Nikitin et al., 2006, 2008).

To test predictions from the model regarding the CGCs' electrical responses to increasing amounts of injected depolarizing current, we carried out new experiments using standard two-electrode current clamp methods in normal physiological saline (see details in Staras et al., 2002; Kemenes et al., 2006; Nikitin et al., 2006). CGCs ($n = 5$) in isolated CNS preparations from laboratory-bred *Lymnaea stagnalis* were impaled with two microelectrodes (filled with 4-M KAc, tip resistance: 8–15 MΩ), one to inject current and another to record voltage responses from the cell. The CGCs were identified on the basis of their size, position in the ganglia and characteristic tonic firing pattern (McCrohan and Benjamin, 1980a,b). At the beginning of each experiment, the membrane potential of the CGC was set at −80 mV by injecting an appropriate amount of steady current. To measure spike frequency increases in response to current injection, we injected increasing amounts of depolarizing current into the cells in the range 0 to 2 nA. Each period of current injection lasted for 10 s with 60 s intervals between successive stimulations to allow the CGC to fully recover from the effect of the preceding test. The average spike frequency (in Hz) at each test current level was calculated from the total number of spikes generated by the cell during the 10 s stimulation period.

RESULTS

The model includes all the previously identified voltage-gated ionic currents in the biological CGCs. For each current, the maximal conductance, reversal potential and activation/inactivation kinetics were estimated from existing voltage- and current-clamp electrophysiological data (Staras et al., 2002; Nikitin et al., 2006). In a first stage, the estimation of 18 of the 43 parameters governing the activation and inactivation kinetics of most currents in the model was possible from voltage-clamp data (**Table 1**). In a second stage, the remaining parameters, including the maximal conductance and reversal potential for each current, were estimated by fitting the whole-cell model against current-clamp data, while most of the parameters

Table 1 | Estimated parameter values for all ionic currents in the fitted CGCs model.

		Activation				Inactivation			
I_{NaT}	g_{NaT}	V_H^m	V_S^m			V_H^h	V_S^h	τ_o^h	δ^h
	1.68	−35.20	9.66			−56.43	−8.41	778.82	0.03
I_{NaP}	g_{NaP}	V_H^r	V_S^r	τ_o^r	δ^r				
	0.44	−47.03†	20.55†	4.01	1.00				
I_A	g_A	V_H^a	V_S^a	τ_o^a	δ^a	V_H^b	V_S^b	τ_o^b	δ^b
	18.82	−36.37†	8.72†	13.28†	0.39†	−83.00†	−6.20†	266.75†	0.83†
I_D	g_D	V_H^n	V_S^n	τ_o^n	δ^n				
	1.20	−59.43	34.79	14.52	0.18				
I_{LVA}	g_{LVA}	V_H^c	V_S^c			V_H^d	V_S^d		
	0.01	−41.35†	5.05†			−64.13†	−4.03†		
I_{HVA}	g_{HVA}	V_H^e	V_S^e	τ_o^e	δ^e	V_H^f	V_S^f	τ_o^f	δ^f
	1.03	−14.25†	6.96†	3.81	0.84	−21.44†	−5.78†	34.68	0.97
	(in mS/cm^2)	(in mV)	(in mV^{-1})	(in ms)		(in mV)	(in mV^{-1})	(in ms)	

†*These parameters were estimated from voltage-clamp data; the rest from current-clamp data.*
††*Reversal potentials are: E_{Na} = +55 mV, E_K = −90 mV and E_{Ca} =+80 mV. Also, C_m = 1 μF/cm^2.*
†††*For details on the meaning of each parameter, see Materials and Methods in Supplementary Material. For an analysis of the variability of these parameters see Section "Tolerance of optimal fitting to variation in parameter values" and* ***Figure 3.***

estimated from voltage-clamp data in the previous step were kept fixed, as explained below. The final values of all parameters in the model are given in **Table 1** along with information on whether their estimation was based on voltage- or current-clamp data. Information on the variability of parameter values is given in Section "Tolerance of optimal fitting to variation in parameter values" and **Figure 3**.

ESTIMATION OF MODEL PARAMETERS FROM VOLTAGE-CLAMP DATA

Parameter estimation in the model from voltage-clamp data is summarized in **Figure 1**. Beginning with the persistent sodium current (I_{NaP}), we utilized information from current traces induced by 800 ms-long voltage steps to membrane potentials in the range from −90 to +30 mV from a holding potential of −110 mV (see Nikitin et al., 2006 for details of methods). The induced current persisted without significant inactivation for the duration of each step. The equilibrium current at the end of each step was modeled as a function of voltage (Eq. S11 in Materials and Methods in Supplementary Material) and it was fitted to the experimental data (**Figure 1Ai**). From the fitted model, the steady-state activation of the persistent-sodium current (r_∞) was derived as a sigmoid function of the membrane potential (**Figure 1Aii**).

For the low-voltage-activated (I_{LVA}) and high-voltage-activated (I_{HVA}) calcium currents, we first computed the steady-state inactivation curves (**Figure 1Bi**; d_∞ and f_∞ respectively) by fitting sigmoid functions of voltage (Eq. S9 in Supplementary Material) to normalized peak currents recorded during voltage steps to 0 mV from holding membrane potentials between −60 mV and +15 mV in the case of I_{HVA} and to −50 mV from holding membrane potentials between −100 mV and −30 mV in the case of I_{LVA} (**Figure 1Bi**, open squares and open circles respectively; Staras et al., 2002). Subsequently, we used the current recorded during a voltage-clamp ramp protocol (voltage change from −100 mV to +30 mV over a time interval of 120 ms; see Staras et al., 2002 for details). The induced current trace had two components (**Figure 1Bii**): (a) one activating at ~ −60 mV and reaching a peak at ~ −45 mV and (b) one activating at ~ −30 mV and peaking at ~0 mV. From the fitted model (Eq. S12 in Supplementary Material; **Figure 1Bii**), we derived the steady-state activation for I_{LVA} and I_{HVA}, c_∞ and e_∞ respectively, as functions of membrane potential (**Figure 1Biii**).

In the case of the delayed rectifier (I_D) and transient potassium (I_A) currents, we utilized the current traces recorded during a voltage step protocol from a holding membrane potential of −90 mV to steps from −20 to +35 mV over a period of 100 ms (**Figure 1Ci**; Staras et al., 2002). This protocol revealed an early transient current corresponding to I_A and a second sustained one, which persisted for the duration of the step (I_D). The model for the total potassium current (Eq. S13–S16 in Materials and Methods in Supplementary Material) was fitted simultaneously to the whole set of potassium data using the full trace method (Willms et al., 1999), as illustrated in **Figure 1Ci**. From the fitted model, we derived estimations for the steady-state activation and inactivation of I_A (**Figure 1Cii**, a_∞ and b_∞ respectively), the steady-state activation of I_D (**Figure 1Cii**, n_∞*), the activation and inactivation relaxation times for I_A (**Figure 1Ciii**, τ_a and τ_b respectively) and the activation relaxation time for I_D (**Figure 1Ciii**, τ^*_n).

ESTIMATION OF MODEL PARAMETERS FROM CURRENT-CLAMP DATA

The results from fitting the whole CGC model against current-clamp data are presented in **Figure 2**. This data took the form of a 1.9 s-long voltage trace recorded using a two-electrode current-clamp protocol from CGCs in the intact nervous system of unconditioned animals in the absence of external stimulation (**Figures 2Ai, ii**). We chose to fit a recording interval with only one spike, because consecutive spikes in the same recording are not exactly the same in terms of amplitude and duration, thus fitting simultaneously all of them using the present method would be problematic. However, the voltage trace we used is representative of the spontaneous CGCs activity and it contains information on both the spike shape and long interspike intervals that characterize this activity. The model was fitted to the data using an iterative method (see Materials and

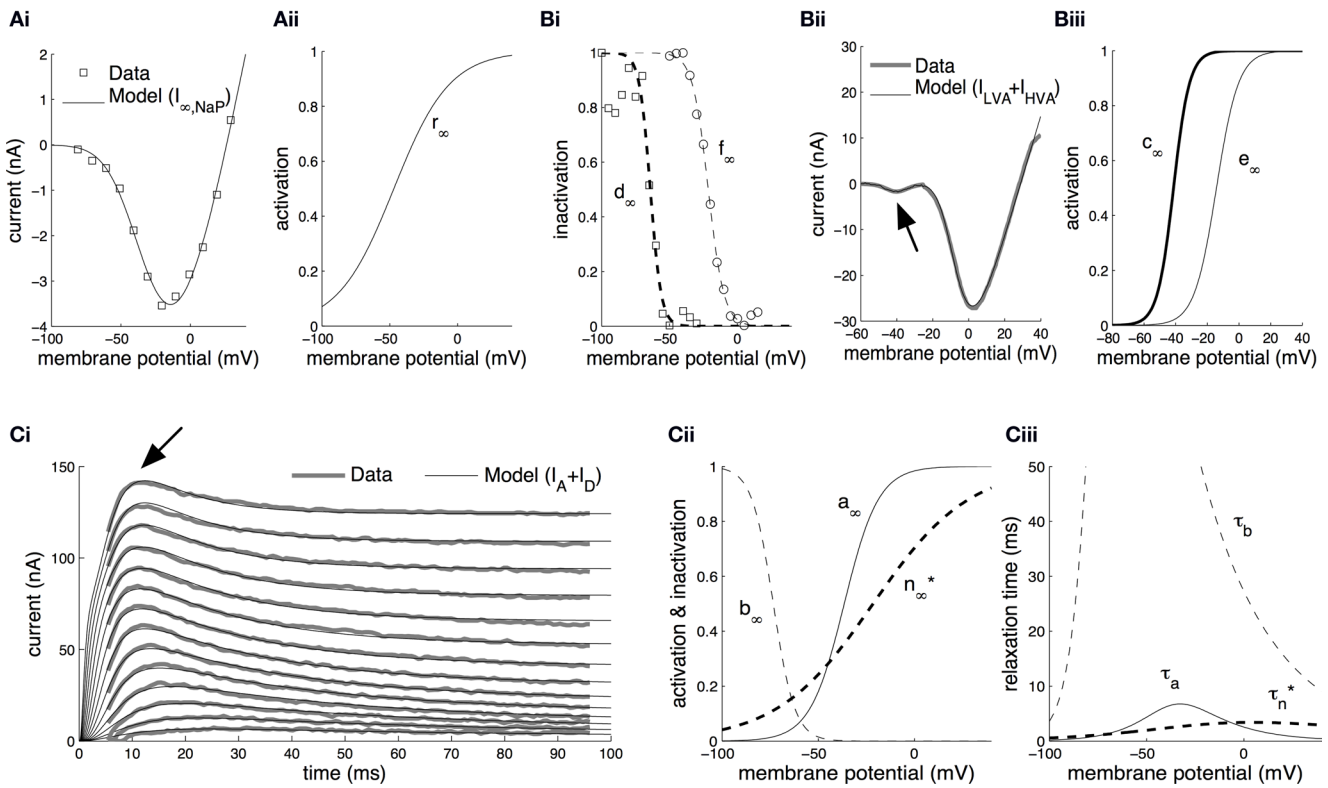

FIGURE 1 | Parameter estimation from voltage-clamp data. (A) Estimation of the steady-state activation of the persistent sodium current. Persistent sodium currents at equilibrium, $I_{\infty,NaP}$, were measured at the end of 800 ms-long voltage steps to membrane potentials in the range from –90 to +30 mV from a holding potential of –110 mV (**Ai**, open squares; Nikitin et al., 2006). The model for $I_{\infty,NaP}$ (Eq. S11 in section Materials and Methods in Supplementary Material) was fitted against this data (**Ai**, solid line) permitting the estimation of the steady-state activation of the current, r_∞, as a sigmoid function of the membrane potential **(Aii)**. **(B)** Estimation of the steady-state activation and steady-state inactivation of the low-voltage-activated and high-voltage-activated calcium currents. The steady-state inactivation for I_{LVA}, d_∞, was computed by fitting a sigmoid curve to normalized peak currents recorded during voltage steps to –50 mV from holding membrane potentials between –100 mV and –30 mV **(Bi**, open squares; Staras et al., 2002). Similarly, for the steady-state inactivation of I_{HVA}, f_∞, we fitted a sigmoid curve to normalized peak currents recorded during voltage steps to 0 mV from holding membrane potentials between –60 mV and +15 mV **(Bi**, open circles; Staras et al., 2002). Subsequently, the model in Eq. S12 in Supplementary Material was fitted against the total calcium current induced during a voltage-ramp protocol changing the membrane potential from –100 mV to +30 mV over a time interval of 120 ms (**Bii**; Staras et al., 2002). The arrow in **Bii** indicates the low-voltage-activated component corresponding to I_{LVA}. From the fitted model, we derived the steady-state activation for I_{LVA} and I_{HVA} (c_∞ and e_∞, respectively) as sigmoid functions of the membrane potential **(Biii)**. **(C)** Estimation of the activation and inactivation kinetics for the transient (I_A) and delayed-rectifier (I_D) potassium currents. The model for the total potassium current under voltage-clamp (Eq. S13–S16 in Supplementary Material) was fitted to current traces induced during 100 ms-long voltage steps from a holding membrane potential of –90 mV to steps from –20 to +35 mV (**Ci**; Staras et al., 2002) using the full trace method (Willms et al., 1999). The arrow in **Ci** indicates the early transient component corresponding to I_A. The fitted model permitted the estimation of the steady-state activation, a_∞, and inactivation, b_∞, for the transient potassium current I_A, and the steady-state activation, n_∞*, for the delayed rectifier as illustrated in **Cii**. The corresponding relaxation times, τ_a, τ_b and τ^*_n, were also derived from the fitted model (**Ciii**). The estimated kinetic parameters for I_D are marked with an asterisk, because they are further modified and receive their final values based on current clamp data.

Methods in Supplementary Material for details), during which the parameter values estimated from voltage-clamp data in the previous stage were kept fixed, with the exception of those controlling the activation kinetics of I_D (i.e. n^*_∞ and τ^*_n in **Figures 1Cii,iii**). These were further adjusted at this stage, which was necessary for obtaining a sufficiently good fit to the experimental data. These and the remaining unknown parameters were left free to vary during the optimization process and progressively attained optimal values, as illustrated by the gradual convergence of the model to the experimental data (**Figures 2Ai,ii**). The estimated parameters at this stage were the maximal conductances for each current (g_{NaP}, g_{NaT}, g_A, g_D, g_{LVA} and g_{HVA}), the activation and inactivation kinetics of I_{NaT} (m_∞, h_∞ in **Figure 2Bi**; τ_h in **Figure 2Bii**), the activation relaxation time for I_{NaP} (τ_r in **Figure 2Biii**), the activation and inactivation relaxation times for I_{HVA} (τ_e and τ_f in **Figure 2Biv**) and the final parameter values for the activation kinetics of I_D (n_∞ in **Figure 2Bi** and τ_n in **Figure 2Bii**). The reversal potentials for each group of currents were given standard values at this stage (sodium, E_{Na} =+55 mV; potassium, E_K = –90 mV; calcium, E_{Ca} = + 80 mV), but they were left free to vary during the optimization at a later stage (see **Figure 3**).

TOLERANCE OF OPTIMAL FITTING TO VARIATION IN PARAMETER VALUES

In order to assess the uniqueness and accuracy of the parameter values that were estimated from current-clamp data, the optimization process against this data was repeated starting from a large number of

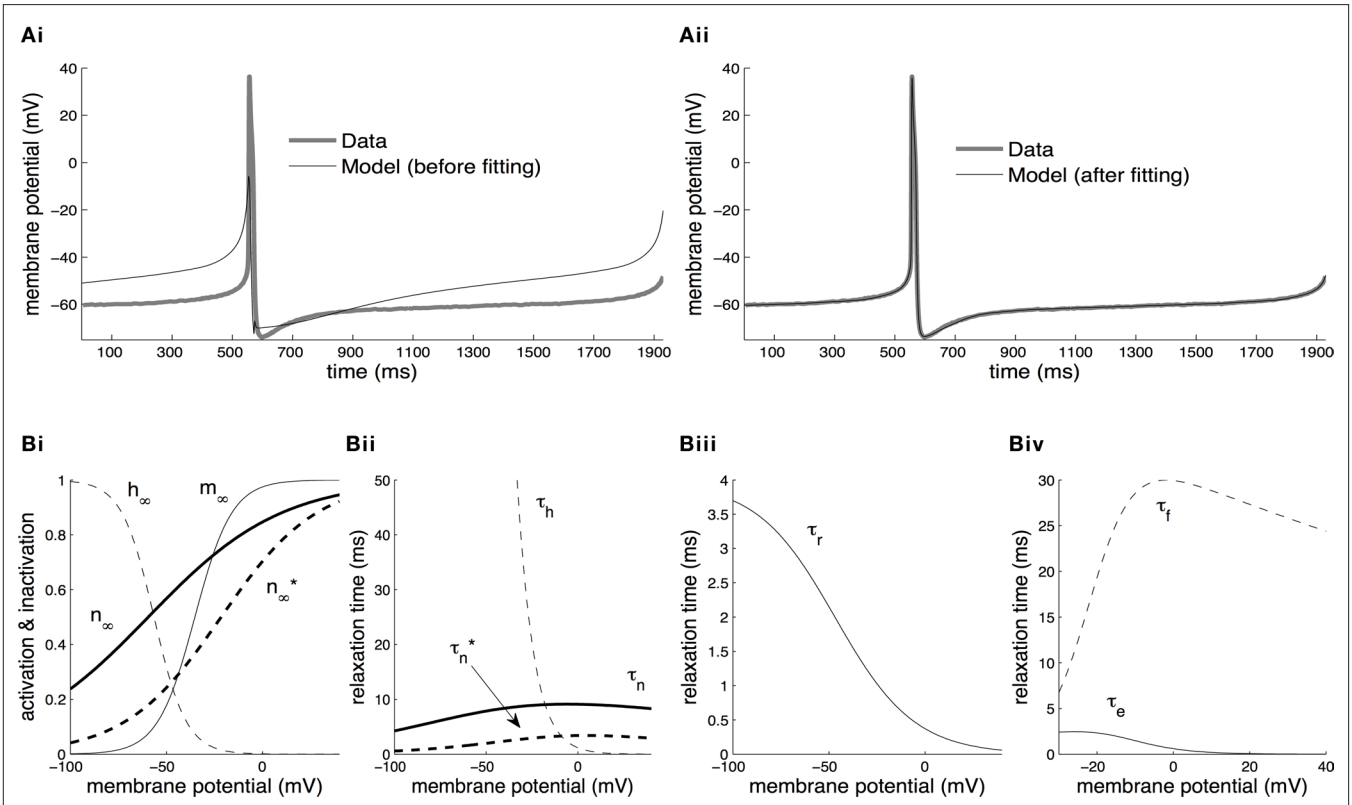

FIGURE 2 | Parameter estimation from current-clamp data. (A) The whole-cell model was fitted against a two-electrode current-clamp recording of duration ~1.9 s from CGCs in the intact nervous system of unconditioned animals. The parameters estimated at the previous stage from voltage-clamp data were kept fixed, with the exception of those referring to the activation kinetics of the delayed rectifier, I_D. During the optimization process, the free parameters in the model progressively attained optimal values, as indicated by the gradual convergence of the model to the data **(Ai, ii)**. **(B)** The steady-state activation, m_∞, and inactivation, h_∞, of the transient sodium current were derived from the fitted model at this stage as sigmoid functions of the membrane potential **(Bi)**. The relaxation times for the inactivation of the transient sodium current, τ_h, for the activation of the persistent sodium current, τ_r, and for the activation, τ_e, and inactivation, τ_f, of the high-voltage-activated calcium current were also derived from the fitted model at this stage as functions of the membrane potential **(Bii–iv)**. Also, the parameters for the steady-state activation of the delayed rectifier, n_∞, and for the associated relaxation time, τ_n, received their final values at this stage **(Bi, ii)**. The dashed lines illustrating n^*_∞ and τ^*_n in the same panels are as in **Figures 1Cii,iii**.

random initial values of the relevant parameters. First, we examined the maximal conductances and reversal potentials for all currents in the model, while the remaining parameters were kept fixed at their previously estimated values. The random initial values for the maximal conductances were uniformly distributed within the rather broad interval of 0.001–120 mS/cm^2. Similarly, the reversal potentials for each group of ionic currents were again uniformly distributed within the following intervals: E_{Na}, +25 to +60 mV; E_K, −95 to −75 mV; E_{Ca}, 25 to 85 mV. A total of 90 random initial values for each of these parameters (10 × number of free parameters) were examined and for each of them, the model converged to an optimal solution (normalized sum of squared residuals 0.0782 ± 0.0004 s.e.m. **Figure 3Ai**). Examination of the maximal conductance and reversal potential values estimated after the end of the process revealed that they were tightly confined, within 15% of their median value (**Figure 3Bi**).

At a second stage, the same process was repeated, but this time the maximal conductances and reversal potentials were kept fixed to their previously estimated values, while the rest of the estimated-from-current-clamp-data parameters (indicated in **Figures 3Bii,iii**) were left free to vary during the optimization. The random initial values were generated by letting parameters V^x_H (x = m,h,n) and τ^x_o (x = h,r,n,e,f) be uniformly distributed within ±10 mV and ±50% of their previously estimated values, respectively. A total of 140 different initial values for each of these parameters were tested (10 × number of free parameters), among which only 63 (45%) converged to the same sufficiently good solution (normalized sum of squared residuals 0.0749 ± 0.0008 s.e.m. **Figure 3Aii**, dashed rectangular region). Examination of the optimal values obtained after the end of the fitting process revealed that the estimated values for parameters V^x_H (x = m,h,n), V^m_S and V^n_S were confined narrowly around the median, with only the values for V^h_S (a parameter controlling the steady-state inactivation of the transient sodium current) showing a broader dispersion (**Figure 3Bii**). The estimated values for parameters τ^x_o and δ^x (x = h,r,n,e,f) showed a broad distribution (**Figure 3Biii**) indicating that tightly constraining these parameters was not critical for optimally fitting the model against the current-clamp experimental data.

OVERVIEW OF THE MODEL NEURON ACTIVITY AND CONTRIBUTION OF IDENTIFIED IONIC CURRENTS TO MEMBRANE EXCITABILITY

An overview of the spike shape and firing activity of the fitted model is given in **Figure 4**. The model was compared to an intracellular current-clamp recording from axotomized cells, which

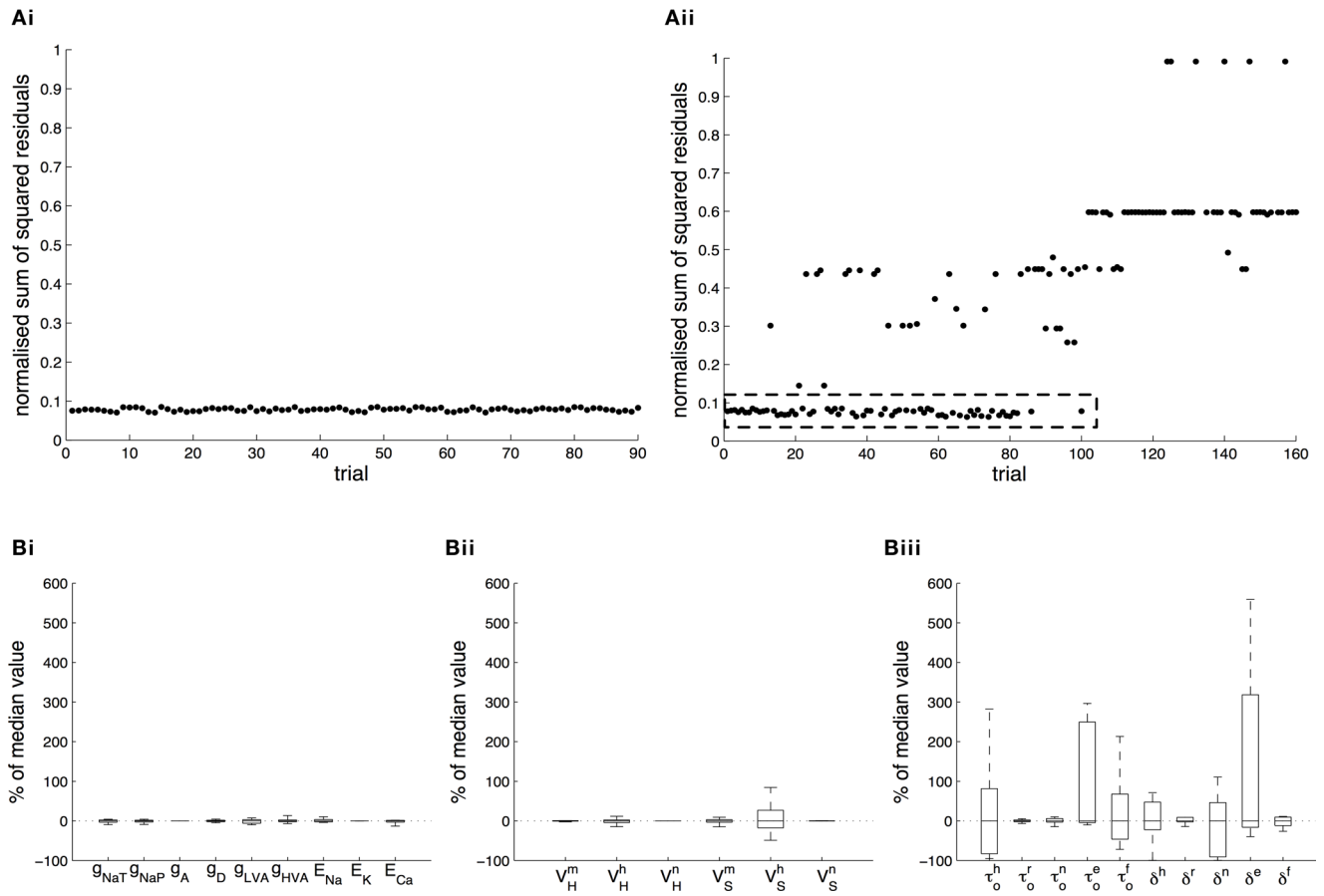

FIGURE 3 | Tolerance of optimal fitting to variation in parameter values. **(A)** Optimization against current-clamp data was repeated from a large number of randomly selected initial values for the maximal conductances and reversal potentials in the model, while the rest of the parameters were kept fixed to their previously estimated values. A total of 90 different initial values for each free parameter were tested, all of which converged to the same equally good fit to the data at the end of the optimization **(Ai)**. Similarly, the optimization based on current-clamp data was repeated for parameters V_H^x and V_S^x (x = m,h,n) and τ_o^x and δ^x (x = h,r,n,e,f), while the rest of the parameters in the model, including maximal conductances and reversal potentials, were kept fixed to their previously estimated values. We tested 140 randomly distributed initial values for each of the parameters V_H^x (x = m,h,n) and τ_o^x (x = h,r,n,e,f), among which only 63 (45%) converged to a sufficiently good fit to the current-clamp data (**Aii**, dashed rectangular region). All 230 points illustrated in **Ai** and **Aii** were normalized by dividing with the largest sum of squared residuals found in these simulations. **(B)** Examination of the estimated maximal conductance and reversal potential values corresponding to the optimal points illustrated in **Ai** revealed that these values were tightly constrained around the median, not exceeding 15% of this value **(Bi)**. Similarly, the optimal values for parameters V_H^m, V_H^h, V_H^n, V_S^m and V_S^n (i.e. those corresponding to the points inside the rectangular dashed region in **Aii**) were tightly constrained around the median with the exception of the values for V_S^h (a parameter controlling the steady-state inactivation of I_{NaT}; see Eq. S9 in section Materials and Methods in Supplementary Material), which were dispersed within 80% of the median value **(Bii)**. On the other hand, the optimal values for most of the examined parameters τ_o^x and δ^x (x = h,r,n,e,f) were rather broadly distributed around the median **(Biii)**. All parameters in **Bi–iii** were centered and normalized with respect to the median. The lower and upper edges of each box in the same plots correspond to the 25th and 75th percentiles, respectively, while the whiskers above and below each box indicate the most extreme parameter values.

demonstrated a close agreement in the spontaneous tonic firing activity (**Figure 4A**), spike shape (**Figure 4B**) and current-frequency response (**Figure 4C**) between the model and the biological neuron. In the absence of synaptic or experimentally applied input both the biological and the model CGCs fire at a mean frequency of ~0.7 Hz (~42 spikes/min; **Figure 4A**). Typically, an action potential starts as a gradual depolarization of the cell membrane that becomes very rapid after a threshold (~−50 mV) is crossed (**Figure 4B**). The spike reaches a peak of approximately +40 mV, which is followed by a repolarization phase with a pronounced "shoulder" (indicated by an arrow in **Figure 4B**). At its most hyperpolarized state, immediately after the spike, the membrane potential reached a value of about −75 mV, before returning gradually to its baseline value (~−60 mV). The spike had a total duration of ~17 ms (measured at −20 mV). Furthermore, when injected with constant currents of increasing amplitude, the model responded with an increase in its firing frequency, which was in close agreement with the response of the biological neuron (**Figure 4C**).

In a next stage, we performed a set of simulations in which we examined the effect of selectively blocking identified ionic currents on the electrical properties of the model cell, thus mimicking the application of specific pharmacological agents in the biological neurons during a series of independent electrophysiological experiments (Staras et al., 2002).

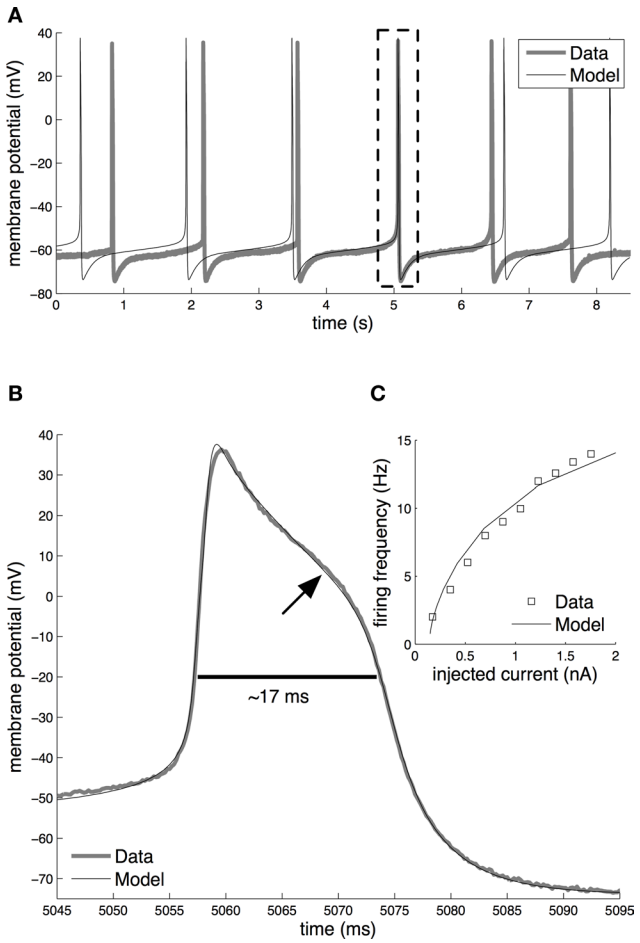

FIGURE 4 | Basic electrical activity in the model and biological CGCs. (A) Spontaneous tonic firing in the fitted model and the biological neuron (Data). In the absence of external or synaptic input, both fire at approximately 0.7 Hz. Dashed rectangle indicates the overlapping real and simulated spike, respectively, shown on an expanded voltage and time scale in **(B)**. **(B)** Shape of the action potential in the model and biological CGCs. In both the model and biological neuron, the shape of the action potential is very similar, with the duration being approximately 17 ms (measured at –20 mV) and amplitude being approximately 115 mV. The arrow indicates the characteristic "shoulder" of the action potential during its repolarisation phase. **(C)** Current-frequency response in the model and the biological neuron. In both, externally injected currents in the range 0–2 nA induce spikes at frequencies up to approximately 15 Hz. To aid clarity, the frequency data shown for the biological neuron come from a single experiment where the same CGC was tested with increasing amounts of current injected into the soma through one electrode while recording spike activity through a second electrode. The same test was repeated with 4 more CGCs in different preparations. The membrane potential of all five neurons tested this way was set at –80 mV prior to each test and so the frequency responses to the same amount of current showed very little variance between the individual neurons.

As a first example, we examined the effect of removing the persistent and transient sodium currents from the model. In the intact nervous system, bathing the preparation in zero-Na^+ saline caused the CGCs to stop firing and hyperpolarize to a very negative potential (**Figure 5Ai**; also see Staras et al., 2002; Nikitin et al., 2008). Artificially repolarizing the cell above the firing threshold failed to evoke action potentials, but washing back into normal saline caused the CGCs to start firing again, with no apparent change in the spike shape (Staras et al., 2002).

We simulated the removal of Na^+ ions in our model by gradually setting the maximal conductance of the persistent and transient sodium currents (g_{NaP} and g_{NaT}, respectively) to zero over a time interval of 60 s. Under these conditions, the model neuron immediately ceased firing and the membrane potential hyperpolarized at a very negative value (~−90 mV), as in the biological neuron (**Figure 5Aii**). In addition, when the model was artificially repolarized above −50 mV by external current injection, the action potentials did not recover, similarly to the biological neuron (data not shown). This result suggests that the total sodium current plays an important role in the generation of action potentials (a function traditionally attributed to its transient component) in both the model and biological neurons. In addition, it constitutes a significant depolarizing force, sufficient to bring the membrane potential above its firing threshold even from a very negative value, thus sustaining spontaneous tonic firing in both the model and the biological CGCs.

Next, we examined the effect of blocking the delayed rectifier potassium current, I_D, to the electrical properties of the neuron. In the intact nervous system, washing the preparation in 50-mM TEA (tetraethylammonium chloride) resulted in a significant depolarization of the membrane potential, the individual spikes became broader and the amplitude of the after-hyperpolarization following each spike became smaller (**Figure 5Bi**). These effects were completely reversed, when the preparation was washed back into control saline (Staras et al., 2002).

In the model, application of TEA was mimicked by reducing the maximum conductance of the delayed rectifier, g_D, by 30% (**Figure 5Bii**). Under these conditions, the model neuron became significantly depolarized, with a spike after-hyperpolarization smaller by ~12 mV and an increased spike duration by ~17 ms. Completely blocking the delayed rectifier in the model by setting its maximal conductance equal to zero resulted in ceasing firing, the membrane potential failed to repolarize and settled at a very positive value (>50 mV; data not shown). These effects are comparable to those recorded from the biological neuron when TEA is present in the saline and they suggest that, in agreement with the role typically attributed to this current, the delayed rectifier is important in repolarizing the membrane during an action potential in both the biological and the model neuron.

As a final example, we examined the effect of blocking the high-voltage-activated calcium current, I_{HVA}, on the electrical properties of the membrane. In neurons from the intact nervous system, washing the preparation into 100-μM $CdCl_2$, a non-specific blocker of the high-voltage-activated calcium current, resulted in the cell becoming depolarized (from −63 mV to −59 mV) and a characteristic narrowing and shortening of the action potential (**Figure 5Ci**), which reversed to normal, when the preparation was washed back to control saline (Staras et al., 2002).

In the model, the application of $CdCl_2$ was mimicked by setting the maximal conductance of the high-voltage-activated calcium current, g_{HVA}, to zero. Similarly to the biological neuron in the presence of $CdCl_2$ in the saline, the spike lost its characteristic "shoulder", becoming narrower and shorter by ~6 ms and ~17 mV respectively

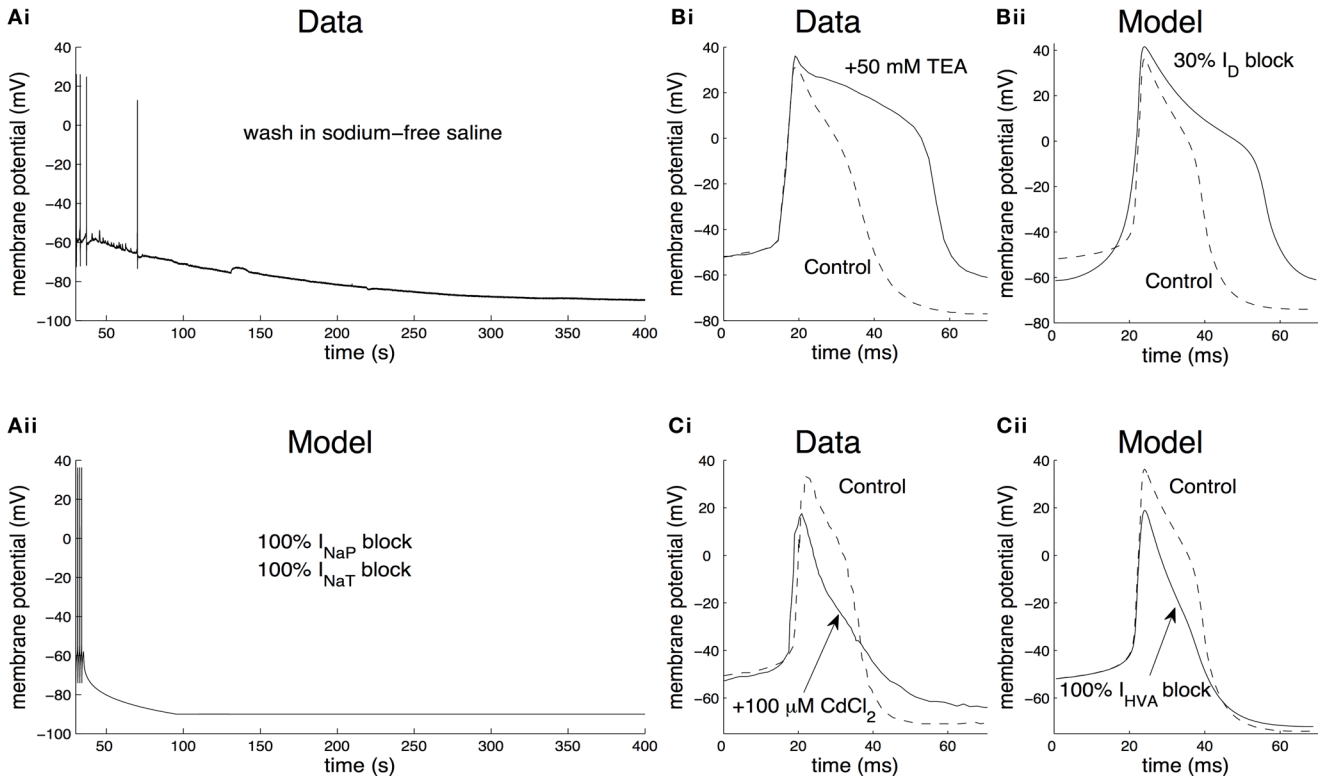

FIGURE 5 | Contribution of identified ionic currents to the electrical properties of the CGCs. (A) Contribution of the total sodium current. When washing the preparation into sodium-free saline, CGCs from the intact nervous system cease to fire and the membrane potential is significantly hyperpolarized (Ai; also see Staras et al., 2002). In the model, washing into sodium-free saline was simulated by gradually setting the maximal persistent and transient sodium conductances (g_{NaP} and g_{NaT}, respectively) to zero over a time interval of 60 s **(Aii)**. This is equivalent to completely removing the transient and persistent sodium currents from the model, inducing the cell to stop firing and the membrane potential to settle at a very negative value (~−90 mV), similarly to the biological neuron. **(B)** Contribution of the delayed rectifier potassium current to spike shape. When blocking I_D by washing the preparation in 50 mM TEA (tetraethylammonium chloride), the duration of the action potentials recorded from CGCs in the intact nervous system increased significantly (**Bi**; also see Staras et al., 2002). Also, the after-hyperpolarization following each spike was reduced in amplitude. In the model, this situation was simulated by blocking the maximal conductance of the delayed rectifier, g_D, by 30% (Bii). This resulted in spikes of longer duration by ~17 ms and a smaller spike after-hyperpolarization by ~12 mV, similarly to washing the biological neuron into saline containing TEA. **(C)** Contribution of the high-voltage-activated calcium current to spike shape. When blocking I_{HVA} by washing the preparation into 100-μM $CdCl_2$, the spikes recorded from CGCs in the intact nervous system became shorter and narrower and the spike after-hyperpolarization was reduced in amplitude (**Ci**; Staras et al., 2002). In the model, blocking I_{HVA} by $CdCl_2$ was simulated by setting g_{HVA}, the maximal conductance of the high-voltage-activated calcium current, equal to zero **(Cii)**. This resulted in spikes losing their characteristic "shoulder" and becoming narrower and shorter by ~6 ms and ~17 mV respectively, similarly to recordings from the biological neuron after the application of $CdCl_2$.

(**Figure 5Cii**). Also, the after-hyperpolarization following each spike became smaller in amplitude by ~2 mV, similarly to the biological neuron. These results suggest that I_{HVA} contributes to the generation of action potentials resulting in higher and broader spikes, in both the model and the biological CGCs.

Blocking I_{LVA} did not have any apparent effect on the firing frequency or spike shape of the model neuron, but enhancing I_{LVA} had a weak effect on the firing frequency, which started becoming significant after a 10-fold increase in the maximal conductance of this current. On the other hand, blocking I_A in the model resulted in increasing both the width and height of the spontaneous spikes. However, these effects were not consistent across different instantiations of the CGC model, i.e., when different parameter combinations were tested. In the absence of a systematic experimental analysis on the effects of blocking I_A or I_{LVA} under current-clamp conditions, which could serve for validating the model, we do not report these results in the present study.

Overall, the effects of blocking identified sodium, potassium and calcium currents on the electrical properties of the CGCs are similar in both the model and the biological cells. These results, in combination with the ability of the model to reproduce accurately the spike shape, spontaneous tonic activity and current-frequency response of the biological CGCs suggest that the model successfully captures essential aspects of the electrophysiological properties of the biological cells and justify its use in a predictive setting.

SIMULATING THE EFFECTS OF CLASSICAL CONDITIONING ON THE ELECTRICAL PROPERTIES OF THE CGCs

During appetitive classical conditioning using a single-trial protocol (Alexander et al., 1984), the resting membrane potential of the CGC soma in trained animals is significantly depolarized at 24 h after conditioning (mean membrane potential increase, 2.5 mV; merged data from left and right CGCs), when compared to measurements taken from unpaired or naïve controls (**Figures 6Ai,ii**; see Nikitin

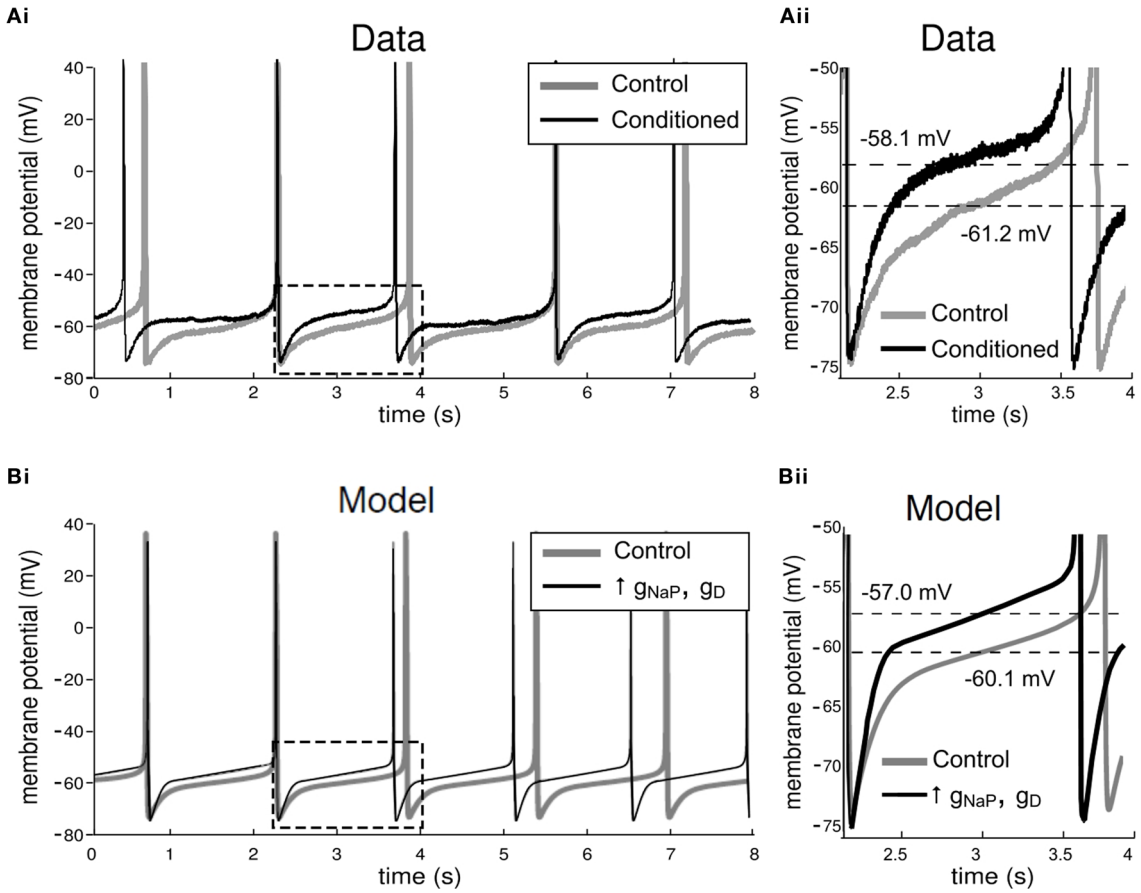

FIGURE 6 | Electrophysiological effects of conditioning in the biological and model CGCs. (A) Effects of conditioning in the biological CGCs. Recordings from CGCs in animals trained using a single-trial classical appetitive conditioning protocol do not show any significant differences in the frequency of the spontaneous firing activity of the cell, when compared to recordings from cells in non-conditioned animals (Example traces shown in Ai, also see Kemenes et al., 2006). However, the membrane potential of CGCs from conditioned animals (measured midway between consecutive spikes and averaged for the whole trace shown) was depolarized, when compared to CGC recordings from non-conditioned control animals (section of **Ai** in dashed rectangle shown in **Aii**). **(B)** Effects of conditioning in the model CGCs. Conditioning in the model was simulated by a balanced increase in g_{NaP} and g_{D}, the maximal conductances of the persistent sodium and delayed rectifier potassium currents respectively. For example, when g_{NaP} was increased by 50%, increasing g_{D} by approximately the same proportion stabilized the spontaneous firing frequency in the model cell at the value it had before increasing the two conductances, i.e., ~0.7 Hz **(Bi)**. A closer inspection of the model CGCs revealed that the membrane potential after the increase was depolarized by 3.1 mV (section of **Bi** in dashed rectangle shown in **Bii**). This increase is comparable to the values measured from the biological cells after conditioning (example in Aii).

et al., 2008 for more details). However, no significant differences were found in the firing frequency of spontaneously generated CGC spikes between trained and control animals (**Figure 6Ai**). Other spike parameters, such as duration, amplitude and after-hyperpolarization also remained unchanged after conditioning (Kemenes et al., 2006; Nikitin et al., 2008).

An interesting question raised during these experiments concerned the ionic mechanisms of the experience-induced changes in the electrical properties of the CGCs, namely the persistent depolarization of the somal membrane potential and the concomitant stabilization of spike frequency and other spike parameters. Here, we have utilized the model developed in the previous sections to test whether specific changes in identified ionic currents are sufficient to explain the electrophysiological effects of conditioning on the membrane of CGCs from trained animals. We initially focused on two ionic currents of the CGC, the persistent sodium current, I_{NaP}, and the delayed rectifier potassium current, I_{D} (Staras et al., 2002). Previous work already has shown that conditioning enhances I_{NaP} (Nikitin et al., 2008). A statistical comparison of the areas under the full I-V curves of I_{D} in CGCs from trained versus control animals has shown that there was no significant global decrease in I_{D} that could have contributed to the learning-induced membrane potential depolarization (Nikitin et al., 2008) but in this previous work I_{D} was not examined in more detail. We therefore now performed pairwise comparisons of discrete I_{D} amplitude data from our original voltage-clamp experiments (Nikitin et al., 2008). This more detailed analysis revealed a consistent learning-induced increase in I_{D} in response to voltage steps from −60 mV to ≥0 mV, reaching statistical significance at +30 mV ($n = 10$ in each group, unpaired t-test, df = 18, $t = 2.43$, $p < 0.03$), indicating a learning-induced increase in the maximal conductance of I_{D}.

In the model, the electrophysiological effects of conditioning were simulated by a balanced increase in the maximal conductances of the persistent sodium and delayed rectifier potassium currents (g_{NaP} and g_D, respectively), such that the firing frequency of the cell before and after the increase remained approximately the same (~0.7 Hz), as was observed experimentally when CGCs from trained and naïve animals were compared (Kemenes et al., 2006; Nikitin et al., 2008). For example, increasing only g_{NaP} by 50% induced a dramatic increase in the firing frequency of the model cell (~15 Hz; data not shown), far beyond the normal operating frequency range of the CGCs. However, if g_D was increased simultaneously by approximately the same proportion, the mean firing frequency of the cell remained stable at ~0.7 Hz (5 spikes over 7 s; **Figure 6Bi**), i.e., within the narrow frequency range between 0.6 Hz and 1 Hz, which is characteristic for CGCs in the type of isolated preparation we used in this study. A closer comparison of the model before and after the enhancement of these two currents revealed that the membrane potential of the model after the enhancement was depolarized by 3.1 mV (**Figure 6Bii**), similarly to biological neurons from trained animals (Nikitin et al., 2008).

The changes in the membrane potential, spike amplitude, spike duration and amplitude of the spike after-hyperpolarization induced by systematically increasing both the persistent sodium and delayed rectifier maximal conductance values in the model are summarized in **Figure 7**. The two conductances were both increased in the range 5% to 50% of their initial values, such that the firing frequency of the cell remained stable at ~0.7 Hz, as explained above (**Figure 7A**). Overall, simulating conditioning by artificially enhancing g_{NaP} and g_D by up to 50% in the model was sufficient to depolarize the membrane by more than 2.5 mV (**Figure 7Bi**), the experimentally measured mean depolarization in axotomized CGCs in isolated CNS preparations from conditioned animals (Nikitin et al., 2008).

The model also faithfully replicated the lack of any significant effect of conditioning on the afterhyperpolarization of CGCs when g_{NaP} and g_D were increased together (**Figure 7Bii**). However, unlike the electrophysiological experiments on CGCs from conditioned and control animals (**Figure 7Ci**, also see Kemenes et al., 2006; Nikitin et al., 2008), when g_{NaP} and g_D were increased together in the model, both the duration and amplitude of the spikes decreased in an approximately linear manner (**Figures 7Biii,iv**). We therefore set up the testable hypothesis that a change in a conductance other than g_{NaP} and g_D may compensate for the changes in spike amplitude and duration caused by these two conductances. When this new hypothesis was tested using the model (**Figure 7Cii**), we found that the change in spike amplitude was completely reversed and the change in spike duration was partially reversed by an appropriate increase (20%) of the identified high-voltage-activated calcium conductance, g_{HVA}, of the CGC (Staras et al., 2002), without affecting the membrane potential (**Figure 7Cii**) or firing frequency of the cell (data not shown).

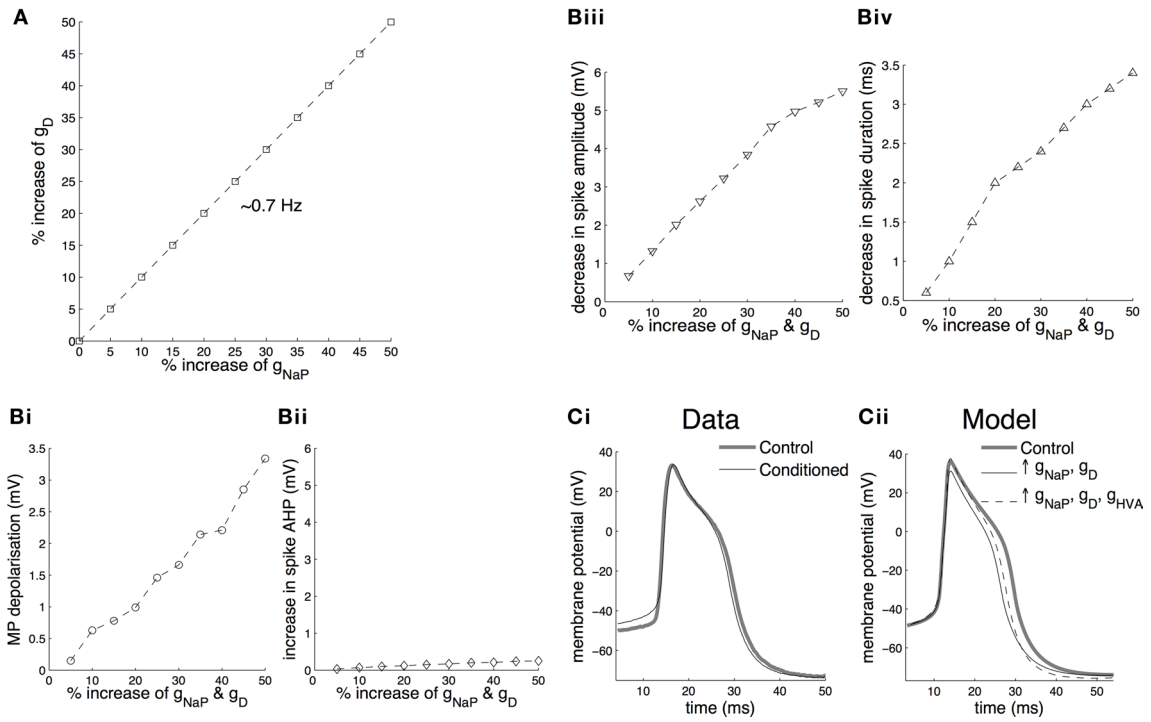

FIGURE 7 | Effects of simulating conditioning on membrane potential and spike shape in the model CGCs. (A) The effect of conditioning in the model CGCs was simulated by a balanced increase in g_{NaP} and g_D such that the spontaneous firing frequency before and after the increase remained approximately the same (~0.7 Hz). Stabilization of the firing frequency after increasing g_{NaP} between 5% and 50% of its initial value required increasing g_D by approximately the same proportion. **(B)** A balanced increase of g_{NaP} and g_D between 5% and 50% of their initial values resulted in a persistent membrane depolarization by more than 2.5 mV (the experimentally observed mean depolarization in axotomized CGCs in isolated CNS preparations; Nikitin et al., 2008) **(Bi)**, virtually no increase in the amplitude of the spike after-hyperpolarization **(Bii)**, a decrease in spike amplitude by ~6 mV **(Biii)** and a decrease in spike duration by ~3.5 ms **(Biv)**. **(C)** Changes in spike amplitude and duration, however, do not occur after classical conditioning (example traces in **Ci**, also see Kemenes et al., 2006). These changes were fully (spike amplitude) or partially (spike duration) reversed by an appropriate increase (20%) of g_{HVA} (**Cii**).

In summary, the above results demonstrate that our model is able to replicate the conditioning-induced persistent membrane depolarization and concomitant stabilization of the firing frequency in the CGCs by a balanced increase of the persistent sodium and delayed rectifier potassium currents. This, in combination with previously published experimental data (Nikitin et al., 2008) and the results of the new analysis described in the Results in Supplementary Material, suggests that there is a causal link between experience-induced changes in these two conductances and the electrical properties of the CGCs that underlie the formation of long-term associative memory in *Lymnaea* during conditioning. Importantly, the application of the model resulted in setting up the hypothesis that the high-voltage-activated calcium current, I_{HVA}, also needs to be modified to fully stabilize the shape of the spike.

DISCUSSION

Although a number of previous papers have already demonstrated learning-induced intrinsic changes in ionic conductances in both vertebrate and invertebrate preparations (Debanne et al., 2003; Magee and Johnston, 2005; Zhang and Linden, 2003), none of them addressed the issue of stability of key neuronal functions after learning, and specifically, after classical conditioning. Here, we established how a neuron can undergo learning-induced intrinsic plasticity of some of its key electrical properties (e.g. membrane potential) without consequent changes in other equally important electrical properties (e.g. spike frequency) essential for its basic network functions. To achieve this goal, we used a computational modeling approach based on both voltage- and current-clamp data obtained in the same identified neuron type in preparations from both classically conditioned and control animals. The CGC neuron of *Lymnaea* was a highly suitable model system to use for this analysis because (i) to fulfill its key modulatory role in the snail feeding network it has to fire in a particular frequency range (between ~ 0.6 and 1 Hz in isolated CNS preparations (Yeoman et al., 1994) and (ii) this firing frequency is retained even after the neuron has undergone plastic changes affecting its membrane potential (Kemenes et al., 2006). Our new work provides the first mechanistic explanation of how this difficult task is achieved by balanced learning-induced changes in specific ionic conductances.

Analysis of the Hodgkin-Huxley-type model of the CGCs we have built revealed that plastic changes in two identified currents, I_{NaP} and I_D, were sufficient to mimic the previously recorded modifications of the intrinsic electrical properties of these neurons during single-trial behavioral conditioning experiments, i.e., a persistent membrane depolarization with a parallel stabilization of the firing frequency (Kemenes et al., 2006). In addition, the model predicted that the high-voltage-activated calcium current, I_{HVA}, is also likely to be enhanced in the CGCs as a result of conditioning. However, we found that if the simulated increase in I_{HVA} exceeded 20%, when it compensated for around 40% of the spike narrowing effect of the enhancement of I_{NaP} and I_D, it also started to affect spike amplitude. It will therefore need to be investigated whether changes in other currents might also contribute to further compensatory spike broadening after increasing I_{HVA}, as well as I_{NaP} and I_D. One possible candidate mechanism is a learning-induced decrease in a putative calcium-activated TEA-insensitive potassium current of the CGC ($I_{K(Ca)}$), which only shows a significant activation at high voltage step levels (from –60 mV to –20 mV and above; Staras et al., 2002). Because of the rare occurrence of this current, it was not characterized in further detail in previous work (Staras et al., 2002) and therefore was not incorporated in our model. Whether I_{HVA} and $I_{K(Ca)}$ are actually modified by classical conditioning, will be tested in future experiments, which can also lead to further refinement of the current model.

There are a number of important previous examples where long-lasting depolarization occurs after *in vitro* or i*n vivo* training or activation of second messenger cascades with no underlying net change in input resistance (e.g., Swandulla and Lux, 1984; Kemenes et al., 1993; Ross and Soltesz, 2001; Jones et al., 2003). A lack of a change in input resistance also was observed in the depolarized *Lymnaea* CGCs after behavioral classical conditioning (Kemenes et al., 2006). Our recent voltage clamp studies (Nikitin et al., 2008) and the computational modeling described in this paper showed that in the CGCs the depolarization is predominantly driven by an enhanced persistent sodium current with increases in potassium and calcium conductances preventing changes in spike frequency and spike shape, respectively. Swandulla and Lux (1984) showed that a cAMP-induced increase in a sodium conductance in *Helix* neurons is compensated for by a decrease in a potassium conductance leading to no net change in input resistance near the resting potential. This is clearly not the case in the CGCs after learning. We therefore have to speculate that a so far unidentified conductance decreases after learning and this decrease compensates for the increase in the persistent sodium conductance. For example, there may be a learning-induced decrease in a chloride conductance resulting from the previously observed depolarization-induced increase in baseline levels of intracellular calcium in the CGC (Kemenes et al., 2006). Previous work already has linked a depolarization-induced rise in intracellular calcium levels in *Lymnaea* neurons to decreases in a chloride conductance (Vulfius et al., 1998) but further experiments will need to be performed to establish if this also happens in the CGCs after classical conditioning.

In the biological CGCs the learning-induced depolarization can be quite variable depending on the type of preparation used and time of test after training. Kemenes et al., (2006) used semi-intact preparations with all the lip chemosensory structures and nerves intact. In these preparations the shift at 24 h after training was ~ 5 mV (with a stable CGC firing rate), whereas in isolated CNS preparations tested at 24 h post-training it was ~ 2.5 mV (Nikitin et al., 2008; the paper that provided the learning-related voltage-clamp data for the model). **Figures 7A,Bi** together show that a balanced change in I_{NaP} and I_D can explain stable firing even when the membrane potential is shifted by more than 2.5 mV. We cannot rule out however that in the semi-intact preparations and indeed, in the intact animals, external modulatory inputs from the chemosensory pathways also contribute to both membrane potential depolarization and spike frequency stabilization. This is particularly likely to be the case at 14 days after training when in semi-intact preparations the shift was in the range of 10 mV (Kemenes et al., 2006).

An important issue in the construction of biologically realistic neuronal models is estimating the values of the various parameters that appear in the model, based on available electrophysiological data (Prinz et al., 2003; Huys et al., 2006; Haufler et al., 2007; Hobbs and Hooper, 2008). In the present paper, we successfully used a combination of voltage- and current-clamp recordings for estimating a large number of unknown parameters in our model, including maximal conductances, reversal potentials and the parameters governing the activation and inactivation kinetics of all the voltage-gated ionic currents that have been identified in the CGCs. The parameter estimation method we applied permitted the construction of a model capable of reproducing the spike shape, spontaneous firing activity and current-frequency response of the biological neurons with high accuracy. In addition, the model simulated successfully the effects of various pharmacological agents on the electrical properties of these cells by selectively blocking identified ionic currents in the model and comparing the results of these simulations to independent experimental data (Staras et al., 2002). These results confirm that the model captures essential aspects of the electrophysiology of the biological CGCs, thus being a useful predictive/analytical tool in mechanistic analyses of neuronal plasticity.

The only other examples of realistic modeling in the *Lymnaea* nervous system refer to the feeding CPG interneurons (Vavoulis et al., 2007) and a feeding motoneuron type, B1 (Vehovszky et al., 2005). In the first case (Vavoulis et al., 2007), two-compartment models of three important feeding CPG interneurons (N1M, N2v and N3t) and an identified modulatory interneuron, the Slow Oscillator (SO), were constructed. These models were then organized into a network that resembled important aspects of the *Lymnaea* feeding CPG, both in terms of network topology and function. In this network model, the individual cells were of the Hodgkin-Huxley type and mimicked sufficiently well the electrical properties of their biological counterparts, but unlike the CGC model presented here, the ionic currents included in these neuronal models were not, in most cases, the result of direct observation (e.g., through voltage-clamp experiments), but rather were inferred from the analysis of an extensive set of current-clamp recordings capturing the characteristic patterns of electrical activity expressed by the biological neurons.

In the case of the B1 motorneuron, the model was constructed in order to study the effects of the biogenic amine octopamine on neuronal excitability (Vehovszky et al., 2005). From a methodological point of view, both the B1 and the CGC models are single-compartment and they follow a similar mathematical formalism. One difference is that the B1 model contained three voltage-gated ionic currents (a persistent potassium current, a transient potassium current and a transient sodium current), which were the major ionic currents found in the B1 motoneurons (Vehovszky et al., 2005). Importantly, in the B1 model the parameters for the individual ionic currents were estimated mainly from analysis of voltage-clamp data, while in the case of the CGC model, we used a combination of voltage-clamp and current-clamp data. This made it possible to replicate the shape of the action potentials and the spontaneous firing activity of the biological neuron with very high fidelity.

COMPARISON WITH OTHER SYSTEMS AND LIMITATIONS OF THE MODEL

One of the early examples of biophysical neuronal modeling in invertebrates was a mathematical model of the lateral pyloric (LP) neuron in the crustacean stomatogastric ganglion (Buchholtz et al., 1992; Golowasch et al., 1992). The model contained all the major ionic currents identified through electrophysiological (voltage-clamp) analysis (Golowasch and Marder, 1992) and it mimicked successfully the basic activity of the biological neuron. A more recent modeling study (Nowotny et al., 2008) employed an automatic optimization method to fit a model of the LP neuron against a rich set of current-clamp data, enabling the simulation of the dynamic behavior of the biological neuron over a wide range of conditions. In the present paper, we first optimized the model against extensive voltage-clamp data, and then fitted a single spike from current clamp data. The ability of our model to reproduce the dynamic behavior of the biological neuron, e.g., its response to injected current and pharmacological agents and classical conditioning, emerged as a result of this initial optimization process against a combination of voltage- and current-clamp recordings.

In vertebrates, detailed computational models were used to study the contribution of a persistent sodium current to membrane excitability in CA1 hippocampal pyramidal cells (Vervaeke et al., 2006) and small dorsal root ganglion neurons (Herzog et al., 2001). In the latter study, the computer simulation showed that the persistent TTX-resistant sodium current, which is very similar to I_{NaP} in the CGCs, had a strong depolarizing influence on the resting membrane potential. Both our computational model and previous experimental work (Nikitin et al., 2008), have demonstrated a similar link between I_{NaP} and membrane potential in the CGCs. These observations suggest that a causal relationship between persistent sodium conductances and membrane potential is conserved between invertebrate and vertebrate neurons.

An important limitation is that the CGC model does not include at the moment any second-messenger cascades or intracellular calcium dynamics. A consideration of these processes will have to be included in further modeling in order to fully understand the mechanisms involved in the more dynamic properties of the CGCs, such as the ability of these cells to keep their firing frequency relatively stable in the face of external and internal perturbations. We hypothesize that this homeostatic capability of the CGCs relies upon activity-dependent mechanisms, which typically employ the concentration of intracellular calcium as an indicator of the level of electrical activity in the neuron (Marder and Prinz, 2002). Also, it has been shown that injection of cAMP in the CGC soma induces prolonged (lasting several hours) plastic changes of the electrical properties of the CGCs, including a significant enhancement of the persistent sodium current, increased bursting, a significant depolarization of the somatic potential and decreased input resistance (Nikitin et al., 2006). Thus, it is likely that processes dependent on the cAMP second-messenger cascade are implicated in the expression of long-term neuronal plasticity in the CGCs. It follows that modeling intracellular calcium dynamics and cAMP-dependent second-messenger pathways, as well as their interactions with ionic currents in the cell membrane will improve the realism of the CGC model and, therefore, should be the subject of future model development.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/behavioralneuroscience/paper/10.3389/fnbeh.2010.00019/

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Balanced plasticity and stability of the electrical properties of a molluscan modulatory interneuron after classical conditioning: a computational study

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MATERIALS AND METHODS

MATHEMATICAL FRAMEWORK

The CGCs were modeled as a single-compartment Hodgkin-Huxley-type neuron containing all the voltage-dependent ionic currents that have been previously identified in these cells (Staras et al., 2002; Nikitin et al., 2006). The current conservation equation for the CGCs model took the following form:

$$C_m \frac{dV}{dt} = -\overbrace{\left(I_{NaT} + I_{NaP}\right)}^{sodium} - \overbrace{\left(I_A + I_D\right)}^{potassium} - \overbrace{\left(I_{LVA} + I_{HVA}\right)}^{calcium} \quad \text{(S1)}$$

where $C_m = 1\ \mu F/cm^2$ is the capacitance of the cell membrane. I_{NaT} and I_{NaP} are the transient and persistent sodium currents, I_A and I_D are the transient A and delayed rectifier potassium currents and I_{LVA} and I_{HVA} are the low-voltage-activated and high-voltage-activated calcium currents, respectively. Each of these currents is modeled as follows:

$$I_{NaT} = g_{NaT} \cdot m_\infty^3 \cdot h \cdot (V - E_{Na}) \quad \text{(S2)}$$

$$I_{NaP} = g_{NaP} \cdot r^3 \cdot (V - E_{Na}) \quad \text{(S3)}$$

$$I_A = g_A \cdot a^4 \cdot b \cdot (V - E_K) \quad \text{(S4)}$$

$$I_D = g_D \cdot n^4 \cdot (V - E_K) \quad \text{(S5)}$$

$$I_{LVA} = g_{LVA} \cdot c_\infty^3 \cdot d_\infty \cdot (V - E_{Ca}) \quad \text{(S6)}$$

$$I_{HVA} = g_{HVA} \cdot e^3 \cdot f \cdot (V - E_{Ca}) \quad \text{(S7)}$$

where E_{Na}, E_K and E_{Ca} are the reversal potentials (in mV) of the sodium, potassium and calcium currents, respectively and g_{NaT}, g_{NaP}, g_A, g_D, g_{LVA} and g_{HVA} are the maximal conductances (in mS/cm^2) of the respective currents.

The dynamic variables *r, a, n, e* and *h, b, f in* Eqs S2–S7 model respectively the activation and inactivation of the corresponding currents and obey first-order relaxation kinetics:

$$\frac{dx}{dt} = \frac{x_\infty - x}{\tau_x} \quad \text{(S8)}$$

where $x = h, r, a, b, n, e$ or f. In the previous equation, the steady-states x_∞ and relaxation times τ_x for each dynamic variable were modeled as functions of the membrane potential (Willms et al., 1999):

$$x_\infty = \left(1 + \exp\left(\frac{V_H^x - V}{V_S^x}\right)\right)^{-1} \quad \text{(S9)}$$

and

$$\tau_x = \tau_o^x \cdot \exp\left(\delta^x \frac{V_H^x - V}{V_S^x}\right) \cdot \left(1 + \exp\left(\frac{V_H^x - V}{V_S^x}\right)\right)^{-1} \quad \text{(S10)}$$

where V_H^x is given in mV; V_S^x has units mV^{-1} and it is positive for an activation variable and negative for an inactivation one; δ^x is given without units and takes values between 0 and 1; τ_o^x is in ms and takes strictly positive values. The fast activation of I_{NaT} (m_∞) and the fast activation and inactivation of I_{LVA} (c_∞ and d_∞, respectively) were modeled using Eq. S9 as sigmoid functions of the membrane potential. The exponent x in parameters V_H^x, V_S^x, δ^x and τ_o^x indicates the dynamic variable these parameters refer

to. For example, the parameter V_H^r refers to the dynamic variable *r*, which models the activation of the persistent sodium current, I_{NaP} (Eq. S3).

MODEL OF THE PERSISTENT SODIUM CURRENT UNDER VOLTAGE-CLAMP CONDITIONS

In a nominally calcium-free saline with blockers for calcium and potassium currents added, voltage steps in the range from −90 mV to +30 mV from a holding membrane potential of −110 mV induce sodium currents in voltage-clamped CGCs from the intact nervous system of *Lymnaea* animals, which persist for the total duration of 800 ms-long steps without showing significant inactivation (Nikitin et al., 2006). The expression for the equilibrium current measured at the end of the 800 ms-long steps is given by replacing r_∞, for *r* in Eq. S3:

$$I_{\infty,NaP} = g_{NaP} \cdot \left(1 + \exp\left(\frac{V_H^r - V}{V_S^r}\right)\right)^{-3} \cdot (V - E_{Na}) \tag{S11}$$

By fitting this expression to the equilibrium currents at the end of the 800 ms-long voltage steps (Figure 1Ai in main text), we estimated the values of the parameters V_H^r and V_S^r and, thus, the steady-state activation of the persistent sodium current, r_∞, as illustrated in Figure 1Aii in the main text.

MODEL OF THE TOTAL CALCIUM CURRENT UNDER VOLTAGE-RAMP CONDITIONS

In zero-sodium saline in the presence of 50 mM TEA and 4 mM 4-AP, application of a voltage-ramp protocol changing the voltage from −100 mV to +30 mV over a time interval of 120 ms induces an inward calcium current with two clearly distinct components in CGCs in the intact nervous system of *Lymnaea* animals (Staras et al., 2002). These two components correspond to the low-voltage-activated and high-voltage-activated calcium currents as illustrated in Figure 1Bii in main text. Assuming that the activation of the high-voltage-activated component is much faster than its inactivation, the expression for the total inward current induced during the voltage ramp, $I_{LVA} + I_{HVA}$, is as follows:

$$\begin{aligned} I_{LVA} + I_{HVA} = \ldots \\ g_{LVA} \cdot \left(1 + \exp\left(\frac{V_H^c - V}{V_S^c}\right)\right)^{-3} \cdot d_\infty \cdot (V - E_{Ca}) + \ldots \\ g_{HVA} \cdot \left(1 + \exp\left(\frac{V_H^e - V}{V_S^e}\right)\right)^{-3} \cdot (V - E_{Ca}) \end{aligned} \tag{S12}$$

By fitting this expression to the experimental data (Figure 1Bii in the main text), we estimated the values of the parameters V_H^c, V_S^c, V_H^e and V_S^e, thus determining the steady-state activation of the low-voltage-activated and high-voltage-activated currents, c_∞ and e_∞ respectively, as illustrated in Figure 1Biii in main text. The steady-state inactivation of these two currents:

$$d_\infty = \left(1 + \exp\left(\frac{V_H^d - V}{V_S^d}\right)\right)^{-1} \quad \text{and} \quad f_\infty = \left(1 + \exp\left(\frac{V_H^f - V}{V_S^f}\right)\right)^{-1} \tag{13}$$

were estimated in advance from independent experimental data, as illustrated in Figure 1Bi in main text.

MODEL OF THE TOTAL POTASSIUM CURRENT UNDER VOLTAGE-CLAMP CONDITIONS

In CGCs from the intact nervous system, voltage-step protocols from a holding membrane potential of −90 mV to voltages from −80 mV to +35 mV reveal two outward currents, an early transient one corresponding to I_A and a delayed persistent one, which lasts for the duration of the step and corresponds to the delayed rectifier, I_D (Figure 1Ci in main text; Staras et al., 2002). The total potassium current induced during a single voltage step to membrane potential *V* is modeled as follows:

$$I_A + I_D = (g_A \cdot a^4 \cdot b + g_D \cdot n^4) \cdot (V - E_K) \tag{S13}$$

where *a, b* and *n* are functions of time given by the following expressions:

$$a(t) = a_\infty + (a_0 - a_\infty) \cdot \exp\left(-\frac{t}{\tau_a}\right) \tag{S14}$$

$$b(t) = b_\infty + (b_0 - b_\infty) \cdot \exp\left(-\frac{t}{\tau_b}\right) \tag{S15}$$

$$n(t) = n_\infty + (n_0 - n_\infty) \cdot \exp\left(-\frac{t}{\tau_n}\right) \tag{S16}$$

In the previous equations, the quantities x_∞ and τ_x (where *x* is *a, b* or *n)* are modeled by Eq. S9 and S10 as follows:

$$a_\infty = \left(1 + \exp\left(\frac{V_H^a - V}{V_S^a}\right)\right)^{-1} \quad \text{and}$$

$$\tau_a = \tau_o^a \cdot \exp\left(\delta^a \frac{V_H^a - V}{V_S^a}\right) \cdot \left(1 + \exp\left(\frac{V_H^a - V}{V_S^a}\right)\right)^{-1}$$

$$b_\infty = \left(1 + \exp\left(\frac{V_H^b - V}{V_S^b}\right)\right)^{-1} \quad \text{and}$$

$$\tau_b = \tau_o^b \cdot \exp\left(\delta^b \frac{V_H^b - V}{V_S^b}\right) \cdot \left(1 + \exp\left(\frac{V_H^b - V}{V_S^b}\right)\right)^{-1}$$

$$n_\infty = \left(1 + \exp\left(\frac{V_H^n - V}{V_S^n}\right)\right)^{-1} \quad \text{and}$$

$$\tau_n = \tau_o^n \cdot \exp\left(\delta^n \frac{V_H^n - V}{V_S^n}\right) \cdot \left(1 + \exp\left(\frac{V_H^n - V}{V_S^n}\right)\right)^{-1}$$

In Eqs S14–S16, a_0, b_0 and n_0 are equal to a_∞, b_∞ and n_∞, respectively for $V = -90$ mV, the holding membrane potential. By fitting Eqs S13–S16 to the experimental data (Figure 1Ci in main text) we estimated parameters V_H^x, V_S^x, τ_o^x and δ^x ($x = a,b,n$), which govern the steady-states and relaxation times for the activation and inactivation of the transient and delayed rectifier potassium currents, as illustrated in Figures 1Cii,iii in main text.

FITTING THE FULL CGC MODEL TO CURRENT-CLAMP DATA

In the model, 18 of the 43 parameters were estimated by exploiting voltage-clamp data as explained above. The values of the remaining parameters (e.g. reversal potential and maximal conductance) however could be estimated more accurately from current-clamp compared to voltage-clamp data. For example, the sodium current reversal potential measured in voltage-clamp tests was around +30 mV (Nikitin et al., 2006), whereas in most current-clamp tests the action potential peaks at around +40 mV, indicating that the true value of the reversal potential is nearer to +50 mV than to +30 mV. The data measured in the voltage-clamp tests at very positive voltage steps may have been affected by the activation of potassium currents, which are almost impossible to block fully, even with a mixture of 4-AP and TEA. These parameters therefore were estimated by fitting the whole-cell model to current-clamp data (Figure 2 in main text), using an efficient non-linear least-squares algorithm, implemented in the function *lsqcurvefit* from the MATLAB® Optimization Toolbox. In summary, at each iteration of the optimization algorithm, the whole-cell model was numerically solved and the calculated membrane potential was compared against an experimentally measured 1.9-s long voltage trace, which was recorded under current-clamp conditions (Figure 2A in main text). Based on the discrepancy between the theoretical and the experimental membrane potential, the algorithm modified the values of the unknown parameters in order to minimize this discrepancy. The process was repeated until a sufficient level of coincidence between the model and experimental data was achieved or a maximum number of iterations was exceeded. The parameter values that were previously estimated from voltage-clamp data were kept fixed, with the exception of the parameters governing the activation kinetics of I_D (V_H^n, V_S^n, τ_o^n, δ^n), which were further adjusted at this stage.

The basic insight in fitting the full CGC model to current clamp data with this method was using the experimentally recorded voltage trace under current clamp as a forcing term (Huys et al., 2006; Haufler et al., 2007), which permitted decoupling the system of ODEs that model the neuron (Eqs S1 and S8) and solving each of them independently and efficiently. Since the least-squares algorithm we used does not guarantee convergence to a global minimum, we repeated the optimization process starting from a large number of random initial values of the unknown parameters in order to exclude finding only local solutions (see Figure 3 in main text and associated text).

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Sucrose acceptance and different forms of associative learning of the honey bee (*Apis mellifera* L.) in the field and laboratory

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The experiments analyze different forms of learning and 24-h retention in the field and in the laboratory in bees that accept sucrose with either low (≤3%) or high (≥30% or ≥50%) concentrations. In the field we studied color learning at a food site and at the hive entrance. In the laboratory olfactory conditioning of the proboscis extension response (PER) was examined. In the color learning protocol at a feeder, bees with low sucrose acceptance thresholds (≤3%) show significantly faster and better acquisition than bees with high thresholds (≥50%). Retention after 24 h is significantly different between the two groups of bees and the choice reactions converge. Bees with low and high acceptance thresholds in the field show no differences in the sucrose sensitivity PER tests in the laboratory. Acceptance thresholds in the field are thus a more sensitive behavioral measure than PER responsiveness in the laboratory. Bees with low acceptance thresholds show significantly better acquisition and 24-h retention in olfactory learning in the laboratory compared to bees with high thresholds. In the learning protocol at the hive entrance bees learn without sucrose reward that a color cue signals an open entrance. In this experiment, bees with high sucrose acceptance thresholds showed significantly better learning and reversal learning than bees with low thresholds. These results demonstrate that sucrose acceptance thresholds affect only those forms of learning in which sucrose serves as the reward. The results also show that foraging behavior in the field is a good predictor for learning behavior in the field and in the laboratory.

Keywords: *Apis mellifera*, sucrose acceptance, color learning, olfactory PER conditioning, retention, hive entrance learning, reward value

INTRODUCTION

In their natural environment honey bees rapidly learn the features and locations of profitable floral sources and are able to remember this information for a long period of time (von Frisch, 1965; Waser, 1986). Associative learning of individual bees is a prerequisite for the exploitation of profitable food sources and plays a pivotal role in the organization and division of labor in a bee colony (for a review see Page and Erber, 2002). Nectar is the main food source of honey bee colonies. It also serves as a reward for individual forager bees and reinforces the association of sensory signals with nectar resources (von Frisch, 1965; Menzel and Müller, 1996). Honey bees are able to discriminate the different sugar components in nectar and prefer sucrose to most other naturally occurring sugars (Barker and Lehner, 1974). Therefore, most learning protocols with bees in the field or in the laboratory use sucrose solutions as the reward.

The physiological mechanisms of learning in the bee have been investigated using different learning protocols under laboratory conditions. In the laboratory bees can be conditioned to olfactory, visual and tactile cues. For olfactory conditioning the proboscis extension response (PER) protocol with harnessed bees has been used for over six decades (Frings, 1944; Vareschi, 1971; Bitterman et al., 1983). This protocol is based on the PER which can be elicited by stimulation of the antennae with sucrose. When presentation of an odor is paired with sucrose stimulation of the antennae and proboscis, the bee responds after 2–5 pairings with the conditioned PER. There exist also different protocols for color conditioning of honey bees in the laboratory which either need a large number of learning trials (Masuhr and Menzel, 1972) or removal of the antennae (Hori et al., 2006) or the flagellae (Niggebrügge et al., 2009). Very effective laboratory conditioning protocols were developed for tactile PER conditioning and for operant conditioning of antennal movements (Erber et al., 1998; Kisch and Erber, 1999; Kisch and Haupt, 2009).

Individual bees can differ significantly in their sensitivities for sucrose which can be estimated under laboratory conditions using the sucrose concentration dependent PER (Page et al., 1998; Scheiner et al., 2003). In these experiments a harnessed bee is stimulated with increasing concentrations of sucrose and it is registered at which concentrations PER occurs (Page et al., 1998). Sensitive bees respond at low sucrose concentrations or even when stimulated with water, while insensitive bees show the PER only for higher concentrations of sucrose. Individual thresholds for sucrose or individual sucrose sensitivity in bees can be estimated using these tests (Page et al., 1998). Sucrose sensitivity is strongly correlated with the foraging task specialization of a bee. Pollen and water foragers have the highest sensitivity for sucrose, while nectar foragers have

Abbreviations: GRS, gustatory response score; PER, proboscis extension response.

a lower sensitivity (Pankiw and Page, 2000). In the field individual nectar bees accept different sucrose concentrations while foraging at a food site (von Frisch, 1927; Núñez, 1966; Mujagic and Erber, 2009). Very sensitive bees collect low sucrose concentrations and even water, while insensitive bees collect only high sucrose concentrations (Mujagic and Erber, 2009). Acceptance for sucrose in the field and sucrose responsiveness of the same bees in the laboratory show only partial correlation (Mujagic and Erber, 2009). Nectar foragers which differ significantly in their sucrose acceptance in the field can show similar sucrose responsiveness in the laboratory, suggesting that acceptance measured in the field is a more sensitive parameter than the concentration dependence of the PER measured in the laboratory (Mujagic and Erber, 2009).

The individual differences of sucrose responsiveness have consequences for learning behavior. As sucrose is the reward in most conditioning protocols in the field and in the laboratory, the reward value during conditioning is different in bees that have low or high sucrose sensitivity. The relations between sucrose sensitivity and learning were analyzed in a series of laboratory studies using different classical and operant conditioning protocols (Scheiner et al., 1999, 2001a,b, 2003, 2005). The laboratory experiments demonstrated that sucrose PER sensitivity is correlated with learning performance. Bees which were highly responsive to sucrose stimuli applied to the antennae had significantly better acquisition and retention than bees with low sucrose sensitivity. These experiments support the hypothesis that the reward value of sucrose during associative learning depends on the individual sensitivity for sucrose (Scheiner et al., 2005). It is not clear at the moment whether the rules between sucrose sensitivity and associative learning that were found under laboratory conditions are also valid in the field. A correlation between sucrose sensitivity in the field and associative learning at a food site would have significant consequences for the division of foraging labor within a colony.

It is the main goal of the present study to analyze the effects of different sucrose sensitivities on color learning in the field. We want to compare color learning at a food site where sucrose serves as a reward with color learning at the hive entrance which functions without sucrose reward. With these experiments we want to test two alternative hypotheses concerning the relations between sucrose sensitivity and learning. If differences in learning performance were only found with a sucrose reward, this would imply that different reward values cause differences in learning performance and memory formation. If differences in learning performance can be found also when sucrose is not the reward, this would imply that different sensory sensitivities also affect the physiological mechanisms of learning. Color learning at a food site is a good protocol to test the effects of sucrose rewards on learning performance in the field. Free flying bees learn color signals very fast. They reach the asymptote of the acquisition curve after 3–10 rewards and can discriminate the conditioned color very efficiently from an unrewarded alternative (Menzel, 1969; Erber, 1975a). Even very short sucrose rewards lasting only 100 ms are sufficient for successful association (Erber, 1975a,b). In the hive entrance color learning protocol sucrose does not serve as a reward. The bees learn that a visual cue signals an open entrance to the hive. The return to the colony functions as the reward in this protocol. This paradigm has been successfully used to analyze visual learning, discrimination and route memory in different hymenoptera (Schremmer, 1941; Beier and Menzel, 1972; Chittka and Menzel, 1992; Harris et al., 2005; Zhang et al., 2006).

It is another goal of this study to investigate whether different sucrose sensitivities in the field have consequences for learning performance in the laboratory. Previous experiments have shown that sucrose sensitivity measured with the PER test affects learning performance under laboratory conditions (Scheiner et al., 1999, 2001a,b, 2003, 2005). It is so far not known whether differences of sucrose acceptance in the field have similar effects on learning performance in the laboratory. For learning studies under laboratory conditions we decided to use olfactory PER conditioning because similar to the conditions in the field, bees can be conditioned to a single sensory cue and then tested with two alternative cues. Recently published color conditioning protocols for the laboratory (Hori et al., 2006; Niggebrügge et al., 2009) could not be used for these experiments because the bees have to be kept for at least 1 day in tubes and the antennae or parts of the antennae have to be cut off before conditioning. Under these conditions it is impossible to measure sucrose responsiveness in the laboratory. It is also impossible to correlate sucrose acceptance in the field with behavior in the laboratory that is measured 1 or 2 days after the field tests.

In the last years laboratory experiments on sensory sensitivity in bees were used to develop hypotheses concerning foraging and learning behavior in a natural environment (Scheiner et al., 2004). With the present study we want to examine whether the findings from the laboratory are valid also under field conditions and whether sucrose sensitivity in the field affects learning in free flying (color learning at food site) and harnessed bees (PER odor conditioning). So far, sucrose served as a reward in all experiments analyzing the relations between sucrose sensitivity and learning. With this study we want to examine for the first time whether bees with different sucrose acceptance also show differences in learning performance when sucrose is not the reward (hive entrance color conditioning). These experiments will bridge the gaps between behavioral studies under laboratory and field conditions. They will also test whether conclusions which were developed for learning under laboratory conditions hold in the natural environment.

MATERIALS AND METHODS

We used three different learning protocols in which bees were either conditioned to a color in the field or to an odor in the laboratory. In all three protocols a single sensory cue is rewarded and discrimination is tested in dual choice tests by offering simultaneously two alternative colors or sequentially two different odors. In a first set of experiments we studied color conditioning at a food site in the field. We measured acquisition, discrimination and 24-h retention in free flying bees which either accepted ≥50% (w/w) or ≤3% (w/w) sucrose in the field. In a second set of experiments we selected free flying bees with sucrose acceptance thresholds ≥50% (w/w) or ≤3% (w/w). The bees were then transferred to the laboratory for experiments using the olfactory PER conditioning protocol. Similar to the learning experiments at the food site, acquisition, discrimination and 24-h retention were analyzed in these bees. In the third set of experiments we tested free flying bees which had different sucrose acceptance (≥30% or ≤3%) in a hive entrance color learning protocol which functions without sucrose reward.

SELECTING BEES WITH DIFFERENT ACCEPTANCE THRESHOLDS

For the color learning experiments in the field and the olfactory PER conditioning experiments in the laboratory we used bees from a large hive. At the beginning of an experiment acceptance thresholds for sucrose were measured in individual bees. Honey bees from the colony were trained to visit a food site in a green house approximately 50 m away from the hive. The bees visiting the feeder were marked individually with color-coded spots on the thorax and abdomen. The feeder contained 50% (w/w) sucrose solution. To determine the individual acceptance threshold, the concentration of the sucrose solution at the feeder was reduced in steps (for details see also Mujagic and Erber, 2009). All the experiments were performed with newly alarmed nectar foragers, while bees belonging to the long-term foraging group that visited the food site regularly, were caught and kept in a box for the duration of the experiment. The newly alarmed bees were offered each of the following concentrations for 20 min: 50%, 30%, 10% and 3% (w/w). The exact sucrose concentration was adjusted using a refractometer (N-50 E, Atago, Tokyo, Japan). It was recorded at which concentrations an individual bee visited the feeder. After testing each concentration for 20 min, the bees were assigned to one of two groups. Bees which accepted sucrose concentrations of 50% (w/w) but no smaller concentrations were assigned to the "high threshold" or "insensitive" group (≥50% acceptance group). Bees which accepted a concentration of 3% (w/w) were assigned to the "low threshold" or "sensitive" group (≤3% acceptance group). Bees which accepted 30% (w/w) or 10% (w/w) but no lower concentrations were not used for the color learning experiments at the food site or the olfactory conditioning experiments. After the tests of acceptance, a single bee was selected if color learning at the food site was analyzed. For the olfactory conditioning experiments in the laboratory several bees with acceptance thresholds of ≥50% or ≤3% were caught at the feeder after drinking for 20 s either the 50% or 3% sucrose solution. The bees were placed in individual glass vials and transported to the laboratory (for details see Experiment 2: Olfactory PER conditioning in the laboratory)

EXPERIMENT 1: COLOR LEARNING AT A FOOD SITE IN THE FIELD

At random a single, newly alarmed bee which belonged either to the ≥50% or the ≤3% acceptance group was chosen and used for the following experiments. All other bees visiting the feeder were caught and kept in a box during the experiment. Similar to the study of Menzel (1967) we used horizontal color cues for conditioning and testing. The cues were produced by LEDs illuminating semitransparent Plexiglas®-plates (10 × 10 cm) from below. Homogenous illumination was produced by placing each plate on a 5 cm high cylinder which had 40 LEDs arranged in two stripes around the inner circumference. The LEDs emitted either green light (λ = 525 nm; LM 10A-T2-LINEARlight Flex, 24V–1200 lm–72W, Osram GmbH, Munich, Germany) or blue light (λ = 470 nm; LM 10A-B2-LINEARlight Flex, 24V–460 lm–48W, Osram GmbH, Munich, Germany). Four independent LED illumination units each driven by a constant current source were used. The experiments were performed on a white table (100 × 100 cm) inside a shady green house which had a window to allow bees to fly in and out. To avoid olfactory cues due to scent marking of the bees, the plates used for conditioning and testing were cleaned regularly with 70% ethanol and water. The illumination units used during the conditioning trials were never used during the unrewarded test trials. For conditioning an illumination unit with green light was placed in the center of the table and a small feeder containing 50% sucrose solution was placed on the plate. The bee could land on the green plate with the feeder and drink the solution *ad libitum*. For the color choice tests two units, one illuminated with green and the other with blue light, were placed approximately 50 cm apart from each other on the table. Each of the units had an empty feeder in the center of the plate.

At the beginning of the experiment spontaneous choice preference for the two colors was tested. Two light sources with green and blue illumination were placed on the table and for 1 min it was registered how often the experimental bee approached each of the two color alternatives. Then the positions of the two color plates were exchanged and behavior was registered again for 1 min. An approach was counted when the bee flew over the illuminated plate or hovered around the feeder in the center. Landings on the plates were counted separately but as bees did not land frequently, this measure was not used for evaluation of the experiments. After measuring spontaneous choice, the bee was conditioned for the first time. Irrespective of the acceptance group to which a bee belonged, it was rewarded with 50% sucrose in the feeder on a green light source positioned in the middle of the table. The bee flew back to the hive and color choice was tested after it returned to the test site in the same way as described for spontaneous choice. Each bee was conditioned seven times and color choice was tested after each conditioning trial when the bee returned to the set-up. After the color test following the seventh trial, the test bee was caught and put in a small queen cage (10 cm × 3 cm × 1.5 cm) which was then placed for 24 h inside the home colony. On the next day the cage was taken from the colony and the bee was released from the cage inside the green house. All tested animals flew back to the hive and usually returned to the green house after 10–30 min. Bees returning to the set-up later than 30 min after release were discarded from the following retention test. In the 24 h retention test color choice of the conditioned bee was measured as described before.

EXPERIMENT 2: OLFACTORY PER CONDITIONING IN THE LABORATORY

Nectar foragers with sucrose acceptance thresholds of ≥50% or ≤3% in the field were selected and tested in an absolute olfactory PER conditioning experiment with additional dual choice test in the laboratory. Similar to color learning with free flying bees in the field, seven conditioning trials and 24-h retention were analyzed. After testing the sucrose acceptance threshold, each bee was caught at the feeder after drinking for 20 s either the offered 50% or 3% sucrose solution. In a previous experiment (Mujagic, 2009) we measured at a feeder in the field the ingested volume/time for sucrose solutions with different concentrations (50% and 3%). We found that bees from the two acceptance groups drink approximately the same volumes/time (≈ 1 μl/s). We also determined the crop content and found no significant differences between the crop volumes for both sucrose concentrations.

In the present study bees were allowed to drink for 20 s which leads to equally filled crops in the tested bees. The exact drinking duration was measured with a stopwatch. All captured bees were kept individually in small glass vials and taken immediately to the

laboratory. The bees were shortly cooled in a refrigerator at 4°C until they showed the first signs of immobility. Then they were placed in small metal holders with strips of adhesive tape attached between head and thorax and over the abdomen. Bees were allowed to recover for at least 15 min before testing sucrose responsiveness. In this test the antennae of the harnessed bee were stimulated with a droplet of water and increasing sucrose concentrations of 0.1%, 0.3%, 1%, 3%, 10%, 30% and 50% (w/w). It was registered at which concentrations PER occurred (for details see Mujagic and Erber, 2009). The temporal interval between stimulations was 2 min. Responsiveness in this test can be used to compare in the same bee sucrose responsiveness in the laboratory with acceptance in the field. The gustatory response score (GRS) is a good measure for responsiveness in the laboratory (Scheiner, 2004). This score is defined as the sum of proboscis extensions elicited by water and the different sucrose concentrations during the sucrose concentration PER test. As eight different gustatory stimuli were applied, the GRS can vary between 0 (a bee not responding with PER to any stimulus) and 8 (a bee responding to all stimuli). Bees that did not respond to any stimulus (GRS 0) were excluded from the experiment.

Olfactory PER conditioning started 15 min after the sucrose concentration PER test. For olfactory PER conditioning we used cineole (≥98%, C8144-Sigma-Aldrich Chemie GmbH, Munich, Germany) as CS+ and clove oil (C8392-Sigma-Aldrich Chemie GmbH, Munich, Germany) as CS–. The experimental bee was placed in front of two tubes (diameter 5 mm) through which a constant airstream from an aquarium pump was blown. An odor was added to the airstream by opening the valve of a channel that contained a piece of cellulose soaked with either 1 µl of cineole or 1 µl of clove oil. The scented air was removed with an exhaust vent placed behind the bee. Olfactory PER conditioning started with a test of spontaneous PER behavior. Each of the two test odors was presented for 5 s to the bee at a stimulus interval of 5 min. Bees responding with PER during the spontaneous test were discarded from the following conditioning assay. The bees were then conditioned in seven trials to the CS+ (cineole). In each trial the CS+ was presented for 3 s before proboscis extension was elicited by applying a droplet of 50% sucrose solution to each antenna. After proboscis extension the bee was allowed to drink for about 1 s from that sucrose droplet. Discrimination between CS+ and CS– was tested by presenting each of the odors in random order after each conditioning trial. The time interval between conditioning and presentation of the first odor was 5 min, the second odor was presented 7 min later. We used this olfactory conditioning protocol because we wanted to apply similar dual choice tests in all three learning experiments. After absolute conditioning to a sensory cue (color or odor) discrimination was tested in dual choice tests. In the olfactory protocol the two odors were offered sequentially. To compare acquisition in different groups of bees, an acquisition score was calculated. This score is the total number of conditioned responses to the CS+ and it has a range between 0 (no conditioned PER) and 7 (conditioned PER after each conditioning trial).

After the last conditioning trial and the following odor tests the bees were fed to satiation with 50% sucrose solution. They were then transferred with their tubes into a humid chamber kept at room temperature until the retention test on the next day. In the 24-h retention test each odor (CS+ and CS–) was presented to the bee at a temporal interval of 5 min in random order and it was registered whether PER occurred. As bees were tested repeatedly after the seventh conditioning trial, an extinction phenomenon reducing the PER in the retention test cannot be excluded.

EXPERIMENT 3: COLOR LEARNING WITHOUT SUCROSE REWARD AT THE HIVE ENTRANCE

In most of the earlier studies visual discrimination at the hive entrance was tested by connecting the hive to two separate entrances. The animals then had to learn that one of the entrances with a specific visual cue gave access to the hive, while the alternative visual cue signaled a blocked entrance. With this paradigm the position of the visual mark signaling the entrance has to be changed frequently to avoid position learning of the cue (Beier and Menzel, 1972; Zhang et al., 2006). We performed an extensive series of pilot experiments over two foraging seasons to define a color learning protocol for the hive entrance. The interference between the experimental bee and other foraging bees returning to the hive can lead to side preferences which are a major problem in these experiments. We also found that experimental bees stop foraging at the feeder when they are tested in dual choice color tests after each visit to the feeder. We developed a hive entrance learning protocol that was similar to the color learning protocol at the food site. In the color learning experiments at the feeder, only the learning color was present during the reward. During an unrewarded test the bee had to choose between the learning color and an alternative. In the hive entrance protocol conditioning and testing was similar to the color learning protocol at the food site. A single bee was conditioned by allowing it to enter the hive marked with a single color plate. In the learning tests the entrance was blocked and the bee could approach and land repeatedly on a plate with the learning color and an alternative.

Similar to the learning protocol at the food site, color learning was tested at the hive entrance in bees which had low or high acceptance thresholds. In this experiment we had to use a small four frame colony to minimize interference between the test bee and other returning foragers of the colony during the choice tests. As the hive entrance was blocked during choice tests, there was no interference with departing bees. Honey bees from the colony were trained to visit a feeder in a small shed 40 m away from the hive. The bees visiting the feeder were marked individually with color-coded spots on the thorax and abdomen. The feeder contained 50% (w/w) sucrose solution. The experiments were performed with newly alarmed bees that had not visited the feeder before. The acceptance threshold of a newly alarmed bee was determined as described above. During the measurement of the acceptance threshold the hive entrance was open and not marked with a color plate. The concentration of the sucrose solution at the feeder was reduced in steps as described above.

The sucrose thresholds of single bees depend on many exogenous and endogenous factors, like the weather, the state of the hive, the amount of available pollen, the foraging specialization of the bee and its genetic background (Page et al., 2006). The color learning experiments at the food site and the olfactory PER conditioning experiments were performed during the foraging season 2008, while the hive entrance experiments were performed during the season 2009. In all the hive entrance experiments with the

small four frame colony only one bee had an acceptance threshold of 50%, while many bees had thresholds at 30%. Therefore, bees which accepted sucrose concentrations of ≥30% were assigned to the "high threshold" or "insensitive group" (≥30% group). In a number of pilot experiments over several foraging seasons we detected no behavioral differences for learning in the field and in the laboratory between bees that had acceptance thresholds of ≥30% or ≥50%. Bees which accepted a concentration of 3% were assigned to the "low threshold" or "sensitive" group (≤3% group). Bees which accepted 10% but no lower concentrations were not used for the experiments.

After testing the acceptance threshold, an individual bee was allowed to collect 50% sucrose at the feeder. Other foragers visiting the feeder were caught and kept for the time of the experiment in a small cage. The following experiments were all done with a single bee by two observers. Color choice behavior of the bee was tested when it returned from the feeder to the hive. A white plate (60 × 40 cm) was positioned in front of the entrance of a small four frame hive. Bees could enter and leave the hive only through a central tube (diameter 2 cm) in the middle of the plate. In the training situation the central tube was open and a colored (yellow or blue) 12 cm × 12 cm plate surrounding the tube marked the entrance. Whenever the bee returned during the learning phase from the feeder to the hive it could enter the hive entrance which was marked by the plate with the learning color. In the test situation the central tube was covered with a white plate so that the returning bees could not enter the hive anymore. A yellow and a blue colored square plate was positioned 24 cm to the right and to the left of the center tube. The plates had a dark round center (diameter 2 cm) which looked like the hive entrance during the training phase. Opaque Plexiglas® GS (3-mm thick; colors "yellow" or "sky blue"; www.modulor.de) was used for the colored plates during training and testing. To avoid odor cues, different plates were used during training and testing and all plates were cleaned every day with ethanol.

Behavior of an individual bee in the choice situation at the hive was recorded with two video cameras (Sony HDR-CX11E) positioned approximately 200 cm from the right and the left colored plate during the test situation. The focal length of each camera was adjusted to record the area of the color plate and approximately 1 cm around the plate. Behavior of the bee during the choice test was recorded for 3 min, then the positions of the two plates were exchanged and behavior was recorded for another 3 min. This test procedure helps to avoid artifacts due to a side preference of the bee. The videos were later analyzed in the slow motion mode and when necessary in the single frame mode. The test bee could be easily distinguished by the color dots on the thorax and abdomen from other foragers of the colony that also returned to the hive. As we used a small colony, there were not many other bees returning to the hive during the color tests. "Approaches" toward each of the two alternative plates were counted for the two tests each lasting 3 min. An "approach" toward the plate was registered whenever the individual test bee was flying in front of the color plate. In addition, the "time" spent hovering in front of a plate or spent sitting on a plate was measured. The times for each plate were added for the two 3 min tests. The proportions of approaches and time between the two alternative plates were calculated and used as measures indicating choice behavior between the two alternatives.

After assigning a single bee to the low or high acceptance threshold group, the learning experiment started. At the beginning of the experiment spontaneous color choice behavior was tested by closing the hive entrance in the middle and offering the bee the two alternative color plates right and left of the entrance. The bee could not enter the hive through the two color plates. Behavior was recorded for 6 min, after 3 min the positions of the plates were exchanged. The initial positions of the two plates were chosen at random. Then the two test plates were removed and a yellow plate was mounted at the hive entrance which was now open again. The test bee was allowed to collect 50% sucrose at the feeder and to return 10 times to the hive through the yellow plate covering the hive entrance. After 10 visits to the feeder and the hive the entrance was closed again and choice behavior of the individual bee was recorded as described above for the spontaneous behavior. Again, the initial positions of the two plates were chosen at random.

Small differences of learning performance in bees are often difficult to detect if only one alternative is conditioned. In tactile conditioning experiments with pollen and non-pollen bees it was shown that minor differences in acquisition and discrimination can be detected with a reversal learning protocol in which the bee is first conditioned to one alternative and then to the other (Scheiner et al., 1999). From a number of pilot experiments at the hive entrance we knew that there exist only small differences in acquisition and discrimination between bees with different acceptance thresholds. Therefore, we used a reversal learning protocol for hive entrance conditioning. After this first learning test the hive entrance was marked by a blue plate to test reversal learning. As before, the bee could visit the feeder and enter the hive 10 times through the blue plate covering the entrance. Choice behavior was tested again for 2 × 3 min after 10 visits to the hive.

The behavior of 15 bees in each of the two acceptance threshold groups was analyzed in the three behavioral tests before learning, after entering the hive 10 times through the yellow plate and after entering the hive 10 times through the blue plate. After the tests with an individual bee, the foraging group, which was kept in a cage, was set free and could forage again at the feeder. During that time the hive entrance was not marked by a color plate. As other bees of the colony could enter the hive during the training sessions of an individual bee, only one learning experiment was performed during a day and a new experiment started on the next day. As bees take about 5 min for a round trip between feeder and hive, one can estimate that individual bees of the colony use the unmarked hive entrance over 100 times during the approximately 10 h of foraging after the previous experiment. It cannot be excluded that a newly alarmed test bee had entered the hive entrance with the blue or yellow conditioning plate on the day before. We minimized this effect by performing only one color learning experiment per day and by keeping the hive entrance open without a color plate for most hours of the day.

STATISTICS

Color learning at a food site

The proportions of approaches toward the green and blue alternatives were transformed using a modification of the arcsin(sqrt) function (Freeman and Tukey, 1950; Zar, 1999). The transformed data were tested for significant deviations from normal distribution

($p < 0.05$; D'Agostino and Pearson omnibus normality test; GraphPad Prism 4). No significant deviations from normal distributions were found. Therefore, parametric methods were used for statistic comparisons. Data of learning trials and the 24-h retention test were statistically compared between individuals of each acceptance groups using a two way ANOVA with repeated measures (GraphPad Prism 4).

Olfactory PER conditioning

Acquisition scores and GRSs for each sucrose acceptance group (≥50% or ≤3%) were tested for significant deviations from normal distributions ($p < 0.05$; D'Agostino and Pearson omnibus normality test; GraphPad Prism 4). Most groups were not distributed normally. Therefore, medians, quartiles and mean values are presented in the figures. Statistic differences of acquisition scores and GRS between the two sucrose acceptance groups were tested with the Mann–Whitney U test (GraphPad Prism 4). Differences in the 24 h retention test between the acceptance groups were analyzed using Fisher's exact probability test (GraphPad Instat 3.06). Statistic differences between the acquisition curves for both acceptance groups were analyzed using a binary logistic regression model for repeated measurements (Wright et al., 2007). Statistics were calculated with the GENLIN command of the statistic software PASW Statistics 18, using a binomial distribution, logit linking function and repeated measurements.

Hive entrance color learning

For statistical analyses proportions of times spent on the yellow or blue alternative were transformed using the arcsin(sqrt(p)) function (Zar, 1999). Proportions of approaches were transformed using a modification of the arcsin(sqrt) function (Freeman and Tukey, 1950; Zar, 1999). Data were tested for deviations from normal distributions ($p < 0.05$; D'Agostino and Pearson omnibus normality test; GraphPad Prism 4). No significant deviations from normal distributions were found.

RESULTS

COLOR LEARNING AT A FOOD SITE IN THE FIELD

In the first experiment free flying nectar foragers with high and low sucrose acceptance thresholds (≥50% and ≤3%) were tested in a color learning protocol at a food site in the field. The acquisition curves and retention after 24 h for bees from the two acceptance groups are shown in **Figure 1**. The acquisition functions clearly differ between bees with acceptance thresholds ≤3% and ≥50%. Bees of the 3% acceptance group show faster acquisition because the choice reaction differs significantly already after the first learning trial from spontaneous choice (Bonferroni posttest; $p < 0.001$), while bees from the 50% group reach this level of significance after the third learning trial. After 3–5 rewards bees of both groups reach stable acquisition plateaus. At the plateau there exist no more statistic differences between consecutive choice tests (Bonferroni posttests; $p > 0.05$; 50% acceptance after the third learning trial; 3% acceptance after the fifth learning trial). Highly significant differences of the choice reactions between the two groups were found for each single learning trial (unpaired t-test, $p < 0.001$). A two way ANOVA for repeated measures shows that acceptance ($F = 64.02$, DF: 1, 304, $p < 0.0001$) and the learning trials ($F = 58.83$, DF: 8,304, $p < 0.0001$) have significant effects. Also the interactions are highly significant ($F = 14.03$, DF: 8, 304, $p < 0.0001$).

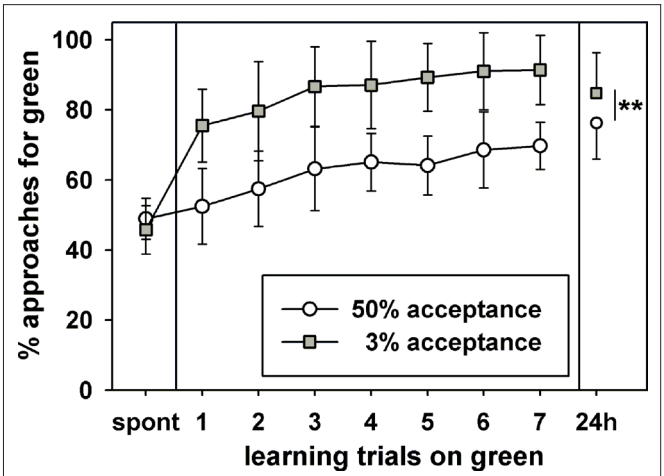

FIGURE 1 | Color learning experiment at an artificial feeder in the field for bees which either had acceptance thresholds ≤3% or ≥50%. The bees could choose between a green and blue alternative (for details see text). The abscissa shows the spontaneous choice (spont), choice tests after seven learning trials on green, and retention after 24 h (24 h). The ordinate shows the mean percentages of approaches with the respective standard deviations for the rewarded green alternative. The stars indicate significant differences of the choice reaction in the 24-h retention test (2-sided t-test, $p < 0.01$); other significant differences are not indicated in the figure, for details see text. 20 bees were tested in each group. Bees in the 50% acceptance group made between 4 and 17 approaches in the tests, resulting in a total of 3143 approaches for this group. Bees in the 3% acceptance group made between 5 and 17 approaches in the tests, resulting in a total of 3370 approaches for this group.

In the retention test after 24 h the percentages of approaches toward the conditioned color were significantly different between the two acceptance groups (2-sided t-test, $p < 0.01$). It is remarkable that the choice reaction of the 3% acceptance group at 24 h was significantly reduced compared to the seventh learning trial (Bonferroni posttest; $p < 0.001$), while for the 50% acceptance group the percentage of correct choices in the retention test at 24 h was significantly larger than the choice reaction after the seventh trial (Bonferroni posttest; $t = 2.374$, $p < 0.05$). These findings demonstrate a convergence of choice performance but still significant differences between the two acceptance groups in the 24-h retention test.

PER RESPONSIVENESS, OLFACTORY PER CONDITIONING AND 24H RETENTION IN THE LABORATORY

The olfactory PER conditioning experiments in the laboratory were done with bees that had either ≥50% or ≤3% sucrose acceptance in the field. At the beginning of the laboratory experiment PER responsiveness for different sucrose concentrations was tested in these bees to compare sucrose acceptance in the field with sucrose PER responsiveness in the laboratory. The sucrose concentration dependent PER curves for the two acceptance groups were very similar and the two GRSs which are a compound measure for responsiveness were not significantly different (**Figure 2**; Mann–Whitney

U Test, $p > 0.05$). We conclude from this experiment that the bees in the two sucrose acceptance groups show similar sucrose responsiveness in the laboratory.

Acquisition curves, discrimination and 24-h retention tests for bees from the two acceptance groups are shown in **Figure 3A**. Bees from the ≤3% acceptance group clearly show better acquisition and

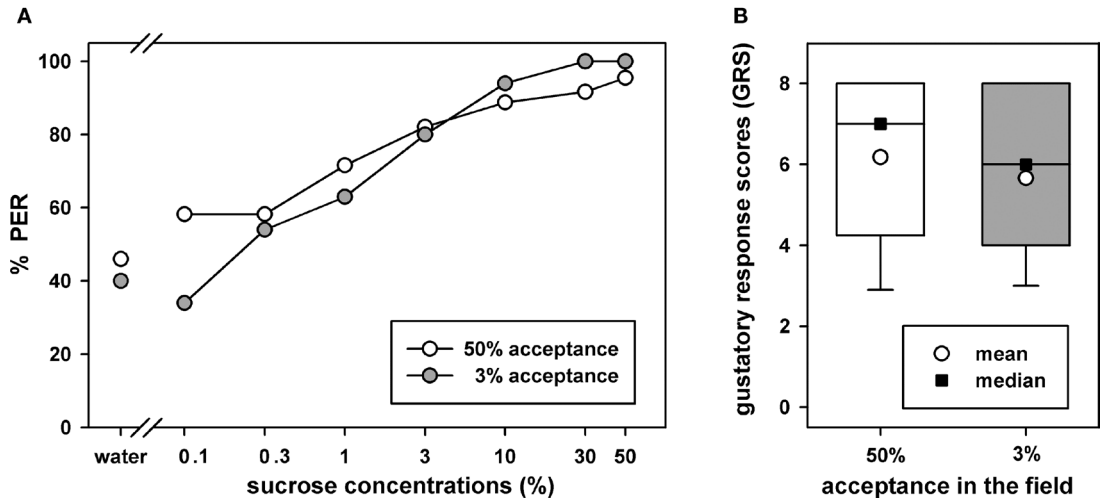

FIGURE 2 | PER responsiveness for different concentrations of sucrose in two groups of bees which either had acceptance thresholds ≤3% or ≥50% in the field. (A) Sucrose concentration dependence of the PER. The abscissa shows the applied stimuli starting from water to 50% sucrose. The ordinate shows the percentages of PER for each stimulus. **(B)** The gustatory response scores for the two groups (for details see text). The box plots show medians, means, quartiles, the whiskers indicate 95% percentiles. 35 bees were tested in the 3% acceptance group and 28 bees in the 50% acceptance group.

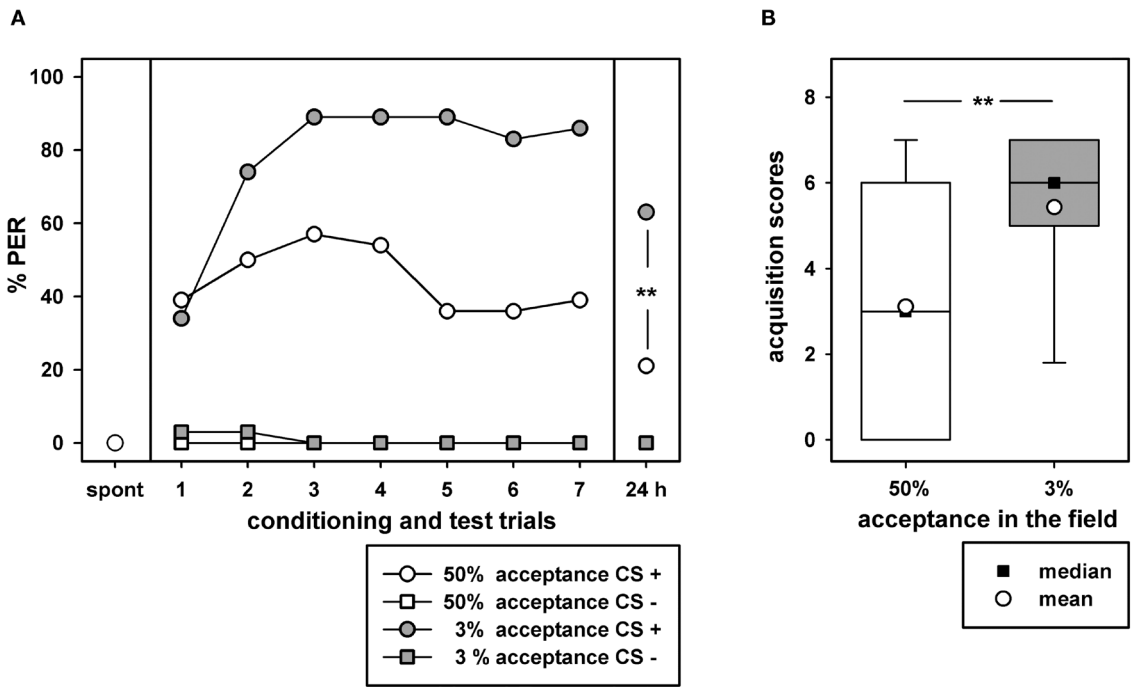

FIGURE 3 | Olfactory PER conditioning for two groups of bees which either had acceptance thresholds ≤3% or ≥50% in the field. (A) The acquisition curves, discrimination, and 24-h retention in the two groups. The abscissa shows spontaneous choice (spont), tests after each of the seven learning trials, and 24-h retention (24h). The ordinate shows the percentages of PER to the conditioned odor cineol (CS+) and the alternative not rewarded odor clove oil (CS–). ** indicates a significant difference for the retention tests (Fischer's exact probability test, $p < 0.01$); other significant differences in the acquisition curves are not indicated (for details see text). **(B)** The acquisition scores for the two groups (for details see text). The box plots show medians, means, quartiles, the whiskers show 95% percentiles. ** indicates a significant difference between acquisition scores (Mann–Whitney U Test, $p < 0.01$). 35 bees were tested in the 3% acceptance group and 28 bees in the 50% acceptance group.

retention than bees from the ≥50% acceptance group. No differences in odor discrimination were found between bees of the two groups. Compared to spontaneous behavior, bees of both groups show significant increase of conditioned PER after the first conditioning trial (Fisher exact probability test, $p < 0.001$). The acquisition curves differ significantly between the two acceptance groups (binary logistic regression model for repeated measurements, Wald-chi-square = 12.89, df = 1, $p < 0.001$). As a consequence of the differences in acquisition also the acquisition scores which are compound measures of acquisition differ significantly (**Figure 3B**; Mann–Whitney U test, $p < 0.01$). The differences in acquisition between the two sucrose acceptance groups are similar to those for color learning with free flying bees from the same acceptance groups (**Figure 1**). Apparently, differences in sucrose acceptance in the field are also correlated with differences in olfactory PER conditioning in the laboratory, although sucrose responsiveness measured with the PER protocol in the laboratory does not differ between the two groups (**Figure 2**). In the 24-h retention tests the percentages of conditioned responses for both groups were lower compared to the response behavior after the seventh conditioning trial but these differences were not significant (Fisher exact probability test, $p > 0.05$). Similar to the results during the conditioning phase, the differences of conditioned PER between the two sucrose acceptance groups were significant also in the 24-h retention tests (Fisher exact probability test, $p < 0.01$). We conclude from these experiments that differences of sucrose acceptance in the field are correlated with differences in acquisition and retention in olfactory conditioning in the laboratory.

HIVE ENTRANCE COLOR LEARNING

The experiments were done with individual bees which either had sucrose acceptance thresholds ≥30% or ≤3%. Bees of the two groups did not differ significantly in the mean times for a roundtrip from feeder to hive and back (30% group: 5.2 ± 0.49 min; 3% group: 4.7 ± 0.23 min; 2 sided *t*-test, $p = 0.43$).

To quantify color choice behavior we measured approaches toward the yellow and blue plates and the time spent in front of the plates or on the plates. In all tests the percentages of approaches and of time were very similar (**Figures 4A,B**). Approaches and times are significantly correlated for both experimental groups when spontaneous choice tests, tests after 10 learning trials on yellow and on blue are used for calculating the correlation (Pearson correlation; 30% group: $r = 0.8388$, $p < 0.0001$; 3% group: $r = 0.6585$, $p < 0.0001$). Also the results of the statistical comparisons within each group and between different phases of the experiment are very similar for the two behavioral measures. The results demonstrate that the two behavioral measures used for the hive entrance learning experiments are closely correlated.

Spontaneously bees of both groups approach the yellow plate less frequently than the blue plate (**Figures 4A,B**; one sample *t*-test, tested against a theoretical value of 50% choices for yellow; 30% acceptance group: $p < 0.05$; 3% acceptance group: $p < 0.0001$). Similar to the approaches, bees of both groups also spend less time on the yellow alternative. Bees of the 3% acceptance group spend significantly less time on the yellow alternative, while this difference is not significant for the 30% group (one sample *t*-test, tested against a theoretical value of 50% time

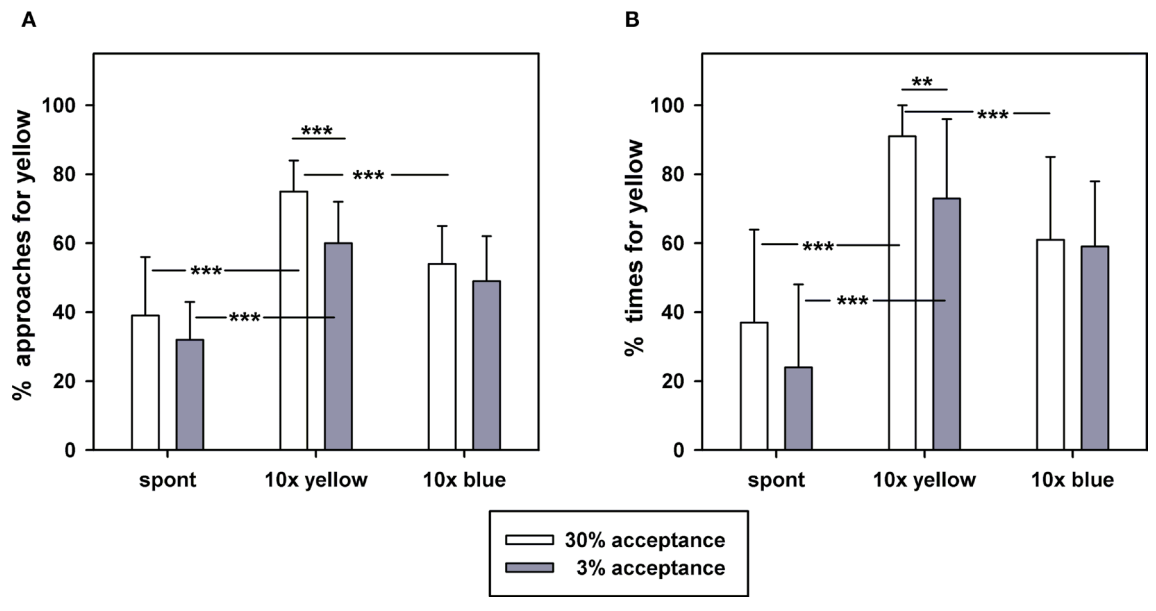

FIGURE 4 | Color learning at the hive entrance for two groups of bees which had acceptance thresholds ≤3% or ≥30% in the field. The abscissa of both graphs show choice behavior before learning (spont), after 10 learning trials with a yellow plate marking the hive entrance (10× yellow), and after 10 reversal learning trials with a blue plate marking the hive entrance (10× blue). **(A)** The ordinate shows mean percentages of approaches and the respective standard deviations toward the yellow plate compared with the approaches toward a blue plate (for details see text). **(B)** The ordinate shows mean percentages of times and the respective standard deviations spent in front or on the yellow plate compared with the times for a blue plate (for details see text). Significant differences of Bonferroni multiple comparison tests (for comparisons within an acceptance group) and of 2-sided *t*-tests (between acceptance groups) are indicated (** $p < 0.01$; *** $p < 0.001$; for details see text). Fifteen bees were tested in each of the two acceptance groups.

spent on yellow; 30% acceptance group: $p > 0.05$; 3% acceptance group: $p < 0.001$). The differences in spontaneous choice both for approaches and times between the two acceptance groups are not statistically significant (*t*-tests; approaches: $p > 0.05$; time: $p > 0.05$). After 10 learning trials using the yellow entrance plate, the percentages of approaches and of time for the yellow alternative are significantly enhanced for bees of both sucrose acceptance groups (**Figures 4A,B**; Bonferroni multiple comparison tests, $p < 0.001$). After 10 learning trials on yellow, bees of the 3% group show significantly lower percentages of approaches and times for the yellow alternative than the 30% group (**Figures 4A,B**; *t*-tests, approaches: $p < 0.001$; times: $p < 0.01$). After reversal learning with the blue entrance plate, bees of the 30% sucrose acceptance group show highly significant reductions of approaches and times for the yellow alternative (**Figures 4A,B**; Bonferroni multiple comparison tests, $p < 0.001$). The reductions of approaches and time for the yellow alternative after reversal learning are not significant for the 3% group (**Figures 4A,B**; Bonferroni multiple comparison tests, $p > 0.05$). We conclude from these experiments that the differences in acquisition that we found for sucrose dependent learning in the field and in the laboratory are not apparent in a learning protocol without sucrose rewards. Bees from the sensitive sucrose acceptance group (acceptance threshold ≤3%) do not show better acquisition than bees from the insensitive group (acceptance threshold ≥30%), quite contrarily, reversal color learning is better in the insensitive bees compared to the sensitive sucrose acceptance group.

DISCUSSION

With our experiments we have tested a number of hypotheses concerning the relations between sensitivity for sucrose and learning. We know now that some of the rules developed in laboratory experiments are valid also in the field and that sucrose acceptance in the field is a sensitive parameter to predict learning performance and retention also in the laboratory. Furthermore we have shown for the first time that the relations between sucrose sensitivity and learning only apply to protocols in which sucrose serves as a reward.

Before we performed the present study, learning experiments in the laboratory using different olfactory, tactile and operant protocols (Erber et al., 1998; Scheiner et al., 1999, 2001a,b, 2003, 2005) led to the hypothesis that high sucrose sensitivity is correlated with better acquisition and retention also in the field. The experiments discussed here support this hypothesis. Nectar foraging bees which accept low sucrose concentrations when visiting an artificial food site show better acquisition and 24-h retention in a color learning assay than bees which accept only high sucrose concentrations.

Generally it is not easy to compare experiments with bees in the field with those in the laboratory because the analyzed behaviors are not identical. The estimation of sucrose sensitivity is a good example for these difficulties. Sucrose sensitivity in the laboratory is determined by stimulating bees with different concentrations of sucrose and by registering at which concentrations an individual bee extends its proboscis. The PER is a relatively simple reaction to a gustatory stimulus. The sucrose sensitivity estimated with this experiment is valid for a stimulus-response relationship under laboratory conditions. In the field sucrose sensitivity is estimated by measuring which sucrose concentrations a bee is willing to accept during foraging. Sucrose acceptance in the field is the result of a complex behavioral sequence in which a bee first has to land on a food site; second it has to probe the offered sucrose solution with the antennae which can lead to extension of the proboscis. At the end of this sequence a bee can either accept the sucrose solution or it can search for another food source (Mujagic and Erber, 2009). In addition, the behavioral contexts between free flying and harnessed bees are completely different which can affect sensory thresholds if the same bee is tested in the field and in the laboratory. Similar arguments can be applied to the different learning protocols in the field and in the laboratory.

Our experiments demonstrate that sucrose acceptance is a sensitive measure of gustatory thresholds and that the measure of PER responsiveness is less sensitive in nectar foragers that accept different sucrose concentrations when foraging in the field. The thresholds of bees accepting low sucrose concentrations (≤ 3%) differed from the thresholds of bees accepting high sucrose concentrations (≥50%) by approximately 1.2 log units of concentration, still the PER concentration-response curves of the same individuals were very similar under laboratory conditions. The experiments also demonstrate that differences of learning performance in olfactory PER conditioning can exist even in bees which show the same PER responsiveness in the laboratory. Earlier studies showed that significant differences in PER responsiveness in the laboratory were correlated with learning performance in tactile or olfactory PER conditioning (Erber et al., 1998; Scheiner et al., 1999, 2001a,b, 2003, 2005). Meanwhile, additional laboratory experiments prove that differences in PER responsiveness are not a necessary prerequisite for differences in PER conditioning. Also other factors like the social role, foraging specialization, foraging age and the state of health (Behrends et al., 2007; Iqbal and Müeller, 2007; Drezner-Levy et al., 2009; Scheiner and Amdam, 2009); can affect learning. In an olfactory PER conditioning experiment with bees that had identical sucrose PER responsiveness, Behrends et al. (2007) demonstrated that old foragers showed inferior learning performance compared to younger foragers. The decrease in learning performance seems not to be related to the chronological age of the animals, but rather to their foraging age and specialization in the field (Behrends et al., 2007; Scheiner and Amdam, 2009). Sucrose acceptance in the field probably is the better predictor for learning performance because this measure is closely related to the foraging specialization in the field (Mujagic and Erber, 2009).

The present experiments clearly show that the individual evaluation of the reward is decisive for learning performance in different protocols that use sucrose as a reward in the field and in the laboratory. Earlier studies showed that associative learning performance in bees is strongly correlated with the concentration of the sucrose reward and that higher sucrose concentrations improve learning performance (Loo and Bitterman, 1992; Couvillon et al., 1994). Laboratory experiments demonstrated that the individual threshold for sucrose and the sucrose concentration in tactile PER conditioning determine the individual value of the reward (Scheiner et al., 1999). By adjusting the reward concentration to the sensory responsiveness of an individual, equal subjective rewards can be generated which lead to similar acquisition and retention in bees that differ significantly in sucrose responsiveness (Scheiner et al., 2005). All these experiments suggest that differences in the perception of sucrose lead to differences in acquisition and retention.

The results of our color experiments at the food site are in good accordance with the learning experiments in the laboratory. Although color learning at a food site and olfactory PER conditioning in the laboratory are two very different forms of associative learning in the bee, the same basic rules between sucrose sensitivity and learning are valid. Bees which accept low sucrose concentrations show better acquisition and retention compared to bees which accept only higher sucrose concentrations. These rules become apparent when the bees with different sucrose sensitivity are rewarded with a high sucrose concentration (50%). Together with the earlier experiments, these results suggest that the subjective reward value is stronger in sucrose sensitive bees than in insensitive bees (Scheiner et al., 2005). Associative learning in the field is dependent on a number of exogenous and endogenous factors which influence learning performance by modulating sucrose sensitivity. The foraging specialization of an individual, the season, the availability of nectar and pollen, the weather and the complex conditions within the colony affect the individual sucrose sensitivity of a foraging bee (for a discussion see Page et al., 2006) and hence learning. We think that the existing data can now be used for modeling the consequences of these different factors for learning under natural conditions.

The hive entrance color learning experiments clearly show that the differences in associative learning which can be found in bees with different sucrose acceptance vanish when sucrose does not represent the reward. This finding supports the hypothesis that differences in associative learning performance and retention in bees are causally related to differences in subjective reward evaluation. Contrary to the learning experiments with sucrose rewards, the results with hive entrance conditioning indicate that acquisition and reversal learning is better for bees which accept only high sucrose concentrations (≥30%). At the moment we do not have a hypothesis concerning these differences. The close relationships between the percentages of approaches and the percentages of time spent in front of the color plates suggest that both measures are robust indicators of color choice. It is possible that the subjective reward values of bees with different acceptance thresholds are different. One factor affecting the subjective reward value in the hive could be the delivery time of the collected sucrose (Lindauer, 1948; Seeley, 1995). As both groups were fed the same sucrose concentration (50%) at the feeder and as the times for roundtrips between food site and hive did not differ between the two groups, this hypothesis is not supported by the experimental data. Clearly, more experiments are necessary to find out in which additional behavioral traits bees with low and high sucrose acceptance thresholds differ. A large number of experiments demonstrate that foraging specialization in bees is correlated with differences in genetic architecture, in phenotypic architecture and in signaling cascades which in turn can affect sensory processing and stimulus evoked responses (Page et al., 2006). There remain many open questions concerning the division of foraging labor and associative learning in the field. Future experiments under natural conditions comparing learning of bees with different foraging specializations are necessary to understand the relations between sensory sensitivity and learning. It would be most interesting to perform a learning experiment comparing water foragers with pollen- and nectar foragers. So far we know that the differences in sucrose acceptance in free flying nectar foragers have effects on associative learning in which sucrose is the reward. For a better understanding of the complex interactions between sensory sensitivity and learning in the field we need similar experiments with foraging bees that collect water or pollen.

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Multiple reversal olfactory learning in honeybees

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In multiple reversal learning, animals trained to discriminate a reinforced from a non-reinforced stimulus are subjected to various, successive reversals of stimulus contingencies (e.g. A+ vs. B–, A– vs. B+, A+ vs. B–). This protocol is useful to determine whether or not animals "learn to learn" and solve successive discriminations faster (or with fewer errors) with increasing reversal experience. Here we used the olfactory conditioning of proboscis extension reflex to study how honeybees *Apis mellifera* perform in a multiple reversal task. Our experiment contemplated four consecutive differential conditioning phases involving the same odors (A+ vs. B– to A– vs. B+ to A+ vs. B– to A– vs. B+). We show that bees in which the weight of reinforced or non-reinforced stimuli was similar mastered the multiple olfactory reversals. Bees which failed the task exhibited asymmetric responses to reinforced and non-reinforced stimuli, thus being unable to rapidly reverse stimulus contingencies. Efficient reversers did not improve their successive discriminations but rather tended to generalize their choice to both odors at the end of conditioning. As a consequence, both discrimination and reversal efficiency decreased along experimental phases. This result invalidates a learning-to-learn effect and indicates that bees do not only respond to the actual stimulus contingencies but rather combine these with an average of past experiences with the same stimuli.

Keywords: learning, multiple reversal, olfaction, honeybee

INTRODUCTION

Adapting to a changing environment requires constant evaluation of action outcomes. Reversal learning (Pavlov, 1927) is an example of how animals can deal with changing environments. In this paradigm, a subject is first trained to discriminate a rewarded stimulus A+ (where A stands for the stimulus and + for the presence of reward) from a non-rewarded stimulus B– (where – stands for the absence of reward) and once the discrimination is mastered, the contingencies are inversed (A– vs. B+) so that the subject has to learn to reverse its response to A and B. Reversals tend to be difficult as there are negative transfer effects; e.g., the individual tends to persist in responding to the stimulus that was originally reinforced. Eventually, however, this tendency becomes weaker, and the response to the alternative stimulus becomes more frequent until it is consistently evoked.

The capacity of animals to solve reversal learning tasks has been extensively studied using different conditioning procedures (for review, see Davey, 1989). In *multiple reversal learning*, successive reversals are performed using the same stimuli (e.g. A+ vs. B–, A– vs. B+, A+ vs. B–). A question underlying this protocol is whether or not animals solve successive discriminations faster (or with fewer errors) with increasing reversal experience. Indeed, a possible outcome of this kind o experiment is that, after extended reversal training, some animals are able to make the next reversal in the sequence faster or in fewer trials. They behave as if they have mastered the abstract concept of alternation or of regular sequence. However, another outcome is also possible if the animal applies a purely associative strategy that averages reinforcements and absence of reinforcements obtained for A and B over trials. If successive conditioning phases are even, animals end-up responding equally to A and B, thus exhibiting an apparent lack of discrimination. This performance results from the fact that A and B acquired the same associative strength across trials.

The honeybee, *Apis mellifera*, constitutes an excellent model for studying the strategies implemented by a relatively simple and yet cognitively sophisticated brain (Menzel and Giurfa, 2001; Giurfa, 2007) to solve multiple reversal learning. In a natural context, honeybees are constant pollinators that remain faithful to a single floral species as long as it provides a profitable nectar and/or pollen reward. The basis for such constancy is the fact that bees learn the floral features (colors, odors, etc) associated with reward (Menzel, 1985, Giurfa, 2007). Changes in food source profitability occur rapidly so that bees have to quickly switch to another floral species to ensure efficient foraging. This scenario could promote, therefore, fast solving of multiple reversal learning and eventually mastering the concept of alternation. On the other hand, it may also be efficient to solve this ecological problem by averaging positive and negative experiences over time, thus deciding whether or not it is timely to switch to another species.

In the laboratory, appetitive learning in honeybees is studied using a Pavlovian conditioning protocol, the olfactory conditioning of the proboscis extension reflex (PER) (Takeda, 1961; Bitterman et al., 1983). In this protocol, a hungry bee that is harnessed and whose antennae are touched with sucrose solution exhibits a PER to reach out and suck the sucrose. Odors to the antennae do not release such a reflex in naive animals. If an odor is presented immediately before sucrose solution (forward pairing), an association is formed that enables the odor to release PER in a following test. Thus, the odor can be viewed as the conditioned stimulus (CS) and the sucrose solution as the unconditioned stimulus (US).

Differential conditioning with two odors, one rewarded and the other not, is also possible in this frame (Bitterman et al., 1983) and thus offers the opportunity to study reversal learning performances in honeybees (Ben-Shahar et al., 2000; Hosler et al., 2000; Ferguson et al., 2001, Komischke et al., 2002).

Here we used the olfactory conditioning of PER to study how bees perform in a multiple reversal paradigm. Our experiment contemplated four consecutive differential conditioning phases involving the same odors, i.e., a first phase of differential conditioning (A+ vs. B–) and three subsequent phases of reversal (A– vs. B+ → A+ vs. B– → A– vs. B+). We asked whether bees would improve their discrimination performance with successive reversals or whether they would generalize their choice to both odors at the end of conditioning as a consequence of equating their associative strengths.

MATERIALS AND METHODS

SUBJECTS

Free-flying honeybee foragers, *Apis mellifera* were caught at the entrance of an outdoor hive situated close to the laboratory building. Bees were placed in small glass vials and cooled in ice until they ceased their movements. The bees were then individually harnessed in small metal tubes so that they could only move their antennae and mouthparts, including the proboscis. Harnessed bees were kept in the dark and high humidity for 2 h. Fifteen minutes before starting the experiment, each subject was checked for intact PER by lightly touching one antenna with a toothpick soaked with 30% (weight/weight) sucrose solution without subsequent feeding. Extension of the proboscis beyond a virtual line between the open mandibles was counted as PER (unconditioned response). Animals that did not show the reflex (<5%) were discarded.

UNCONDITIONED AND CONDITIONED STIMULI

The US was 30% (w/w) sucrose solution delivered to the antennae and mouth parts for 3 s. As the bees' ingestion rate for sucrose solution is 1 μl/s (Núñez, 1966), in each reinforced trial, bees received approximately 2–3 μl of sucrose solution. The CSs were the odorants 2-hexanol and 2-octanone (Sigma-Aldrich, Lyon, France), which are well learned and discriminated by the bees in olfactory PER conditioning (Guerrieri et al., 2005). Four microliters of pure odorant were applied onto a fresh strip of filter paper. The paper strip was then placed into a 1-ml plastic syringe and mounted in an odor-supplying device. When the bee was placed in front of the device, it received a gentle, constant flow of clean air provided by a standard aquarium pump. Computer-driven solenoid valves (Lee Company) controlled airflow delivery. During periods of odorant delivery, the airflow was shunted through a syringe containing the odorant. Each CS presentation lasted 4 s. An exhaust system was mounted behind the bees to remove odor-laden air.

CONDITIONING PROCEDURE

Bees were trained along four consecutive differential conditioning phases. In the first phase, bees were presented with an A+ vs. B– discrimination. In the second phase, the contingencies were reversed so that they had to learn an A– vs. B+ discrimination. In the third phase, the contingencies were again reversed and bees had to discriminate A+ vs. B–. Finally, in the fourth phase a last reversal was proposed so that bees had to discriminate A– vs. B+. Thus, bees experienced two contingency inversions between phases: A+ → A– and B– → B+ from the first to the second phase, A– → A+ and B+ → B– from the second to the third phase, and A+ → A– and B– → B+ from the third to the fourth phase.

Within each phase, reinforced and non-reinforced odorants were given five times (5 CS+ vs. 5 CS–), each in a pseudo randomized sequence. At most, two reinforced/non-reinforced trials succeeded each other within a conditioning phase. This experimental sequence was also varied from one day to the next. In all cases the intertrial interval (interval between two consecutive CS presentations, within or between phases) was 10 min. Thus, each conditioning phase lasted 90 min and the complete experiment, implying four conditioning phases also separated by 10 min, 6:30 h. Two independent groups of bees were trained along these four phases in order to balance 2-hexanol and 2-octanone as odorants A and B (see **Table 1**).

CONDITIONING TRIALS

The onset and offset of each trial as well as of CS and US delivery were controlled and signaled by a computer that was programmed to emit tones of different frequencies for each event. Each trial lasted 60 s. At the beginning of each trial the subject was placed in front of the odor-supplying device for 30 s to allow familiarization with the training situation. Thereafter the CS was presented for 4 s. In reinforced trials, the US onset occurred 3 s after CS onset. Both antennae were lightly touched with a toothpick soaked with the sucrose solution and after proboscis extension the bee was allowed to feed for 3 s. Therefore, the interstimulus interval was 3 s and the overlap between CS and US was 1 s. After US delivery, the bee was left in the setup until completing 60 s and then returned to its resting position. Non-reinforced trials consisted of CS presentations without US and lasted also 60 s.

RESPONSE MEASUREMENT

We recorded whether or not a bee extended its proboscis within 3 s after onset of the odor (CS). Responses in this interval could not be elicited directly by the US so that we measured conditioned responses to the odorants. Multiple responses during a CS were counted as a single PER. After completing the experiments, all animals were again checked for PER. If an animal did not respond (<5%) it was discarded.

STATISTICAL ANALYSIS

We measured the percentage of conditioned responses (% PER) in reinforced and non-reinforced trials. Repeated-measurement analysis of variance (ANOVA) was used for between-group and within-group comparisons. Although parametric ANOVA is usually not allowed in case of dichotomous data such as those of the PER, Monte Carlo studies have shown that it is permissible to use ANOVA for a dichotomous dependent variable under certain conditions (Lunney, 1970), which are met by our data: equal cell frequencies and at least 40 degrees of freedom of the error term. To provide a quantitative account of reversals we computed for each bee an excitatory reversal score (Δ_e) as the difference in responses to the CS+ between the fifth and the first trial of a reversal phase ($\Delta_e = CS+_{trial5} - CS+_{trial1}$), and an inhibitory reversal score (Δ_i) as the difference in responses to the CS– between the first and the fifth

trial of a reversal phase (Δ_i = CS–$_{trial1}$ – CS–$_{trial5}$). Wilcoxon test was used to compare excitatory and inhibitory reversal scores. ANOVA for repeated measurements was used to compare Δ_i and Δ_e values between conditioning phases. A further index was computed for each bee to quantify the amount of discrimination reached at the end of each conditioning phase. Such a discrimination index (Di) was calculated as the difference between the responses to the CS+ minus the responses to the CS– in the last trial (Di = CS+$_{trial5}$ – CS–$_{trial5}$). ANOVA for repeated measurements was used to compare Di values between conditioning phases. The alpha level was set to 0.05 (two-tailed) for all analyses.

RESULTS

Two independent groups of bees were trained along four consecutive differential conditioning phases involving two odorants, 2-hexanol and 2-octanone, and three reversals. In order to balance odor contingencies, Group 1 (n = 57 bees) was trained to discriminate 2-hexanol as odor A from 2-octanone as odor B, while Group 2 (n = 54 bees) was trained to discriminate 2-octanone as odor A from 2-hexanol as odor B (see **Table 1**). We first compared the performance of both groups along conditioning phases. Within each phase, there were no significant differences between Groups 1 and 2 as shown by 2 × 2 × 5 (group × stimulus A/B × trial) ANOVA for repeated measurements (**Table 2**). Thus data from both groups could be pooled.

Figure 1 shows the pooled performance of bees in our experiment (n = 111 bees). In the 1st phase (A+ vs. B–), bees successfully learned the discrimination. A 2 × 5 (stimulus A/B × trial) ANOVA for repeated measurements yielded significant stimulus ($F_{1,109}$ = 157.87; P < 0.0001) and trial ($F_{4,436}$ = 82.13; P < 0.0001) effects as well as a significant interaction effect ($F_{4,436}$ = 80.04; P < 0.0001) showing that responses to odors followed different significant trends during trials depending on their association with sucrose reward. In the 2nd phase, bees successfully mastered the first reversal as shown by the significant stimulus ($F_{1,109}$ = 6.37, P < 0.05) and trial ($F_{4,436}$ = 10.16; P < 0.0001) effects. Inversion of conditioned responses occurred in the 4th trial, thus yielding a significant stimulus × trial interaction ($F_{4,436}$ = 76.21, P < 0.0001). In the 3rd phase, bees again successfully reversed their conditioned responses to odors as shown by the significant stimulus × trial interaction ($F_{4,436}$ = 46.44, P < 0.0001). In this case, conditioned responses were inversed in the 3rd trial. Stimulus and trial effects were, however, not significant (stimulus effect: $F_{1,109}$ = 0.98, NS; trial effect: $F_{4,436}$ = 1.97, NS), probably because both curves were symmetrical, thus leading to a canceling effect for trial and stimulus. Finally in the last phase, a similar situation as in the 3rd phase was found. Bees successfully reversed their conditioned responses as shown by the highly significant stimulus × trial interaction ($F_{4,436}$ = 32.86, P < 0.0001). In this case inversion of conditioned responses was visible on the 4th trial. As in the previous, 3rd phase, stimulus and trial effects were not significant (stimulus effect: $F_{1,109}$ = 0.44, NS; trial effect: $F_{4,436}$ = 0.61, NS). Thus, bees mastered the original discrimination and the three consecutive reversals. However, **Figure 1** shows that effective discrimination decreased along successive conditioning phases. Indeed the differentiation achieved at the end of each phase decreased along the four phases.

To provide a quantitative analysis of this effect, we computed for each phase a reversal score. Reversal discrimination learning is successful if there is an increase of conditioned responses to the CS+, based on its excitatory properties acquired through association with sucrose reward, *and* a decrease in responding to the CS–, based on its inhibitory properties related to the absence of reward. The excitatory component of reversal (Δ_e) can be quantified as the difference in responses to the CS+ between the fifth and the first trial of a reversal phase (Δ_e = CS+$_{trial5}$ – CS+$_{trial1}$). The inhibitory component (Δ_i) can be quantified as the difference in responses

Table 1 | Two independent groups of bees were trained along four consecutive olfactory reversal discriminations, using 2-hexanol (2-Hex) and 2-octanone (2-Oct) as odorants to be discriminated. The contingencies (+: reinforced with sucrose solution; – : non-reinforced) of the odorants were varied systematically from one phase to the next. Groups were balanced with respect to odorant contingency.

	1st Phase	2nd Phase	3rd Phase	4th Phase
Group 1	2-Hex+ vs. 2-oct–	2-Hex– vs. 2-oct+	2-Hex+ vs. 2-oct–	2-Hex– vs. 2-oct+
Group 2	2-Hex– vs. 2-oct+	2-Hex+ vs. 2-oct–	2-Hex– vs. 2-oct+	2-Hex+ vs. 2-oct–

Table 2 | Fisher statistic values from repeated measurement ANOVA performed within conditioning phases to compare the performances of Groups 1 and 2 (see Table 1). All values were non-significant so that performance of both groups could be pooled.

	1st Phase	2nd Phase	3rd Phase	4th Phase
Group	$F_{1,109}$ = 0.07	$F_{1,109}$ = 0.02	$F_{1,109}$ = 0.01	$F_{1,109}$ = 0.18
Group × stimulus	$F_{1,109}$ = 0.12	$F_{1,109}$ = 0.08	$F_{1,109}$ = 0.001	$F_{1,109}$ = 0.03
Group × trial	$F_{4,436}$ = 0.04	$F_{4,436}$ = 0.31	$F_{4,436}$ = 0.17	$F_{4,436}$ = 0.37
Group × stimulus × trial	$F_{4,436}$ = 0.05	$F_{4,436}$ = 0.03	$F_{4,436}$ = 0.27	$F_{4,436}$ = 0.34

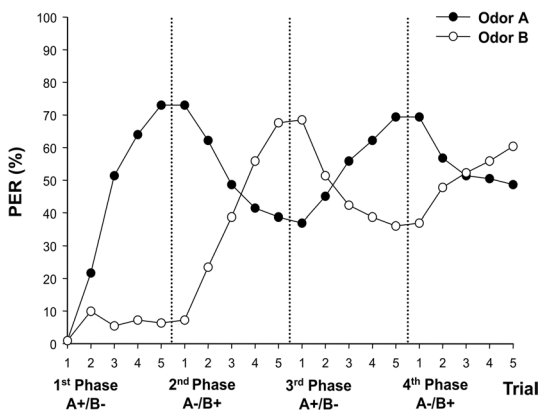

FIGURE 1 | Conditioned responses during multiple reversal learning in honeybees. Proboscis extension responses (% PER) to odors A and B during four consecutive differential conditioning phases. Bees experienced two contingency inversions between phases: A+ → A- and B- → B+ from the first to the second phase, A- → A+ and B+ → B- from the second to the third phase, and A+ → A- and B- → B+ from the third to the fourth phase. n = 111 bees.

to the CS– between the first and the fifth trial of a reversal phase (Δ_i = CS–$_{trial1}$ – CS–$_{trial5}$). Both scores were computed for each bee and reversal phase (2nd, 3rd and 4th phases). **Figure 2** shows the average Δ_e and Δ_i scores obtained (n = 111). In the 2nd phase, in which bees experienced the first reversal, the mean excitatory score Δ_e was significantly higher than the mean inhibitory score Δ_i (Δ_e = 0.60; Δ_i = 0.34; Wilcoxon test: Z = 7.11, P < 0.0001). This result indicates that after achieving the first olfactory discrimination (1st phase), bees were better in increasing responses to the formerly non-rewarded odor than in extinguishing responses to the formerly rewarded odor. In the 3rd phase, excitatory and inhibitory scores were the same (Δ_e = 0.32; Δ_i = 0.32; Wilcoxon test: Z = 0, NS), thus confirming the symmetric performance. Finally, in the 4th phase, excitatory and inhibitory scores were also equivalent (Δ_e = 0.23; Δ_i = 0.21; Wilcoxon test: Z = 0.45, *NS*). Excitatory and inhibitory scores significantly decreased along consecutive reversal phases (Δ_e: $F_{1,220}$ = 20.41, P < 0.0001; Δ_i: $F_{1,220}$ = 3.17, P < 0.05). The excitatory score of the 2nd phase was significantly higher than those of the 3rd and 4th phases (Tukey test: P < 0.0001 in both cases), which did not differ between them. Similarly, the inhibitory score of the 2nd phase was significantly higher than that of the 4th phase (P < 0.05) but not of the 3rd phase. Inhibitory scores of the 3rd and 4th phase did not differ significantly.

Thus, multiple olfactory reversals lead to a progressive decrease in the bees' ability to reverse the reinforcement contingencies. As a consequence, differentiation levels reached at the end of each conditioning phase also decreased. **Figure 3** shows the values of a differentiation index (Di) computed for each bee based on its responses in the fifth trial of each conditioning phase. This index was calculated as the difference between the responses to the CS+ minus the responses to the CS– in the last trial (Di = CS+$_{trial5}$ – CS–$_{trial5}$). A comparison between Dis calculated for each phase showed a significant decrease of differentiation from the 1st to the 4th phase of the experiment ($F_{3,330}$ = 11.34, P < 0.0001). *Pos-hoc* comparisons (Tukey test) showed that the Di of the 1st phase was significantly higher than those of the other three phases (1st vs. 2nd phase: P < 0.001; 1st vs. 3rd phase: P < 0.01; 1st vs. 4th phase: P < 0.0001), while the Dis of the 2nd and the 3rd phase did not differ significantly. The difference between the Dis of the 3rd and 4th phase was marginally non-significant (P = 0.055). Thus, although bees managed to reverse the learned contingencies along three reversal phases, their success progressively decreased and odorant discrimination was achieved with increasing difficulty.

Figure 1 shows the global responses of the entire population of bees tested. As such, it may mask differences in individual strategies applied to solve multiple reversal learning. In order to evaluate the success of an individual in multiple reversal learning, two elemental conditions have to be met: (a) the bee has to master the first olfactory discrimination (1st conditioning phase) because asking about reversal learning is meaningless if the very first learning task was not achieved; (b) the bee has also to succeed in the first reversal (2nd phase) because only then further reversal can be studied. Taking this into account, we classified bees in three categories: (1) bees that were not able to solve the very first discrimination (i.e., discrimination of the 1st phase; n = 35 bees); (2) bees that mastered the very first discrimination, but were unable to solve the subsequent reversal discrimination of the 2nd phase (n = 42 bees); (3) bees that solved the discriminations of the 1st and the 2nd phase (n = 34 bees). The 1st category represents bees that did not meet condition (a) (see above); the 2nd category represents bees that met condition (a) but not condition (b); finally, the 3rd category represents bees that met conditions (a) and (b), which were, therefore, those for which the question of success in further reversal learning was pertinent. The criterion used to define success in solving each phase was the presence of a dual correct response in the last (fifth) trial, i.e., PER to the CS+ and absence of PER to the CS–.

Figure 4 shows the performance of the three categories of bees. Per definition, bees of the 1st category did not master the original discrimination (A+ vs. B–) of the 1st phase and this effect was not

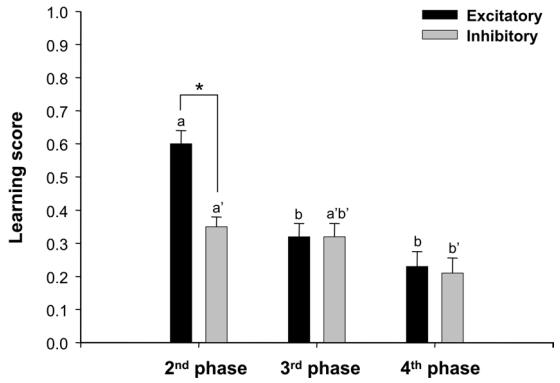

FIGURE 2 | Average excitatory (Δ_e) and inhibitory (Δ_i) reversal learning scores (+S.E.) computed for three consecutive reversal phases (2nd, 3rd, and 4th conditioning phases). Δ_e was calculated as the difference in responses to the CS+ between the fifth and the first trial of a reversal phase (Δ_e = CS+$_{trial5}$ – CS+$_{trial1}$); Δ_i was the difference in responses to the CS– between the first and the fifth trial of a reversal phase (Δ_i = CS–$_{trial1}$ – CS–$_{trial5}$). Statistical comparisons of excitatory scores between phases are indicated by letters (e.g. a, b). Comparisons of inhibitory scores between phases are indicated by letters with prime (e.g. a', b'). Asterisks indicate significant difference between excitatory and inhibitory scores within a phase. n = 111 bees.

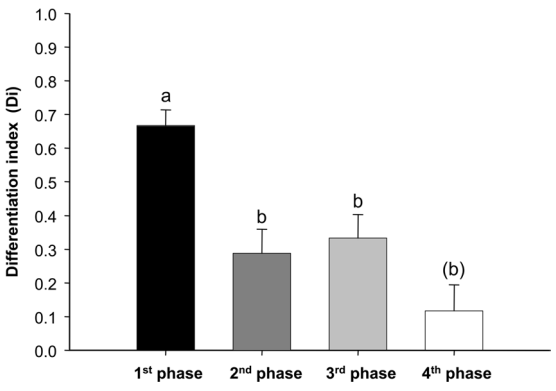

FIGURE 3 | Average differentiation index (Di) obtained in the four consecutive differential conditioning phases (+ S.E.). Di was calculated as the difference between the responses to the CS+ minus the responses to the CS– in the last conditioning trial of each phase (Di = CS+$_{trial5}$ – CS–$_{trial5}$). The difference between the Dis of the 3rd and 4th phase was marginally non-significant (P = 0.055). n = 111 bees.

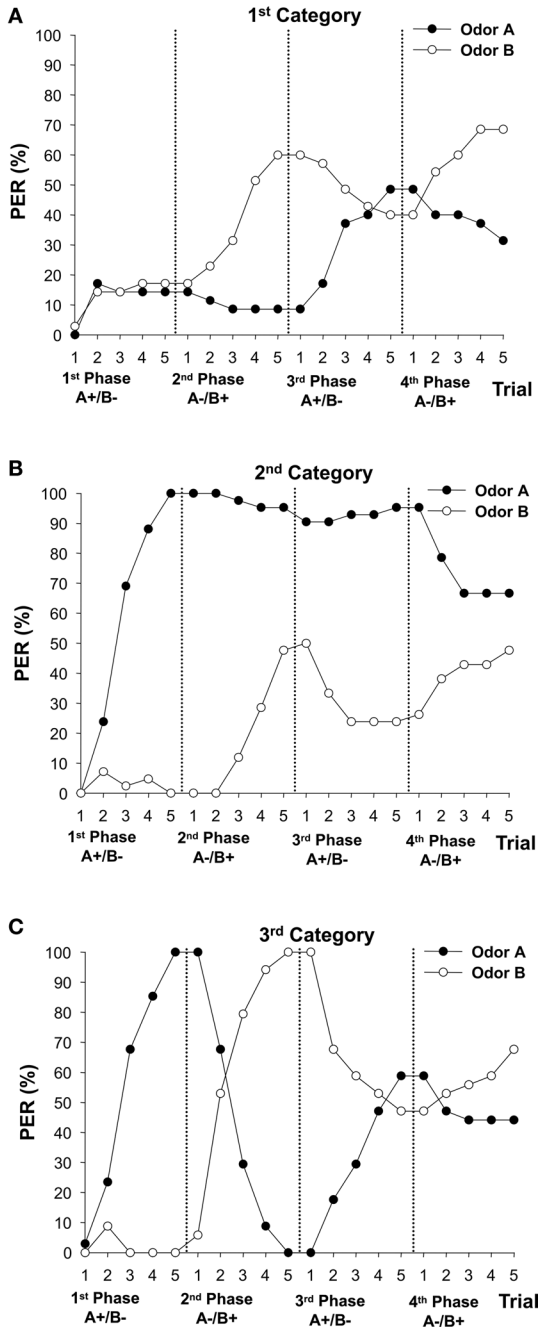

FIGURE 4 | Conditioned responses during multiple reversal learning in three categories of honeybees. Proboscis extension responses (% PER) to odors A and B during four consecutive differential conditioning phases. Categories were defined by determining individual success in solving the 1st and the 2nd conditioning phases. The criterion used to define success in solving each phase was the presence of a dual correct response in the last (fifth) trial, i.e., PER to the CS+ and absence of PER to the CS–. **(A)** First category ($n = 35$ bees) included individuals that were not able to solve the very first discrimination of the 1st phase (A+ vs. B–). **(B)** Second category ($n = 42$ bees) included individuals that mastered the very first discrimination, but were unable to solve the subsequent reversal discrimination of the 2nd phase (A– vs. B+). **(C)** Third category ($n = 34$ bees) included individuals that solved the discriminations of the 1st and the 2nd phase, for which, therefore, the question of success in further reversal learning (3rd and 4th phases) was pertinent.

limited to the fifth trial (**Figure 4A**: $F_{4,136} = 1.14$, NS). The 2nd category, which per definition mastered the discrimination of the 1st phase (**Figure 4B**: $F_{4,164} = 80.46$, $P < 0.0001$), was however unable to master the first reversal task in the 2nd phase. Although these bees responded differently to the odors ($F_{4,164} = 24.36$, $P < 0.0001$), they seemed unable to revert their response to the formerly rewarded (now non-rewarded) odor A (odor A × trial ANOVA: $F_{4,164} = 1.69$, NS). They varied, nevertheless, their responses to the formerly non-rewarded (now rewarded) odor B (odor B × trial ANOVA: $F_{4,164} = 20.92$, $P < 0.0001$). Bees of the 3rd category (**Figure 4C**) were successful in solving the discriminations of the 1st ($F_{4,132} = 63.52$, $P < 0.0001$) and the 2nd phases ($F_{4,132} = 60.86$, $P < 0.0001$). It is, therefore, possible to analyze in this group whether solving a first reversal (2nd phase) improves or not reversal efficiency in the subsequent reversals (3rd and 4th phases).

To answer this question, for all three categories we computed excitatory (Δ_e) and inhibitory (Δ_i) scores for each reversal phase. **Figure 5** shows the mean Δ_e and Δ_i scores calculated for each of category. Even if bees of the 1st category were not able to solve the first discrimination task during the 1st phase, some individuals were able to discriminate odors during the 2nd phase (A– vs. B+), and solved reversal tasks during the 3rd and 4th phases (**Figure 4A**). Their mean excitatory score Δ_e (**Figure 5A**) was significantly higher than their mean inhibitory score Δ_i in the 2nd phase ($\Delta_e = 0.43$; $\Delta_i = 0.09$; Wilcoxon test: $Z = 2.64$, $P < 0.01$). Although Δ_e values were also higher than Δ_i values in the 3rd and 4th phases, this difference was not significant (3rd phase: $\Delta_e = 0.41$, $\Delta_i = 0.24$, $Z = 1.52$, NS; 4th phase: $\Delta_e = 0.28$, $\Delta_i = 0.17$, $Z = 1.12$, NS). Excitatory Δ_e and inhibitory Δ_i scores (**Figure 5A**) did not vary significantly between phases as shown by (score × phase) ANOVA for repeated measurements (Δ_e: $F_{2,68} = 0.48$,NS; Δ_i: $F_{2,68} = 1.48$, NS). These results underline what seems to be a characteristic feature of these bees: after the first conditioning phase, where no learning was visible, they were more responsive to rewarded than to non-rewarded stimuli (see 2nd, 3rd, and 4th phases in **Figure 4A**), thus generating asymmetric curves for both kind of stimuli. This asymmetry, which is particularly visible in the 2nd phase (first reversal) could be seen, however, as a consequence of category sorting. Given that bees of the 1st category were, per definition, those not mastering the original discrimination (A+ vs. B–) of the 1st phase, one can argue that inhibitory learning in the 2nd phase has to be necessarily low because bees start from a low PER level due to the lack of excitatory learning in the 1st phase.

In the case of bees of the 2nd category (**Figure 4B**), mastering reversal tasks was impossible because these bees were unable to revert their original (1st phase) responses to the rewarded odor A even if they reverted their original (1st phase) responses to the non-rewarded odor B (**Figures 4B and 5B**). Thus, in the 2nd phase, their inhibitory score was close to 0 ($\Delta_i = 0.05$) but their excitatory score was, on the contrary, positive ($\Delta_e = 0.48$), and the difference between scores was significant (Wilcoxon test: $Z = 3.36$, $P < 0.001$) thus showing that the absence of reversal was highly associated to the lack of extinction of the formerly rewarded odor A and not to the capacity to revert the learning about the formerly non-rewarded odor B (**Figure 5B**). The reversal being impossible in the 2nd phase, the 3rd phase prolonged this situation as the original, non-reversed learning (A+ vs. B–) was again reinforced. The excitatory score in

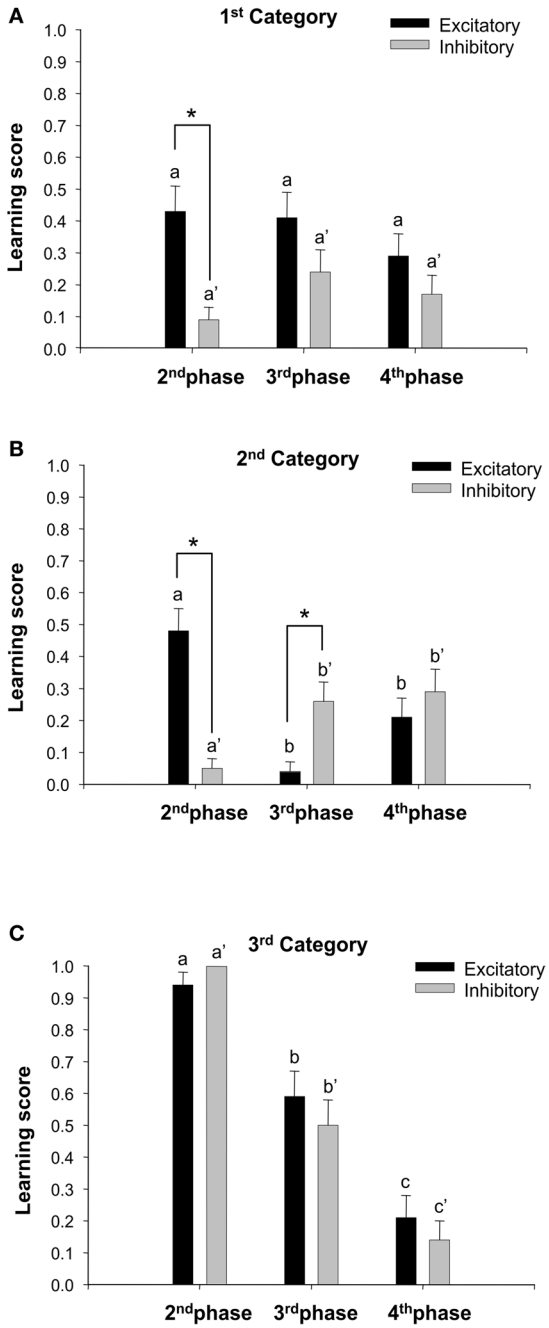

FIGURE 5 | Average excitatory (Δ_e) and inhibitory (Δ_i) reversal learning scores (+ S.E.) computed for the three categories of bees, for the three reversal phases (2nd, 3rd, and 4th conditioning phases). (A) First category (n = 35 bees) included individuals that were not able to solve the very first discrimination of the 1st phase (A+ vs. B–). **(B)** Second category (n = 42 bees) included individuals that mastered the very first discrimination, but were unable to solve the subsequent reversal discrimination of the 2nd phase (A– vs. B+). **(C)** Third category (n = 34 bees) included individuals that solved the discriminations of the 1st and the 2nd phase, for which, therefore, the question of success in further reversal learning (3rd and 4th phases) was pertinent. Statistical comparisons of excitatory scores between phases are indicated by letters (e.g., a, b). Comparisons of inhibitory scores between phases are indicated by letters with prime (e.g. a′, b′). Asterisks indicate significant difference between excitatory and inhibitory scores within a phase.

the 3rd phase was, therefore, close to 0 (Δ_e = 0.04) as bees could not improve their already high responsiveness to the rewarded odor (**Figure 5B**). The inhibitory score in this 3rd phase was, nevertheless, more important (Δ_i = 0.26), and the difference between Δ_e and Δ_i was again significant (Wilcoxon test: $Z = 2.21$, $P < 0.05$), showing again that these bees could eventually revert their conditioned responses to an odorant that was only partially learned (odor B in the 2nd phase). Finally, in the 4th phase, bees were again unable to revert the A+ vs. B– discrimination reinforced in the 3rd phase. Their excitatory and inhibitory scores were equivalent (Δ_e = 0.21; Δ_i = 0.29; Wilcoxon test: Z = 0.68, NS), thus showing a delayed and low tendency to start modulating their responses to A and B only in the last phase of the experiment (**Figure 5B**; see also **Figure 4B**). A (score × phase) ANOVA for repeated measurements showed significant changes both in excitatory Δ_e and inhibitory Δ_i scores along phases (Δ_e: $F_{2,82}$ = 11.98, $P < 0.0001$; Δ_i: $F_{2,82}$ = 4.72, $P < 0.001$). The excitatory score of the 2nd phase was significantly higher than those of the 3rd and 4th phases (Tukey test: 2nd × 3rd phase, $P < 0.001$; 2nd × 4th phase, $P < 0.01$), which did not differ between them. At the same time, the inhibitory score of the 2nd phase was significantly lower than that of the 3rd and 4th phases (Tukey test: $P < 0.01$ in both cases). Inhibitory scores of the 3rd and 4th phase did not differ significantly. These results clearly reflect the high influence of negative transfer effects in the 2nd phase.

Finally, bees of the 3rd category (**Figure 4C**), which successfully mastered the original learning (A+ vs. B–) and the first reversal (A– vs. B+), allowed analyzing whether further reversals were improved by these achievements. Differently from the other two categories (**Figure 5C**), both excitatory and inhibitory scores (2nd phase: Δ_e = 0.94, Δ_i = 1.00; 3rd phase: Δ_e = 0.59, Δ_i = 0.50; 4th phase: Δ_e = 0.21, Δ_i = 0.14) were equivalent within each reversal phase (Wilcoxon test; 2nd phase: Z = 0.00, NS; 3rd phase: Z = 0.80, NS; 4th phase: Z = 0.63, NS). Thus, the capacity of bees to extinguish responses to the formerly rewarded odor was the same as the one to increase responses to the formerly non-rewarded odor in all reversal phases (**Figure 5C**). As for the global analysis, Δe and Δi values of 3rd-category bees significantly decreased along consecutive reversal phases (Δ_e: $F_{2,66}$ = 32.04, $P < 0.0001$; Δ_i: $F_{2,66}$ = 50.41, $P < 0.0001$). All possible comparisons between Δ_e or Δ_i scores corresponding to two different phases yielded significant difference (Tukey test: Δ_e, $P < 0.001$ in all cases; Δ_i, $P < 0.001$ in all cases). Thus, the analysis of the 3rd category, which included bees that were actually effective in solving olfactory reversals, shows that a progressive decrease in the ability to reverse reinforcement contingencies occurred along successive reversal phases.

DISCUSSION

The present work shows that bees can master multiple olfactory reversals involving the same two odorants. In doing this, they do not improve their successive discrimination performances but rather tend to generalize their choice to both odors at the end of conditioning so that both discrimination levels and reversal efficiency (measured through excitatory and inhibitory scores) decreased along experimental phases. This result invalidates the hypothesis of a learning-to-learn effect, in which case a significant improvement of reversal efficiency should be evident in successive reversal phases.

Comparable results were obtained by Menzel (1969) who studied multiple reversal learning in free-flying honeybees trained with two colors, orange and blue. Using a differential conditioning protocol, Menzel (1969) trained honeybees to land five times on one of these colors to get sucrose reward and not on the alternative color that was non-rewarded. Once the first discrimination was mastered, the color contingencies were inversed as in our experiment. After three reversals, both colors were equally chosen at the end of the training procedure. Discrimination recovered only after bees were kept locked up in the hive for a day.

Our results differ in part from those of Menzel (1969) because after three reversals, we still observed a significant discrimination between the two trained odorants even if differentiation decreased and bees tended to respond equally to both odors. Though this difference may be due to different learning dynamics and accuracy in the case of color and olfactory cues and/or to the fact that our bees were restrained in the laboratory while they freely flew in Menzel's (1969) experiments, we cannot exclude that adding further reversal phases results in full generalization and equivalent choice levels for both odorants in our experiments. A more important distinction between Menzel's (1969) work and our study is the demonstration provided in our case that not all the bees are equivalent in terms of the strategies they implement when confronted with a multiple reversal learning problem. An analysis of excitatory and inhibitory scores associated with the responses generated by the CS+ and the CS–, respectively, showed that bees differed in the weight assigned to these two components. Efficient reversers exhibited comparable excitatory and inhibitory scores within each conditioning phase (**Figure 5C**), thus showing that they can equally invert their responsiveness toward excitatory and inhibitory stimuli. On the contrary, less-efficient reversers were characterized by an asymmetric weight between excitatory and inhibitory components (**Figures 5A,B**), which accentuated responses to one (either the CS+ or the CS–) of the stimuli that had to be discriminated. As a consequence, multiple reversal was partial (**Figure 4A**) or did not take place (**Figure 4B**) in these bees. The fact that bees of the same hive differed dramatically in the way they evaluate the CS+ and the CS–, and thus in the way they change their response to them, may be related to their different sensitivities to appetitive and aversive stimuli (Page et al., 1998; Roussel et al., 2009; see Page et al., 2006 for review). It has been suggested that appetitive and aversive behavioral syndromes coexist in a honeybee hive (Roussel et al., 2009). In other words, while some bees exhibit a biased responsiveness to appetitive stimuli (including sucrose and other sensory cues related to the foraging context), other bees exhibit biased responsiveness to aversive stimuli. These interindividual differences, which may determine different excitatory and inhibitory scores, may underlie the different performances observed in our multiple reversal experiment. This hypothesis can be easily tested by measuring in individual bees their responsiveness thresholds to appetitive sucrose solution of different concentrations (Page et al., 1998) and to aversive stimulation with electric shocks of different voltages (Roussel et al., 2009), measuring in each case the appropriate response, PER and sting extension reflex (SER), respectively. In this framework, we predict that bees having comparable sensitivity to appetitive and aversive stimuli will be efficient reversers.

Previous work on olfactory reversal learning in honeybees suggested that a learning-to-learn effect may account for the performance of honeybees trained to solve successive olfactory differential conditionings tasks involving different overlapping pairs of odorants (Komischke et al., 2002). Bees that had experienced three previous reversals were better than bees with no previous reversal experience in solving the final reversal task (Komischke et al., 2002). Although we did not find such an effect, the results of Komischke et al. (2002) cannot be directly compared with those of our study. Indeed, while we only used two odorants (A, B) whose valences were *simultaneously* inversed from phase to phase, Komischke et al. (2002) used four odorants (A, B, C, D), and from the two that had to be discriminated within a phase, only the valence of one was inversed at a time, thus reducing the ambiguity of the problem (e.g. A+ vs. B–, B+ vs. C–, C+ vs. D–, D+ vs. A–). As discussed by Komischke et al. (2002), configural learning may have accounted for the bees' performance in their experiment. When odor pairs are different (AB, BC, CD, DA) bees can learn each odor pair in terms of a unique configuration in which the specific odor combination determines the appropriate choice. For instance, bees may learn that in the context of B, A is the rewarded odor, in the context of C, B is rewarded, in the context of D, C is rewarded, etc. Although bees may use this form of non-elemental processing when solving olfactory discriminations (Deisig et al. 2001, 2002, 2003; Komischke et al., 2003), it cannot help solving the multiple reversals involving just two odorants, in which the outcome of a given configural unit AB changes from phase to phase.

Bees that could reverse their response to odors A and B along the consecutive phases of our experiment tended to generalize their response to both odors after extensive training. It seems, therefore, that they determined their response to a given odorant not only based on its actual contingency, but taking also into account previous experiences with it. Averaging positive and negative experiences along time would yield the progressive decrease in reversal and discrimination observed in our work, which becomes evident at the end of the 4th phase. This result shows that actual, novel experiences do not erase previous memories but are rather integrated into an updating process that allows reevaluation of the associative strength of a stimulus at any encounter. This result is consistent with analyses of memory dynamics in honeybees foraging on a patch of artificial feeders providing different rewards (Greggers and Menzel, 1993). It was shown that in these experimental circumstances, honeybee decisions are controlled by both short-term memories initiated by the reward just experienced and specific long-term memories of individual feeders within the patch. In our case, updating previous memories derived from a conditioned phase (e.g. A+, B–) with short-term memories from a subsequent reversal phase (e.g. A–, B+) may lead progressively to equivalent associative strengths for both odorants. Further reversals may enhance this effect thus resulting in a random choice for both stimuli.

Focusing on the olfactory circuit is necessary to understand the neural basis of multiple reversal learning as studied in our work. The olfactory pathway (CS pathway) has been well described in honeybees: axons of olfactory receptor neurons located on each antenna project to the antennal lobes where they synapse with approximately 4000 local interneurons and 800 projection neurons. Each antennal lobe is made of 166 glomeruli, which are the contact

sites of these different neuron classes. Projection neurons convey the processed information via two principal tracts to higher brain structures, the mushroom bodies and the lateral horn. Mushroom bodies have been traditionally related with learning and memory phenomena (Menzel, 1999; Giurfa, 2007). Specifically, it has been suggested that mushroom bodies are required for solving problems of higher complexity but not necessarily for elemental problems (Giurfa, 2003; Komischke et al., 2005; Devaud et al., 2007; Giurfa, 2007). Devaud et al. (2007) focused on simple olfactory reversal learning in honeybees and showed that reversible blocking of mushroom body signaling via a local injection of procaine impaired olfactory reversal (e.g. bees having learned to discriminate A+ from B− were unable to reverse to A− vs. B+); however further differential conditioning with two additional odors was left intact (e.g., bees having learned to discriminate A+ from B− could learn to discriminate C+ from D−). This led to the suggestion that mushroom body activity may be required to solve conflicts between contradictory CS–US associations (Devaud et al., 2007). If mushroom bodies were required for single reversal learning, it seems logic to suggest that their participation is of fundamental importance for the multiple reversal learning studied in our work as it involves the sequential processing of consecutive contradictory information about associations between CS and US. Obviously, if mushroom body blocking through local injection of procaine impedes the reversal of a learned discrimination, we expect it to also affect further reversals.

Reversal learning could be the appropriate tool to elucidate the control of neural plasticity in the olfactory circuit. Recent experiments have shown that following olfactory learning and the formation of a long-term olfactory memory (3 days after conditioning), structural changes are visible at the level of the antennal lobe where some glomeruli increase their volume in an odor-specific manner (i.e., depending on the odor conditioned; Hourcade et al., 2009). These changes may be due to an increase in synaptic branching for certain glomeruli, resulting from selective gene expression and protein synthesis following long-term memory formation. However, this mechanism has to be subjected to forms of cellular control as bees learning several flower species throughout their life as foragers, may not be subjected to continuous increases in glomerular volumes within the limits of their head capsule. One possibility is that switching to another floral species implies a concomitant decrease in those glomeruli that increased previously as a consequence of a first associative experience, together with an increase in the glomeruli that are pertinent for the novel species exploited. This hypothesis could be tested using reversal learning protocols. In this case, specific glomerular increases are expected for the first conditioning phase in the case of odor A+ but not for B− (Hourcade et al., 2009); however, the critical question is what happens to these glomeruli when A+ is reversed to A− and B− is reversed to B+. This experiment could be done to understand the neural mechanisms underlying reversal plasticity in the olfactory domain.

In an ecological context, honeybee foragers should be prone to reverse efficiently information learned about food sources. Honeybees are flower constant and exploit, therefore, the same floral species as long as it provides profitable nectar and/or pollen reward (Grant, 1950, Chittka et al., 1999). In temperate biotopes, which are characteristic of European bees, different flower species follow predictable, well-defined flowering periods. In this context, worker bees must deal with fast changes in pollen or nectar resources and should be prepared to adapt their foraging behavior to changes in stimulus–reward contingencies. Indeed, when food-source profitability changes, the ability of workers to rapidly switch to another food source will maximize colony productivity. One can, therefore, argue that reversal learning is an important component of colony fitness. However, strictly speaking, the protocol of multiple reversal learning conducted in our work would be hardly conceivable in a natural context. Indeed, in temperate biotopes, where flowering species are replaced one by the another, the scenario of two flower species A and B that would alternatively change their nectar/pollen reward multiple times is unrealistic. This may explain the progressive decrease in discriminative performance exhibited by the efficient reverser bees, which at the end tended to generalize between both odorants.

This argument does not exclude the possibility that in a natural scenario, bees do indeed "learn-to-learn," i.e., learn to perform better, when switching between species that follow each other in successive flowering periods. In this case, ambiguity would be reduced, thus favoring reversal strategies. In other words, rather than concluding that a learning-to-learn effect does not exist in honeybees, we should state that the particular learning conditions imposed by the natural environment or the experimenter may overshadow or make emerge the "learning-to-learn" effect. This conclusion is supported by experiments on reversal learning in bumblebees (Chittka, 1998). In these experiments, bumblebees were trained to collect sucrose solution in a small T-maze so that they had to choose the right arm when the entrance was marked blue and left when it was yellow. After a second reversal, bees chose directions randomly for several hundred trials, thus showing interference between the information learned in the first training and in the reversal, consistently with some of our findings. However, a single bumblebee trained with multiple reversals showed a performance that could be interpreted as a "learning-to-learn" effect; this bee displayed a poor performance until, after more than seven reversals, it detected in an extremely fast way that a reversal took place thus improving dramatically its choices (Chittka, 1998). Although this example is based on a single individual and has to be taken, therefore, cautiously, it suggests that an extensive training schedule may make emerge the "learning-to-learn" effect.

Note, however, that in our case, an extensive training schedule would not have the same effect given the important difference between the T-maze experiments with freely-moving bumblebees and our experiments with honeybees in contention. The latter, contrarily to bumblebees, do not return to the hive to unload the sucrose reward that is provided to them during the training. As a consequence, feeding sucrose reward during hundreds of trials is not possible because the honeybee's crop has a limited capacity of 60 μl (Núñez, 1966, 1982) and when this capacity is reached and bees are satiated, they do not exhibit the appetitive PER anymore, thus impeding the prosecution of the experiment. We cannot exclude, nevertheless, that in a free-flying bee protocol of multi-reversal learning, similar to that used by Menzel (1969) but with increasing number of reversals and trials, bees would be able to improve their reversal performance as the bumblebee did it in the experiments of Chittka (1998).

The observation that an increase in the number of trials may lead to the emergence of the "learning-to-learn" effect is consistent with the so-called "overlearning reversal effect" (Menzel, 1969). This effect, which determines that in a dual-choice situation reversal to the other alternative is increasingly favored with increasing number of trials, is interpreted either as a loss of US strength or a loss of attention to the conditioned stimuli as a result of a general decrease in motivation (Rescorla and Wagner, 1972). In experiments with free-flying bees (Menzel, 1969) or walking bumblebees (Chittka, 1998), the general motivation of the bees does not change throughout the series of learning trials because, otherwise, they would not come back to the feeding site on their own. This might indicate a decrease in the associative strength in predicted US presentations as a mechanism to explain the switch to the alternative stimulus in a dual-choice situation.

The comparison between our experiments and those using freely moving animals, which in turn may also differ depending on variables such as number of reversals and/or number of trials per training phase, reveals that the strategy employed the bees to respond to the problem that is posed to them depends greatly on the design of the experiment and the conditioned stimuli used. The limitation of PER conditioning for questions on multiple reversal learning derives from the harnessing situation and the fact that bees are not allowed to unload the reward successively delivered to them, thus affecting appetitive motivation if hundreds of trials are required to uncover a "learning-to-learn effect." From that point of view, controlled experiments using visual stimuli and free-flying bees are appealing; the experimenter has only to have the persistence to test bees over much longer periods than those already used (Menzel, 1969), which proved already to be insufficient to uncover such an effect, if any.

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Searching for learning-dependent changes in the antennal lobe: simultaneous recording of neural activity and aversive olfactory learning in honeybees

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Plasticity in the honeybee brain has been studied using the appetitive olfactory conditioning of the proboscis extension reflex, in which a bee learns the association between an odor and a sucrose reward. In this framework, coupling behavioral measurements of proboscis extension and invasive recordings of neural activity has been difficult because proboscis movements usually introduce brain movements that affect physiological preparations. Here we took advantage of a new conditioning protocol, the aversive olfactory conditioning of the sting extension reflex, which does not generate this problem. We achieved the first simultaneous recordings of conditioned sting extension responses and calcium imaging of antennal lobe activity, thus revealing on-line processing of olfactory information during conditioning trials. Based on behavioral output we distinguished learners and non-learners and analyzed possible learning-dependent changes in antennal lobe activity. We did not find differences between glomerular responses to the CS+ and the CS– in learners. Unexpectedly, we found that during conditioning trials non-learners exhibited a progressive decrease in physiological responses to odors, irrespective of their valence. This effect could neither be attributed to a fitness problem nor to abnormal dye bleaching. We discuss the absence of learning-induced changes in the antennal lobe of learners and the decrease in calcium responses found in non-learners. Further studies will have to extend the search for functional plasticity related to aversive learning to other brain areas and to look on a broader range of temporal scales.

Keywords: learning, memory, aversive conditioning, antennal lobe, calcium imaging, honeybee, *Apis mellifera*, sting extension reflex

INTRODUCTION

A general question in the study of associative learning and memory is how stimulus-specific and outcome-related information is stored in the nervous system. Neural correlates of memory traces are difficult to delimit because changes in neural activity resulting from even simple learning forms may be distributed among different structures and regions of the brain. This renders difficult the definition of which traces are important for the expression of behavior, at which time they are operative and how they relate to each other (Thompson et al., 1986; Squire, 1987). Even more difficult is the technical challenge of visualizing the neural activity corresponding to a memory trace while simultaneously recording behavioral responses revealing this memory trace (Gottfried et al., 2002).

Invertebrate models are especially suited to tackle this challenge because they learn and memorize relevant information of their environments and because their nervous systems present a reduced number of neurons accessible to different recording techniques (Giurfa, 2007a; Menzel et al., 2007). Both levels of analysis can be combined as invertebrates are robust enough to facilitate parallel access to behavioral responses and neural recordings using various invasive techniques (Giurfa, 2007a).

A standard invertebrate model for the study of learning and memory is the honeybee *Apis mellifera*. This insect exhibits a rich behavioral repertoire, in which learning and memory play a fundamental role in a natural context (Menzel and Giurfa, 2001; Giurfa, 2007b). The study of honeybee learning benefited from careful anatomical and physiological descriptions of the honeybee nervous system (Menzel, 1999, 2001) and from a conditioning protocol that reproduces in the laboratory the natural situation of olfactory learning in a foraging context. The protocol exploits the fact that hungry bees exhibit, even when they are harnessed, an unconditioned response (UR), the proboscis extension reflex (PER), triggered by stimulation of their antennae with sucrose solution (Takeda, 1961; Bitterman et al., 1983). Sucrose acts, therefore, as an unconditioned stimulus (US) replacing nectar reward. Presentation of a neutral odor does not elicit PER in naïve bees; however, pairing the odor and sucrose reward results in the establishment of an associative link between both stimuli so that the odor becomes a conditioned stimulus (CS) capable of eliciting PER after successful learning (conditioned response or CR). Neural pathways for CS (olfactory pathway) and US processing (sucrose pathway) have been partially characterized in the honeybee brain so that olfactory PER conditioning allows recording of behavioral performances (PER) and of neural activity in the bee brain, usually before and after conditioning.

The olfactory pathway (CS pathway) has been well described in honeybees: axons of olfactory receptor neurons located on each antenna project to the antennal lobes where they synapse

with approximately 4000 local interneurons and 800 projection neurons. Each antennal lobe is made of 166 glomeruli, which are the contact sites of these different neuron classes. Projection neurons convey the processed information via two principal tracts to higher brain structures, the mushroom bodies and the lateral horn. The sugar pathway (US pathway) is less known. Sucrose gustatory receptors located on gustatory appendages of the head (i.e., antennae and mouth pieces) send their projections to the subesophagic ganglion (de Brito Sanchez et al., 2007). Activity of a neuron whose cell body is located in the subesophagic ganglion – the VUMmx1 neuron (from Ventral Unpaired Median neuron 1 of the maxillary neuromere) – is thought to represent the neural correlate of sucrose reward (Hammer, 1993). This neuron arborizes in the antennal lobes, the calyces of the mushroom bodies and the lateral horn, which constitute sites of convergence between CS and US pathways.

In the honeybee, optophysiological recordings of neural activity based on imaging of intracellular calcium levels have been particularly useful to study learning-induced modifications in two of these convergence sites, the antennal lobe and the mushroom bodies (antennal lobe: Faber et al., 1999; Sandoz et al., 2003; Peele et al., 2006; Fernandez et al., 2009; mushroom bodies: Faber and Menzel, 2001; Szyszka et al., 2005, 2008). In the antennal lobe, odors give rise to odor-specific glomerular activation patterns (Joerges et al., 1997) that are conserved between individuals (Galizia et al., 1999a; Sachse et al., 1999). Learning-dependent changes of such patterns have been found in different variants of the olfactory conditioning of PER (Faber et al., 1999; Sandoz et al., 2003; Fernandez et al., 2009). Basically, learning leads to a decorrelation of the glomerular patterns corresponding to the odors that need to be discriminated. However, a recent work, using a different staining technique, could not replicate some of these findings (Peele et al., 2006). These studies suffered from an important limitation, which is the impossibility of recording simultaneously calcium activity and PER as a readout of learning success. Such a dual recording is especially difficult because proboscis extension involves a set of muscles placed under the brain so that their contraction during PER induces brain movements that prevent recording calcium signals. This is why studies on learning-dependent neural plasticity in the antennal lobe had to record behavioral responses independently of imaging recordings (i.e., before or after conditioning but never during it).

This problem could be overcome by the advent of a new conditioning protocol in which this problem is absent. In this novel protocol, bees learn to associate an odor (CS) with a mild electric shock (US) eliciting the UR of the sting extension reflex (SER) (Vergoz et al., 2007; Giurfa et al., 2009). Bees learn the association between the odor and the shock and then respond to the odor with a SER (CR). Learning is indeed aversive as shown by the fact that bees trained in this way and transferred to the operant context of a Y-maze, where they can freely walk and choose between the shock-associated odor and a non-shock-associated odor, explicitly avoid the punished odor and choose the non-shock-associated odor (Carcaud et al., 2009). Since SER involves the contraction of abdominal muscles, it is now possible to ask whether the antennal lobe of honeybees hosts an aversive memory trace while simultaneously recording CR confirming the presence of such memory trace. Contrarily to PER, SER does not introduce undesired movements of brain preparation. In this way, movement-free recordings of brain activity can be obtained during SER conditioning with a freely responding animal.

Here we achieved simultaneous recordings of odor-evoked calcium signals in the antennal lobe and of behavioral responses (SER) during conditioning. Based on SER, we could separate learners from non-learners so that we could relate brain activity to learning success and ask whether odor-induced calcium signals in the antennal lobe are modified by the formation of an odor-shock association, consistent with the presence of a memory trace.

MATERIALS AND METHODS

HONEY BEE PREPARATION

Apis mellifera L. workers were taken from a hive and brought to the laboratory where they were fixed in a Plexiglas recording chamber using low-temperature melting wax. The preparation followed the standard method used for calcium imaging recordings performed at the level of the antennal lobe of worker honeybees (Galizia et al., 1997; Sandoz et al., 2003; Deisig et al., 2006). The recording chamber was nevertheless modified to allow visual access to the SER during imaging. To this end, a hole was drilled in the frontal part of the chamber so that the abdomen could be passed through this hole. In this way, the abdomen tip protruded in front of the experimenter and the SER could be visually recorded. The abdomen was isolated from the rest of the body by means of a piece of transparent plastic to prevent the bee from smelling eventual emissions of its own sting alarm pheromone during the recordings. The efficiency of such isolation was checked by presenting isopentyl acetate (IPA) – the main component of the sting alarm pheromone – to bees prepared in this way and by recording antennal lobe activity. Under these circumstances, it is possible to record the glomerular pattern normally activated by IPA (Wang et al., 2008); the glomerular patterns recorded for the odors used during conditioning did not coincide with that elicited by IPA showing that our isolation method was effective.

Two metal plates smeared with E.E.G. gel (Spectra 360 Electrode Gel, Parker Laboratories) were inserted at the back of the chamber to create a contact with the anterior and the posterior parts of the thorax. The bee established a bridge between the plates, which were connected to the shock delivery system (**Figure 1A**). Odor and shock delivery were controlled by the imaging computer so that CS and US presentations could be temporally paired for conditioning (see below). The antennae were fixed to the front of the chamber using cactus spikes and two-component epoxy glue (Red Araldite, Bostik Findley S.A., France). Small pieces of plastic foil were then waxed to create a small pool around the brain region. A rectangular window was cut in the head cuticle between the eyes on the sides, behind the antennae to the front and the median ocellus at the back. Glands and trachea were removed to expose the brain. The brain was then washed thoroughly with saline solution (in mmol/l: NaCl, 130; KCl, 6; $MgCl_2$, 4; $CaCl_2$, 5; sucrose, 160; glucose, 25; Hepes, 10; pH 6.7, 500 mOsmol; all chemicals from Sigma-Aldrich, Lyon, France). The saline solution was gently removed, and the brain was bathed with 20 µl of calcium green-2 AM solution. The dye consisted of 10 µg Calcium Green-2 AM dissolved with 4 µl Pluronic F-127 (20% in dimethylsulfoxide, DMSO) (all from

Molecular Probes, Invitrogen, OR, USA) in 160 μl saline. The bee was left for 45 min on ice. After staining, the brain was thoroughly washed with saline.

OPTICAL RECORDINGS OF ODOR-EVOKED ACTIVITY

In vivo calcium imaging recordings were carried out using a T.I.L.L. photonics imaging system (Martinsried, Germany). Stained bees were placed under an epifluorescent microscope with a 10× water-immersion objective (NA 0.3), and the head region was immersed in saline solution. The preparation was slightly tipped to the front to offer a view of the antennal lobe surface.

Images were taken using a 640 × 480·pixel 12-bit monochrome CCD-camera (T.I.L.L. Imago) cooled to −12°C. Each measurement consisted of 100 frames at a rate of 5 frames/s (interval between frames 200 ms); the mean integration time was 40–120 ms. Light was shut off between frames. Pixel image size corresponded to 4.8μm × 4.8 μm after 4 × 4 binning on chip. Monochromatic excitation light at 475 nm was applied using a monochromator (T.I.L.L Polychrom IV). The filter set on the microscope was composed of a 505 nm dichroic filter and a LP 515 nm emission filter.

ODOR STIMULATION

Under the microscope, a constant air-stream, into which odor stimuli could be injected, was directed to the bee's antennae (distance 2 cm). Odor presentation started at frame 15 and lasted for 5 s. During odor stimulation, a secondary airflow was diverted from the main airflow and passed through an interchangeable glass pipette containing the odor source. Stimulations were controlled by the computer of the imaging system.

The odors used for conditioning were 1-hexanol and 1-nonanol, which can be easily discriminated by honeybees (Guerrieri et al., 2005). In addition, octanal was used before conditioning to test the preparation for good-quality calcium signals. Odor sources were prepared by applying 5 μl of substance onto a 1 cm^2 piece of filter paper inserted in a Pasteur pipette. Pipettes containing a clean piece of filter paper (air control) were presented to the bees before and after conditioning trials. All odors were obtained from Sigma-Aldrich.

CONDITIONING

After successful recording of calcium signals upon presentation of octanal (see above), we started the conditioning procedure. The protocol was inspired by the olfactory aversive conditioning developed by Vergoz et al. (2007) and adapted to the constraints of optical imaging. It consisted of a differential conditioning procedure involving eight trials. In four trials an odor was associated with an electric shock (CS+) while in four other trials a different odor was presented without shock (CS−). CS+ and CS− were presented in a pseudo-randomized sequence starting with the CS+ in half of the animals and with the CS− in the other half. The aversive US was a 5 V electric shock. The odor was presented for 5 s; in CS+ trials, the electric shock was given during the last 2 s of odor presentation. The interstimulus interval was therefore 3 s, and is therefore in the range of ISIs promoting the most robust aversive memories in honeybees (Giurfa et al., 2009). A red light was directed toward the abdomen and away from the microscope objective, so that sting extensions upon odorant and electric shock presentations could be visually recorded. We scored sting extensions during the first 3 s of odor presentation, before shock delivery (i.e., CR). Bees that did not respond with SER to the electric shock were discarded. For half of the bees, 1-nonanol was the CS+ and 1-hexanol the CS−; for the other half odor valence was reversed.

ANATOMICAL STAINING

During optical imaging, the glomerular structure of the antennal lobes is not visible and fluorescence is homogeneous over the whole antennal lobe surface. To reveal the glomeruli, the brain was first bathed with protease (from *Bacillus licheniformis* in propylene glycol; Sigma-Aldrich) during 45 min. It was then rinsed with saline and bathed with neutral red solution (4%, diluted in water) during 20 min. Afterwards the brain was again carefully washed with saline solution. Fluorescence photographs were taken at different focal planes (around 50) using 530 nm excitation light provided by a monochromator and a filter set composed of a 570 nm dichroic filter and LP 590 nm emission filter. We could then identify individual glomeruli using the atlas of the honeybee antennal lobe developed by Galizia et al. (1999b). In all bees we identified the same 21 glomeruli (**Figure 1B**). Physiological responses of these glomeruli account for the behavior of odor-conditioned bees (Guerrieri et al., 2005).

ACTIVITY MAPS

Calcium imaging data were analyzed using custom-made software written in IDL (Research Systems Inc., Boulder, CO, USA). Each odor recording corresponded to a 3-dimensional array with two spatial dimensions (x, y pixels of the area of interest) and a temporal dimension (100 frames). Three steps were carried out to calculate the signals: first, to reduce photon (shot) noise, the raw data were filtered in spatial and temporal dimensions using a median filter with a size of 7 pixels. Second, relative fluorescence changes ($\Delta F/F$) were calculated as $(F - F0)/F0$, taking as reference background $F0$ the average of three frames before any odor stimulation (here frames 5–7). Third, to correct for bleaching and possible irregularities of lamp illumination in the temporal dimension, a subtraction was made at each pixel of each frame, of the median value of all the pixels of that frame. Such a correction stabilizes the baseline of the recordings, without removing pertinent signals. Odor-evoked signals were the typical stereotyped biphasic signals obtained upon bath application of Calcium Green. They showed a first, fast fluorescence increase followed by a slow fluorescence decrease below baseline (Galizia et al., 1997; Stetter et al., 2001; Sandoz et al., 2003). The maximum signal was obtained 1.8 s after odor delivery and the minimum before odor application. For visual observation of the signals, activity maps are shown with the best possible spatial definition of odor-induced signals. Each pixel represents the mean of three frames after 1.8 s minus the mean of three frames just before odor presentation. Activity maps are presented in a false-color code, from dark blue (no signal) to red (maximum signal).

SIGNAL AMPLITUDE CALCULATION

For a quantitative analysis of signal amplitude, we focused on the fast (positive) signal component evoked by odor stimulation (Galizia et al., 1999a; Sachse et al., 1999; Sandoz et al., 2003). This calcium increase upon odor stimulation can be ascribed to an intracellular calcium

increase from the extracellular medium, directly related to neuronal activity (see also Galizia and Kimmerle, 2004). In the antennal lobe, it reflects most probably presynaptic neuronal activity from OSNs (Galizia et al., 1998; Sachse and Galizia, 2003; see Deisig et al., 2010 for discussion on the signal origin). Moreover, studies recording neuronal responses downstream of the antennal lobe showed that these neurons (projection neurons and clawed Kenyon cells, Sachse and Galizia, 2002; Szyszka et al., 2005) respond well within the first second after odor application. Therefore, relevant neuronal activity should be measured early after odor application. Finally, as during conditioning trials, the reinforced odorant is presented with an electric shock, we had to analyze the amplitude of activation in the 3 s during which the odor was presented alone, and before the presentation of the electric shock (US), in order to obtain a neural response to the CS only.

For each glomerulus, the time course of relative fluorescence changes was calculated by averaging 25 pixels (5 × 5) (**Figure 1B**). The amplitude of odor-induced responses was calculated as the mean of 3 frames after odor onset (frames 23–25), minus the mean of 3 frames just before the odor stimulus (frames 11–13). This value was used in all computations.

THE SEARCH FOR LEARNING-INDUCED MODIFICATIONS OF ODOR RESPONSES

As we had access to behavioral responses during conditioning, we could classify each bee as a learner or as a non-learner. Bees showing more responses to the CS+ than to the CS–, were considered as learners (n = 18) whereas other bees were considered as non-learners (n = 17). In this separation, we excluded spontaneous responses in the first trial given that bees were naïve at this stage. We compared antennal lobe activity in both groups as they experienced the same conditioning procedure, leading to different results in each case. We also compared, within each group, the evolution of calcium responses to the CS+ and the CS–.

Different variables were used to quantify antennal lobe activity. We analyzed the following *global measures*: (1) *the total activation of the antennal lobe,* i.e., the sum of the amplitudes of calcium signals in the 21 glomeruli, both for the CS+ and CS–, and for learners and non-learners; (2) *the number of activated glomeruli,* i.e., the number of glomeruli that are activated by a given odor; a glomerulus was considered to be activated when the amplitude of the calcium signal was above 2 SD of the signal calculated before stimulus presentation, i.e., between frames 1 and 14; this variable was compared between learners and non-learners and within groups, between CS+ and CS–; (3) *the similarity between CS+* and CS–; i.e., the Euclidian distance between odor-response patterns when represented in a putative neural space of 21 dimensions defined by the 21 glomeruli identified in all bees (Sandoz et al., 2003). The Euclidian distance (d_{ij}) between odors i and j was calculated as:

$$d_{ij} = \sqrt{\sum_{k=1}^{p} \left(X_{ik} - X_{jk}\right)^2}$$

Where p is the number of measured glomeruli (corresponding to the dimensionality of the Euclidian space, in our case 21) and X_{ik} and X_{jk} are the calcium responses to odor i and j, respectively, in glomerulus k. Similarity between CS+ and CS– measured in this way could be compared between learners and non-learners.

Besides measures of global activity, we also analyzed (4) *possible changes in individual glomerular activation.* To this end, we quantified the amplitude of activation in each of the 21 glomeruli along the conditioning trials for learners and non-learners, and for the CS+ and the CS–. Learning-induced plasticity may differentially affect individual glomeruli. We paid particular attention to those glomeruli that were maximally activated by each odorant (glomerulus 28 for 1-hexanol and 17 for 1-nonanol). We analyzed the evolution of the amplitude of activation of these two glomeruli along trials for learners and non-learners, when their corresponding odors were presented as CS+ or CS–. The same analysis was performed on the 19 remaining glomeruli for both odorants.

Finally, to determine whether variations in individual glomerular responses were not determined by fitness differences or by a high bleaching of the calcium dye, we quantified the basal level of fluorescence *before* odor presentation (mean level of fluorescence taken from frame 5 to 14) for each glomerulus, for learners and non-learners and for both odorants, 1-hexanol and 1-nonanol, when presented as CS+ or CS–.

STATISTICAL ANALYSIS

All variables were compared between groups or within group along conditioning trials, using repeated measurement ANOVAs. Within each group, we wanted to compare the responses to the CS+ and to the CS–. However, since different odors were used as CS+ and CS–, absolute measures of antennal lobe activation before conditioning were different and could not be directly compared. For instance, 1-hexanol and 1-nonanol, used as CS+ and CS–, do not induce the same global activation of the antennal lobe. For these reasons, all values for a given odor were normalized with respect to the value obtained at the first trial with that odor, i.e., when the bees were still naïve. In most cases, we subtracted the absolute value at the first trial from the absolute values at each subsequent trial. Thus, in the graphs, the normalized value at trial 1 was 0. The only exception was the number of activated glomeruli, which was normalized by dividing the value at each trial by the average number of activated glomeruli at trial 1. This normalization allowed direct comparison of the evolution of responses between CS+ and CS–, which was otherwise not possible.

RESULTS

BEHAVIORAL RECORDINGS

We recorded simultaneously odor-induced calcium signals in the antennal lobe and behavioral responses in 35 honeybees subjected to differential olfactory conditioning of the SER. Examples of simultaneous calcium imaging and recordings of SER during conditioning trials are shown in **Figure 1C**. Access to behavioral responses allowed determining whether or not bees learned the association between odorant and shock. To reveal possible modifications of odor representations in the antennal lobe resulting from conditioning, we divided bees into "*learners*" (n = 18) and "*non-learners*" (n = 17). While the former exhibited more responses to the CS+ than to the CS– during conditioning, the latter did not exhibit preferential responses to the CS+ despite having experienced the same conditioning procedure (**Figure 1C**).

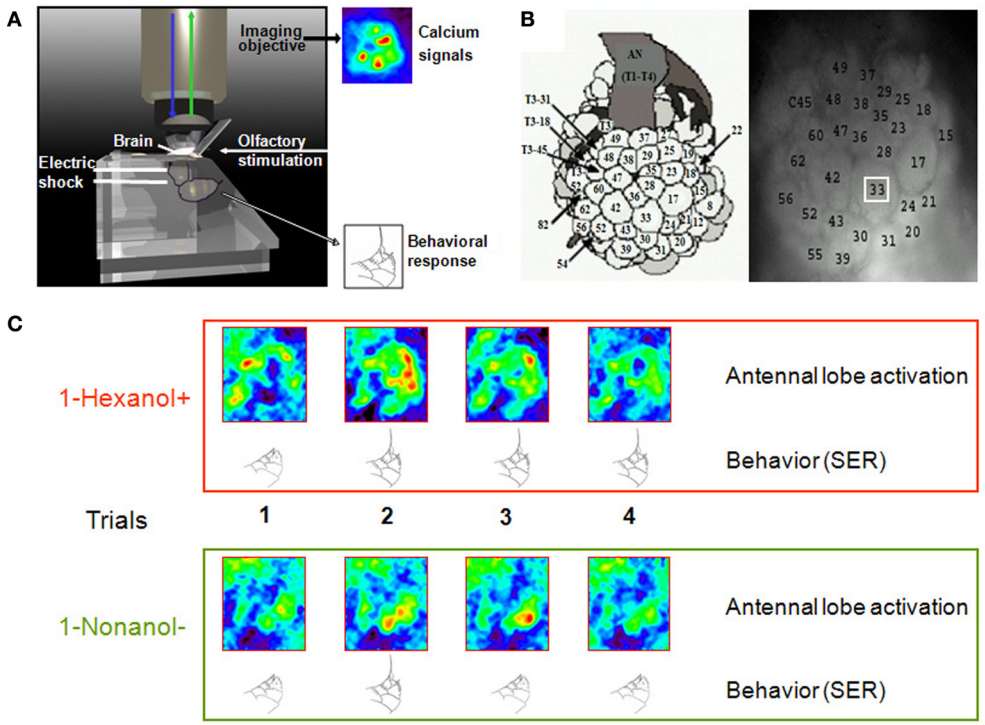

FIGURE 1 | Simultaneous recording of calcium signals in the bee brain and behavioral responses during aversive conditioning. (A) The bee is fixed in the recording chamber, with its brain exposed and bathed with Ringer, and placed under the imaging objective. The antennae can freely move so that they can be stimulated with odorants. Odorant presentations can be paired with electric shocks delivered to the thorax, by means of two conducting plates at the back of the chamber. A hole at the front of the chamber allows visualizing the end of the abdomen, so that the experimenter can observe the behavioral response of sting extension and score behavioral performance during conditioning. Brain activity is monitored throughout the experiment. **(B)** Left, atlas of the honeybee antennal lobe developed by Galizia et al. (1999b) and right, anatomical staining of an antennal lobe allowing recognition of individual glomeruli. The white square is an example of the area in which calcium signal amplitude is calculated for each glomerulus. **(C)** Example of on-line recordings of calcium signals and behavioral responses of individual bees during conditioning. In this experiment, 1-hexanol was paired with shock and 1-nonanol was not paired with shock. During the four reinforced and four non-reinforced trials, we were able to record calcium activity in the antennal lobe upon odor presentations while the bee was learning the associations. We had simultaneous access to the behavioral responses of the bee (sting extensions); in the example shown, the bee started responding with a sting extension to the punished odorant from the second trial on and responded incorrectly to the non-reinforced odorant once in the second trial.

For both learners and non-learners, we found no differences between the behavioral responses of the two subgroups trained respectively with 1-nonanol+ vs. 1-hexanol– or with 1-nonanol– vs. 1-hexanol+ (ANOVA for repeated measurements; learners: $F_{1,16} = 0.28$, NS; non-learners: $F_{1,14} = 1.58$, NS) so that results were pooled within each group. **Figure 2** shows the resulting learning performances of learners and non-learners. Given the classification criterion used for individual bees, learners obviously mastered the discrimination between the CS+ and the CS– (stimulus effect: $F_{1,34} = 40.51$, $p < 0.001$) while non-learners did not (stimulus effect: $F_{1,32} = 0.11$, NS). Moreover, learners and non-learners behaved differently along trials (group × stimulus × trial ANOVA, group effect: $F_{1,33} = 33.81$).

CALCIUM IMAGING RECORDINGS

Parallel to behavioral measurements of SER, we recorded odor-induced calcium signals in the antennal lobe of learners and non-learners. We were, therefore, able to determine whether neural activity changes as a consequence of learning and whether such changes, if any, allow better decorrelation between CS+ and CS–. To this end we asked whether variables characterizing neural activity in the antennal lobe uncover differences between learners and non-learners, and more specifically, between CS+ and CS–.

Does the global amplitude of activation in the antennal lobe vary between learners and non-learners and between CS+ and CS–?

We first compared the global amplitude of activation of the antennal lobe between learners and non-learners, both for the CS+ and the CS– (**Figure 3A**). To this end, we measured the sum of the responses recorded in the 21 measured glomeruli, and we normalized them to the global intensity of activation recorded for odors at the first trial, when bees are still naïve (0 level). This procedure allows discarding possible variations in basal intensity of activation so that CS+ and CS– curves, and learners and non-learners, have a common starting point allowing a better appreciation of variations due to learning.

For both learners and non-learners, we did not find any significant difference in the global amplitude of activation recorded for CS+ and CS– (*learners*; stimulus effect: $F_{1,34} = 0.58$, NS; interaction stimulus × trial: $F_{3,102} = 0.23$, NS; *non-learners*; stimulus effect:

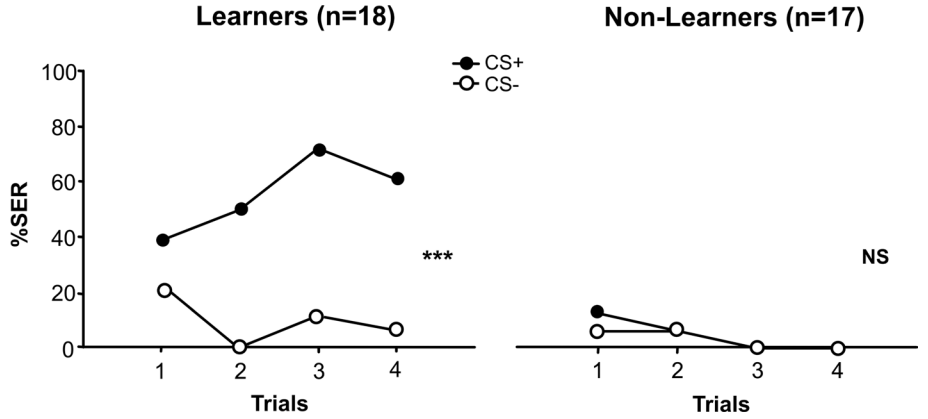

FIGURE 2 | Learning curves of conditioned bees. We distinguished learners from non-learners. Learners mastered the olfactory discrimination as they responded more to the CS+ than to the CS– during conditioning trials (*stimulus* effect: ***$p < 0.001$); non-learners, on the contrary, were unable to master the discrimination (*stimulus* effect: p = NS).

$F_{1,32}$ = 0.058, NS; interaction stimulus × trial: $F_{3,96}$ = 0.03, NS). Although learners definitely managed to discriminate the CS+ from the CS– at the end of training (see **Figure 2**), neural activity elicited by the CS+ and the CS– did not reflect such a differentiation, at least for the global variable we considered. In the case of non-learners, which did not master the behavioral discrimination, the fact that there was no difference between CS+ and CS– in terms of global activation was not surprising. However, in their case, the total intensity of activation decreased along trials both for the CS+ and for the CS– (**Figure 3A**, right panel; trial effect: $F_{3,96} = 6.42$, $p < 0.001$) while it remained constant for learners (**Figure 3A**, left panel; trial effect: $F_{3,102}$ = 1.84, NS). Yet, differences between groups (learners vs. non-learners) were not high enough to reach significance (group effect: $F_{1,33} = 2.48$, NS; interaction group × *stimulus*: $F_{1,33} = 0.56$, NS; interaction group × trial: $F_{3,99} = 1.19$, NS). Thus, the fact that some bees learned to differentiate the CS+ and the CS–, while other bees did not, was not reflected by differences in the global amplitude of activation of the antennal lobe. This variable differed, however, between learners and non-learners in a non-specific way, so that non-learners exhibited a general decrease of activation along trials which learners did not show.

Does the number of activated glomeruli vary between learners and non-learners and between CS+ and CS–?

Even if the global amplitude of activation was not modified by learning, the number of activated glomeruli could have been modified in a CS–specific manner. One may hypothesize that in the case of CS+ activation patterns of learners, there would be more glomeruli activated with smaller amplitude of activation, or less activated glomeruli with higher amplitude of activation. We, therefore, calculated, for learners and non-learners, the number of glomeruli activated by the CS+ and the CS– along conditioning trials. To correct for the fact that different odors activate different numbers of glomeruli in naïve bees, we normalized the data to the number of glomeruli activated by each odor in the first trial (100%) (**Figure 3B**). This procedure determines a common starting point for both CS+ and CS– curves, and for learner and non-learner curves, so that their variation along trials can be compared.

For both learners and non-learners, we found that the number of activated glomeruli did not change between CS+ and CS– (**Figure 3B**; *learners*; stimulus effect: $F_{1,34} = 1.27$, NS; interaction stimulus × trial: $F_{3,102} = 0.86$, NS; *non-learners*; stimulus effect: $F_{1,31} = 0.79$, NS; interaction stimulus × trial: $F_{3,93}$ = 0.55, NS). Similarly, there were no significant differences within groups related to a trial effect (*learners*; trial effect: $F_{3,102}$ = 1.39, NS; *non-learners*: $F_{3,93}$ = 1.05, NS). A comparison between groups (learners vs. non-learners) was also not significant (group effect: $F_{1,32} = 0.40$, NS; interaction group × stimulus: $F_{1,32} = 0.01$, NS; interaction group × trial: $F_{1,96} = 0.60$, NS). Even if learners mastered the discrimination between CS+ and CS– (see **Figure 2**), the number of glomeruli activated by the CS+ and the CS– did not reflect such a differentiation.

Does the similarity between CS+ and CS– vary between learners and non-learners along conditioning trials?

Even if no global changes of neural activity were detected for the CS+ and the CS– in either learners or non-learners, we analyzed whether the similarity between CS+ and CS– was modified in learners vs. non-learners during conditioning. To this end, we calculated the Euclidian distance between CS+ and CS– activity patterns, for both learners and non-learners, in the putative neural space of 21 dimensions defined by the 21 glomeruli under study (Sandoz et al., 2003; Deisig et al., 2006, 2010). Euclidean distance between two odors in this olfactory space provides a good estimation of their perceptual similarity: odors whose loci lie close to each other in the space are perceptually more similar than odors whose loci are separated (Deisig et al., 2006, 2010). In our case, the analysis should reveal if the distance between the CS+ and the CS– increases in learners, consistently with their increased discrimination. We first analyzed whether the distance between CS+ and CS– on trial one predicts behavioral performance, i.e., whether separability between CS+ and CS– *before training* accounts for the learner or non-learner status of a bee. To this end, we compared the Euclidian distance between 1-hexanol and 1-nonanol for learners and non-learners and found no difference between groups (*t*-test: t_{33} = 1.81, NS). This means that this parameter cannot predict whether a bee will be successful in mastering or not the discrimination between CS+

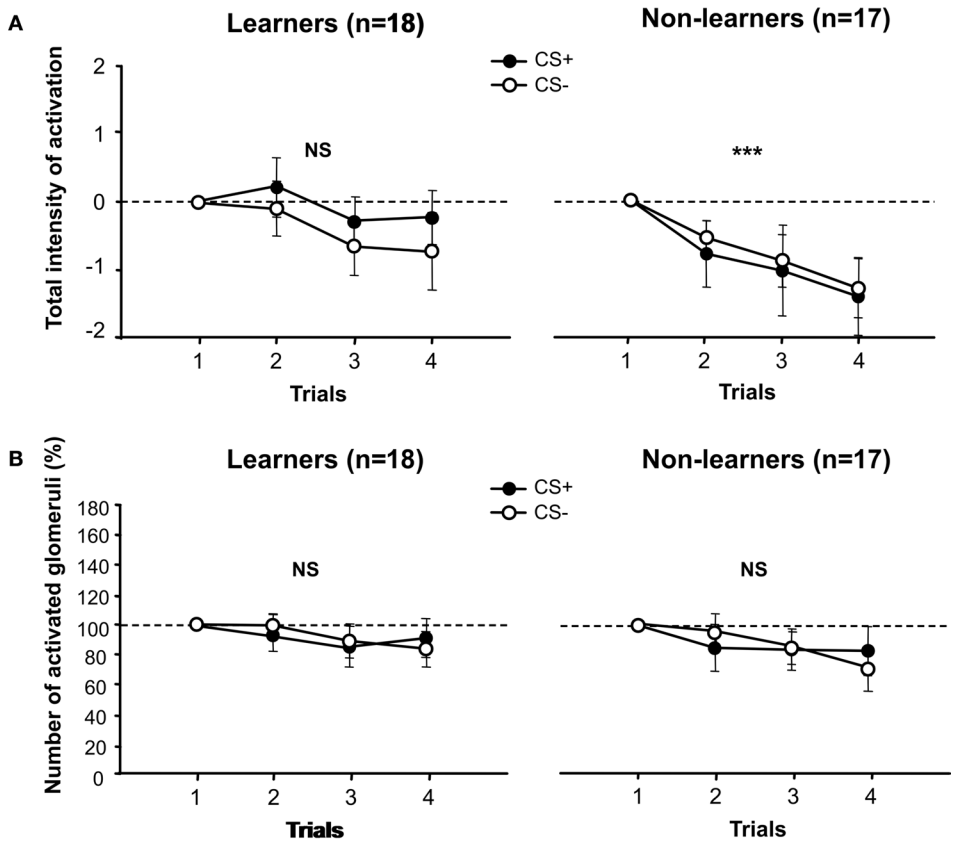

FIGURE 3 | Calcium activity in the antennal lobe. (A) *Total intensity of activation of the antennal lobe.* Amplitudes are normalized to the global intensity of activation recorded for odors in the first trial, when bees are still naïve (0 level). *Left*: for learners, no difference was found between CS+ and CS– during conditioning trials (stimuli effect, p = NS). *Right*: the same lack of differentiation was observed for non-learners, but these bees exhibited a significant decrease of the total antennal lobe activation during conditioning trials (trial effect, $***p < 0.001$). Learners did not exhibit such a decrease (trial effect, p = NS). **(B)** *Number of activated glomeruli.* To get rid of that different odors activate different numbers of glomeruli in naïve bees, we normalized the data to the number of glomeruli activated by each odor in the first trial (100%). *Left*: for learners no difference was found between the number of glomeruli activated by the CS+ and the CS– during conditioning trials (*stimulus* effect, p = NS). *Right*: for non-learners, there was also no difference between the number of glomeruli activated by the CS+ and the CS– during conditioning trials (*stimulus* effect, p = NS). Aversive conditioning does not modify global activity of the antennal lobe in a learning-dependent manner.

and CS–. The original distance between odors before conditioning (i.e., at the first trial) was then normalized to 0 to facilitate comparisons between learners and non-learners. An increase of distance (positive values) along trials reflects better discrimination between odors, while a decrease (negative values) reflects a reduction of the capacity to distinguish odors.

Figure 4 shows the variation of Euclidean distance between CS+ and CS– during conditioning trials, both for learners and non-learners. The trial effect was highly significant showing that there were changes in distance during conditioning ($F_{3,99} = 5.88$, $p < 0.001$); however, neither the group effect (learners vs. non-learners: $F_{1,33} = 0.13$, NS) nor the interaction ($F_{3,99} = 1.25$, NS) were significant. Separated analyses performed on each group showed that learners did not exhibit a significant variation of the distance between CS+ and CS– along trials (trial effect: $F_{3,51} = 1.31$, NS), while non-learners did ($F_{3,48} = 8.09$, $p < 0.001$). However, this difference was not high enough to render significant the group effect of the two-factorial repeated measurements ANOVA (see above). Due to this, we will not further insist on this difference. Note, however, that a reduction of the distance between CS+ and CS– would be difficult to understand in the case of learners, which successfully managed to differentiate these odors.

Does the amplitude of activation of individual glomeruli vary between learners and non-learners and between CS+ and CS–?

As global measures of antennal lobe activity did not reveal any CS-specific effect, neither in learners nor in non-learners, we focused on activity within single glomeruli. We analyzed whether learning-dependent variations that were possibly masked by global measures, could occur at this level. We compared, for learners and non-learners, the amplitude of activation of individual glomeruli in their responses to the CS+ and the CS– along conditioning trials. Data were normalized to the amplitude of activation recorded at the first trial, when bees are still naïve (0 level).

Given that 1-hexanol and 1-nonanol activate a different set of glomeruli, responses were compared separately. We first focused on the two glomeruli that are most strongly activated by each odor, glomerulus 28 (**Figure 5A**) for 1-hexanol, and glomerulus

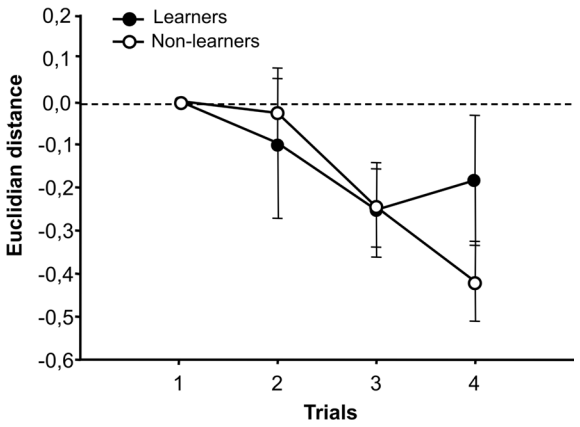

FIGURE 4 | Similarity between activity patterns of CS+ and CS−. The Euclidian distance between CS+ and CS− activity patterns was calculated both for learners and non-learners in the putative neural space with 21 dimensions defined by the 21 glomeruli under study. This distance is a measure of perceptual similarity between odors (larger distance: less similarity, shorter distance: more similarity). The original distance between odors before conditioning (i.e., in the first trial) was normalized to 0 to facilitate comparisons between learners and non-learners. We found no changes in perceptual similarity between CS+ and CS− activity patterns in the course of training between groups (group effect: p = NS) but whereas the similarity between CS+ and CS− did not change along trials for learners (trial effect, p = NS), the similarity decreases for non-learners (trial effect, $^{***}p < 0.001$).

17 (**Figure 5B**), for 1-nonanol. We quantified, for learners and non-learners, the amplitude of activation of these glomeruli along conditioning trials. Note that for each bee, the maximally activating odorant of each glomerulus was either CS+ or CS−. Therefore, this factor was added to the analysis.

For glomerulus 28 (**Figure 5A**), there were no significant differences in the amplitude of activation when 1-hexanol was used as CS+ or as CS−, for both learners (left panel) and for non-learners (right panel) (*learners:* stimulus effect: $F_{1,16} = 1.09$, NS, interaction stimulus × trial: $F_{3,48} = 0.48$, NS; *non-learners*: stimulus effect: $F_{1,15} = 0.034$, NS; interaction stimulus × trial: $F_{1,45} = 0.18$, NS). No differences were found between learners and non-learners with respect to the amplitude of activation of glomerulus 28 (group effect: $F_{1,15} = 0.001$, NS; interaction group × stimulus: $F_{1,31} = 0.59$, NS; interaction group × trial: $F_{3,93} = 0.15$, NS).

Similarly, for glomerulus 17 (**Figure 5B**), there were no significant differences in the amplitude of activation when 1-nonanol was used as CS+ or as CS− both for learners (left panel) and for non-learners (right panel) (*learners*: stimulus effect: $F_{1,16} = 0.01$, NS, interaction stimulus × trial: $F_{3,48} = 0.07$, NS; *non-learners*: stimulus effect: $F_{1,15} = 1.22$, NS, interaction stimulus × trial: $F_{3,45} = 1.04$, NS). There were no differences between learners and non-learners with respect to the amplitude of activation of glomerulus 17 (group effect: $F_{1,15} = 1.98$, NS; interaction group × stimulus: $F_{1,31} = 0.72$, NS; interaction group × trial: $F_{3,93} = 0.6$, NS).

Thus, the amplitude of activation of the glomeruli that were maximally activated by the odorants used did not yield any significant learning-dependent effect. It may be, however, that glomeruli exhibiting less activation upon odor stimulation are those showing significant learning-dependent changes. The same analysis was then performed on the remaining 19 glomeruli and the same results were found both for learners and non-learners, i.e., for each glomerulus, there were no significant differences in the amplitude of activation when 1-hexanol or 1-nonanol were used as CS+ or CS− (not shown).

Finally, we analyzed whether non-learner responses were affected by a problem of fitness or a high bleaching of the dye. To answer this question, we determined and compared the basal level of fluorescence *before* odor presentation (mean level of fluorescence taken from frame 5 to 14) for each glomerulus, for learners and non-learners and for odorants, 1-hexanol and 1-nonanol, when presented as CS+ or CS−.

The basal level of fluorescence increased significantly during conditioning trials (data not shown) in all glomeruli, both for learners and non-learners, and for both odors used as CS+ and CS−. We found no difference between groups (learners vs. non-learners), odors (1-hexanol vs. 1-nonanol) and CS (CS+ vs. CS−). This suggests that although the basal level of fluorescence increased during trials for all bees, non-learners exhibited a progressive decrease of calcium responses (see **Figure 3A**) that was specific to odor delivery. In any case, this decrease was neither due to a fitness problem nor to an abnormal bleaching of the dye.

DISCUSSION

In the present work, we achieved the first simultaneous recording of conditioned and optophysiological responses in honeybees, successfully linking the behavioral and neurobiological levels. We coupled the novel olfactory aversive conditioning of the SER (Vergoz et al., 2007; Giurfa et al., 2009) with calcium imaging recordings of the antennal lobe, which is the first olfactory center of the honeybee brain. The possibility of having a simultaneous behavioral readout (SER) allowed us to separate honeybees that mastered the discrimination between a punished (CS+) and a non-punished odor (CS−) (learners) from those that did not learn the task (non-learners). As these two groups received exactly the same conditioning procedure, they would in principle be well-suited for detecting learning-specific modifications of odor-evoked responses in the antennal lobe. To this end, our analysis focused on a population of glomeruli that is commonly accessed in such recordings (Sachse et al., 1999; Deisig et al., 2006, 2010) and whose responses effectively predict perceptual measures of odor similarity in honeybees (Guerrieri et al., 2005).

Our calcium imaging recordings did not yield evidence for learning-dependent changes in neural activity at the level of the antennal lobe during aversive SER conditioning. Specifically, no differences between glomerular responses to the CS+ and to the CS− could be detected in learners although these bees showed consistent differentiation between these odors. We found however an unexpected effect in non-learners, namely a progressive decrease in physiological responses to odors, irrespective of their valence, in the course of conditioning. While learners exhibited a rather constant level of responses to odors throughout conditioning, non-learners showed a significant decrease of calcium responses both for the CS+ and the CS− (see **Figure 3A**). As decreases in responses in a neurophysiological experiment are often due to a worsening of the animals' condition, one should be careful in analyzing such a result. However, the effects observed in non-learners did not seem to be

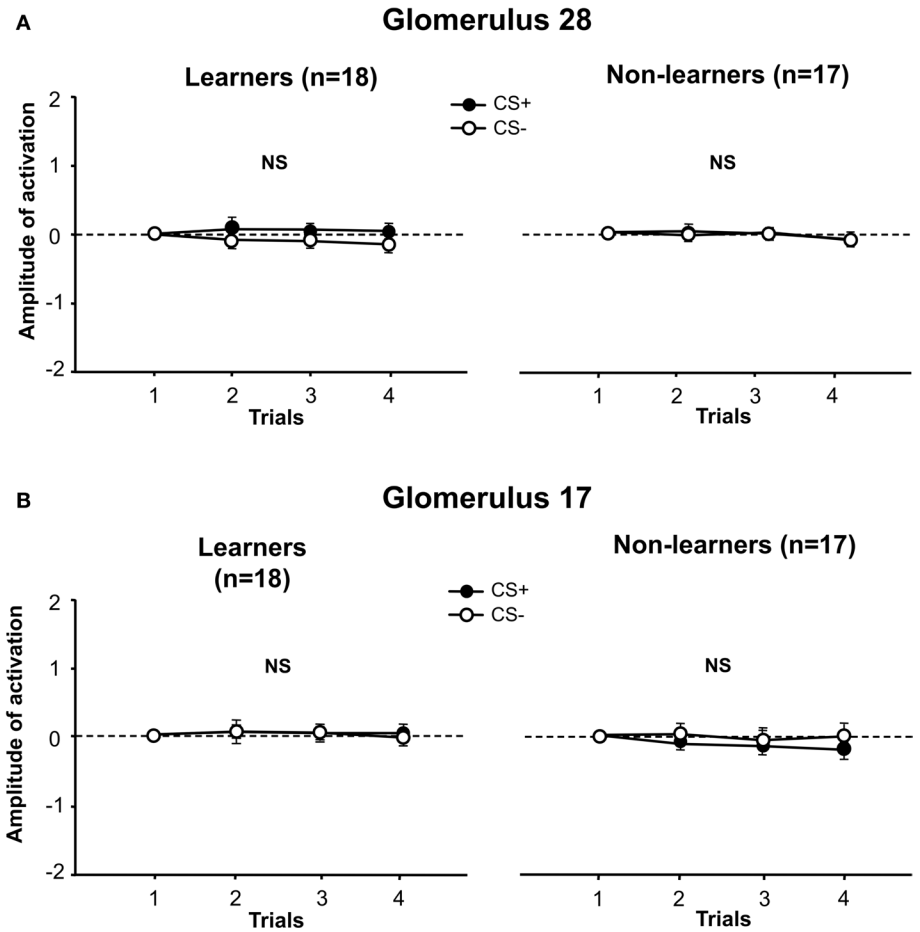

FIGURE 5 | Activation amplitude of individual glomeruli during conditioning. (A) For learners and non-learners, in glomerulus 28, the amplitude of activation did not change along conditioning trials and there was no difference between groups (*stimulus* effect: p = NS, group effect: p = NS). **(B)** For learners and non-learners, in glomerulus 17, the amplitude of activation did not change along conditioning trials and there was no difference between groups (*stimulus* effect: p = NS, group effect: p = NS).

due to a fitness problem because all the bees responded with a SER to all shock presentations throughout conditioning. Additionally abnormal dye bleaching can also be excluded because calcium levels before stimulus delivery were identical between learners and non-learners throughout conditioning (data not shown).

ABSENCE OF A NEURAL CORRELATE OF OLFACTORY DISCRIMINATION DURING AVERSIVE SER CONDITIONING

The differential odor-shock association established through aversive SER conditioning in learners did not lead to any measurable modification in the neural activity of the antennal lobe in response to the CS+ and the CS–. The question therefore arises as to whether experience-dependent plasticity should be expected at this level. Given that our recordings emphasize the responses of olfactory receptor neurons (see above), one could argue that modifications of neural activity resulting from learning should not be visible at a presynaptic level but rather at a postsynaptic level, for instance at the level of 2nd-order neurons such as projection neurons conveying the olfactory message reshaped by the antennal lobe network to higher-order centers (mushroom bodies and lateral horn). From this perspective, the lack of a neural correlate for the differentiation between CS+ and CS– in learners would not be surprising.

This argument can be, however, partially questioned by previous findings, which found experience-dependent neural plasticity at the presynaptic, olfactory receptor level in the honeybee. Two studies on appetitive olfactory PER conditioning used the same dye and staining method as in our work and found learning-dependent changes in calcium activity in the antennal lobe on a medium-term basis. In one study (Faber et al., 1999), differential conditioning in which one odor was paired with sucrose (CS+) and another odor with absence of reinforcement (CS–) resulted in medium-term (10–30 min post-conditioning) quantitative changes in the glomerular pattern of the CS+, which became more intense. The glomerular pattern of the CS– remained unaffected. The result of this variation was that the activation patterns of CS+ and CS– could be decorrelated (Faber et al., 1999). In the other study, Sandoz et al. (2003) used the same staining and imaging procedure to detect learning-dependent modifications of antennal lobe activity in a side-specific olfactory discrimination. Bees were differentially conditioned using two odors

CS1 and CS2. When odorants were delivered to one antenna, CS1 was rewarded with sucrose and CS2 was not (CS1+ vs. CS2−), while it was the opposite when odors were delivered to the other antenna (CS1− vs. CS2+). Imaging calcium responses in both antennal lobes simultaneously showed that in naïve bees, odor-response patterns were highly symmetrical, suggesting that before conditioning, the same odorant elicited the same activation pattern in both antennal lobes. In conditioned bees, topical differences between sides were found. After side-specific conditioning, the left and right representations of the same odorant became slightly different (Sandoz et al., 2003). This form of discrimination resulted, therefore, in a decorrelation of the representations of the conditioning odors between sides.

No such effect was found in our recordings, which used the same imaging procedure (Calcium Green-2 AM staining). One may be tempted to conclude that appetitive PER and aversive SER conditioning have different neuronal substrates, and that a form of peripheral plasticity (involving receptor neuron input) is found only in the case of appetitive PER conditioning. This conclusion would be, however, premature. Firstly, the medium-term changes in glomerular activity reported for olfactory PER conditioning were observed between 10 and 30 min after the last acquisition trial (Faber et al., 1999) or 24 h after conditioning (Sandoz et al., 2003) while in our case measures of glomerular activity were obtained "on-line" *during* conditioning trials. Secondly, the medium-term qualitative changes in glomerular activation reported for olfactory PER conditioning (Faber et al., 1999) have been contradicted by a recent study (Peele et al., 2006) that failed to demonstrate the same modifications in the medium term following similar differential conditioning. Peele et al. (2006) stained one of the tracts of projection neurons conveying information from the antennal lobe toward higher-order brain centers (lateral antenno-cerebralis tract, l-ACT), and found no changes within 15 min after appetitive differential conditioning. These authors concluded that the l-ACT may serve reliable and stable odor-coding while other projection neuron tracts (like the medial or mediolateral tracts, m- and ml-ACT) might be involved in carrying plastic changes to other brain areas. However, this idea was again contradicted very recently, as 24 h after differential conditioning with binary mixtures, a decorrelation between CS+ and CS− representations was found within l-ACT projection neurons (Fernandez et al., 2009). In electrophysiological experiments, differential conditioning leads to both increases and decreases in spike rates of projection neurons for all odors, including the CS+, the CS− and a control odor introduced to test odor generalization (Denker et al., 2010).

Despite these contradictory results, a number of studies indicate that the honeybee antennal lobe is subject to plastic changes following appetitive learning. The formation of long-term appetitive memories leads to structural changes in the antennal lobe which are odor-specific (Hourcade et al., 2009). Specifically, 3 days after PER conditioning, a period that corresponds to the presence of long-term memory in successfully conditioned bees, certain glomeruli increase significantly their volume in an odor-specific manner in comparison to pseudo-conditioned bees that did not establish such a long-term memory. Furthermore, an intracellular calcium increase at the time of appetitive PER conditioning is both necessary and sufficient for inducing transcription-dependent olfactory long-term memory (Perisse et al., 2009). How this calcium increase takes place in the brain, and how long it lasts is still unknown, but the modified calcium response observed by Faber et al. (1999), Sandoz et al. (2003), and Fernandez et al. (2009) could correspond to different phases of this process. In this case, the changes recorded by these authors would not correspond to a change in odor representation, but would rather be the by-product of molecular cascades leading to the long-term storage of appetitive information. At this moment, it is still unknown whether the formation of an aversive olfactory memory also depends on such early calcium signaling phenomenon. To answer this question we would need to search for this kind of signaling in a temporal window similar to that used by Faber et al. (1999), Sandoz et al. (2003), and Fernandez et al. (2009) in their recordings, i.e., 10–30 min and 24 h after the last acquisition trial, respectively.

These arguments attribute an inherent plasticity to the antennal lobe which would reflect the location of a memory trace within its network. A different view can be, however, proposed, suggesting that the olfactory memory trace generated by olfactory conditioning would be located downstream the antennal lobe, for instance, in the mushroom bodies (Heisenberg and Gerber, 2008). Within this conceptual framework, experience-dependent plasticity as occurring in the antennal lobe would be due to feedback processes from the mushroom bodies to the antennal lobes. Interestingly such processes exist in the form of feedback neurons (ALF-1 neurons) connecting mushroom bodies and antennal lobes (Kirschner et al., 2006). Studying if and how these neurons modulate the activity of the antennal lobe network following olfactory learning should help clarifying the issue of olfactory memory location.

A DECREASE OF CALCIUM SIGNALS UPON ODOR STIMULATION IN NON-LEARNERS

In order to understand the significant effect found in our work, it is necessary to have in mind what our calcium imaging recordings really represent when it comes to analyze neural activity of the antennal lobe. We used a permeable calcium sensitive dye, Calcium Green-2 AM, to stain the antennal lobe. This dye can potentially stain all neuronal populations of the antennal lobe: afferents of olfactory receptor neurons, local interneurons, projection neurons and glial cells. However, the signals recorded at the level of the antennal lobe are thought to mainly represent the contribution of olfactory receptors (Galizia and Vetter, 2005; Deisig et al., 2006, 2010). This is due to the numeric overrepresentation of olfactory receptors with respect to other neuronal types in the antennal lobe and to the fact that recorded signals are highly stereotyped and never show any spontaneous activity or any inhibitory responses, which are typical for local interneurons and projection neurons (Sachse and Galizia, 2002). The participation of local interneurons or projection neurons in the compound signal recorded is thought to be negligible (Deisig et al., 2006, 2010). A significant part of the signal may come from glial cells surrounding each glomerulus but even if glial cells participate in the calcium signal their response would be directly correlated to olfactory receptor signals. Thus, the calcium signals recorded in our study mainly represent the signals conveyed by olfactory receptor neurons to the antennal lobe.

Why should these signals decrease (**Figure 3A**) upon olfactory stimulation during conditioning trials in non-learners? Clearly this effect was common both for the CS+ and the CS− and does not seem to be attributable to a fitness problem. Learners received the same amount of electric shocks and did not show such a decrease in calcium responses. Moreover, and most importantly, the decrease was specific to the olfactory stimulations as basal levels of fluorescence prior to odor delivery evolved in the same manner in learners and non-learners. It cannot thus be argued that non-learners exhibited a general, non-specific decrease in neural activity consistent with depressed levels of responsiveness.

In this context, the decrease in calcium signals upon olfactory stimulation may reflect inhibitory modulation of olfactory receptor neuron activity. In lobsters, presynaptic inhibition of olfactory receptor neurons has been demonstrated by recording from the afferent nerve terminals (Wachowiak and Ache, 1997, 1998). A preparation using the isolated but intact brain of the spiny lobster in combination with voltage-sensitive dye staining has allowed recording stimulus-evoked responses of olfactory receptor axons with optical imaging methods. The cellular mechanism underlying presynaptic afferent inhibition appears to be a reduction of action potential amplitude in the axon terminal via two inhibitory transmitters, GABA and histamine, which can independently mediate presynaptic inhibition. GABA- and histaminergic interneurons constitute dual, functionally distinct inhibitory pathways that are likely to play different roles in regulating primary olfactory input to the lobster olfactory lobe (Wachowiak et al., 2002). Interestingly, the same two inhibitory networks – GABAergic and histaminergic – have been found in the honeybee antennal lobe (Sachse and Galizia, 2002; Barbara et al., 2005; Sachse et al., 2006). One may hypothesize that the decrease of calcium responses upon olfactory stimulation in non-learners may be the result of an increased, maximized inhibitory action of inhibitory neurons, which would span the entire antennal lobe, affecting both CS+ and CS− processing. This effect would be different from less drastic increases in antennal lobe inhibition, which result in better olfactory discrimination performances (Stopfer et al., 1997). In our scenario, increased global inhibition affecting all or most glomeruli may have as a consequence a progressive reduction in perceptual distances between CS+ and CS−, which would impede discrimination. Why should inhibition progressively increase in non-learners? So far, we have no answer for this question. It could be seen, nevertheless, as a dysfunction of the olfactory network that prevented non-learners to provide adaptive responses in an olfactory discrimination.

CONCLUSION

Our results show how difficult the search for the neural correlates of associative learning can be. As these changes appear to be highly dependent on the time after conditioning, and sometimes correspond to a very short time-window, the search for learning-induced plasticity has to be carried out on a large scale. This temporal analysis should span numerous periods after conditioning, from the moment in which the association is formed to the latest stages of long-term memory (Berry et al., 2008). Moreover, different brain structures should be considered (Haehnel et al., 2009) in order to determine where the learning trace is located at different post-association periods. The development of this new tool to study aversive learning-induced modifications in the insect brain may be applied to such a large scale study.

Furthermore, it will be possible for the first time to perform comparative analyses between appetitive and aversive learning in honeybees, both at the behavioral and the cellular levels. Besides the established fact that US reinforcing properties are mediated by different aminergic systems in these two learning forms (octopaminergic system for sucrose reward, and dopaminergic system for electric-shock punishment), questions about the kind of discrimination problems that can be solved within these two experimental frameworks, the specific location of aversive vs. appetitive memories, and the nature of CS representation in both forms of conditioning can be now raised and answered.

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An alarm pheromone modulates appetitive olfactory learning in the honeybee (*Apis mellifera*)

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In honeybees, associative learning is embedded in a social context as bees possess a highly complex social organization in which communication among individuals is mediated by dance behavior informing about food sources, and by a high variety of pheromones that maintain the social links between individuals of a hive. Proboscis extension response conditioning is a case of appetitive learning, in which harnessed bees learn to associate odor stimuli with sucrose reward in the laboratory. Despite its recurrent use as a tool for uncovering the behavioral, cellular, and molecular bases underlying associative learning, the question of whether social signals (pheromones) affect appetitive learning has not been addressed in this experimental framework. This situation contrasts with reports underlining that foraging activity of bees is modulated by alarm pheromones released in the presence of a potential danger. Here, we show that appetitive learning is impaired by the sting alarm pheromone (SAP) which, when released by guards, recruits foragers to defend the hive. This effect is mimicked by the main component of SAP, isopentyl acetate, is dose-dependent and lasts up to 24 h. Learning impairment is specific to alarm signal exposure and is independent of the odorant used for conditioning. Our results suggest that learning impairment may be a response to the biological significance of SAP as an alarm signal, which would detract bees from responding to any appetitive stimuli in a situation in which such responses would be of secondary importance.

Keywords: honeybee, learning, pheromone, stress, alarm, olfactory, modulation, insect

INTRODUCTION

Social insects have evolved sophisticated communication systems, which include behavioral displays such as the honeybee dances (Frisch, 1967) and chemical signals that play a crucial role in the coordination of individual behaviors inside a colony. Bees, ants, and wasps release a high variety of chemical compounds that act as pheromones, thus ensuring intraspecific chemical communication and adaptive responses to a variety of stimuli across different timescales (Wilson, 1971; Vander Meer et al., 1997; Wilson and Hölldobler, 2009). While primer pheromones induce long-lasting changes in physiology and behavior, releaser pheromones trigger rapid and short-term behavioral responses (Wilson and Hölldobler, 2009).

Honeybee pheromones have been the subject of intensive studies (Free, 1987) which have focused on their multiple behavioral and physiological consequences. Among the pheromones released by worker bees, the sting alarm pheromone (SAP), a releaser pheromone contained in the sting chamber, prompts stinging and fast recruiting of nest-mates to defend the resources of the colony when released by guards facing a potential danger (Free, 1987). Several studies have shown that SAP acts as a modulator of the sensitivity to environmental stimuli, as assessed by the quantification of reflex responses. In particular, exposure to some of its main components changes the responsiveness (as usually measured by the threshold value of a given stimulus eliciting a response) to appetitive or nociceptive stimuli: it decreases responsiveness to sucrose (Balderrama et al., 2002) and, depending on the nature and dose of the SAP compound, increases or decreases responsiveness to electric shocks (Núñez et al., 1998; Balderrama et al., 2002).

It has been proposed that response thresholds are a key parameter of social regulation in insects (Robinson and Page, 1989; Rueppell et al., 2006). For example, the probability to forage for pollen outside the hive is linked to increased responsiveness to sucrose, and possibly to light (Page and Erber, 2002; Pankiw, 2003, 2005; Ben-Shahar, 2005; Page et al., 2006). Since pheromones modulate sensory response thresholds, they can also affect the probability of performing certain behaviors. Moreover, pheromones can also affect plastic behaviors such as learning, as shown recently by studies on queen mandibular pheromone (QMP) (Vergoz et al., 2007a). This pheromone blocks aversive associative learning in young bees (Vergoz et al., 2007a), in addition to triggering reflex responses such as feeding and grooming the queen (Free, 1987). However, QMP does not affect appetitive learning in young bees, thus specifically preventing aversive experiences that young bees could have in the vicinity of their queen.

Appetitive learning is particularly important during foraging, as it requires to associate floral aromas with the presence of pollen or nectar (Giurfa, 2007). SAP can rapidly induce bees to quit foraging and to reduce recruitment of other bees for foraging by producing a vibrational "stop signal" (Nieh, 2010). Hence, we wondered whether this effect might be accompanied by a change in appetitive learning, which drives foraging activities (Menzel, 1985; Giurfa, 2007). Appetitive learning can be easily studied in controlled conditions using proboscis extension response (PER) conditioning (Takeda, 1961; Bitterman et al., 1983). In this Pavlovian task, restrained bees are trained to associate an odorant (conditioned stimulus, CS) with a sucrose reward (unconditioned stimulus, US). After 3–5

trials consisting in paired odorant-sugar presentations, most bees display a conditioned PER to the odorant, which indicates that the association was learnt. Based on its inhibitory effect on foraging activities, we hypothesized that SAP would impair olfactory appetitive learning in the laboratory. Our results show that exposure to SAP or to its main component isopentyl acetate (IPA) does indeed impair appetitive learning, that this effect is SAP-specific and is independent of the odorant used for conditioning.

MATERIALS AND METHODS

ANIMALS

Honeybees from the strain *Apis mellifera ligustica* were caught at the hive in the morning of each experimental day, cold-anesthetized and restrained in individual harnesses that allowed free movements of the mouthparts and antennae (Bitterman et al., 1983). They were then fed with 5 μL of 50% w/w sucrose solution and maintained 2 h in a dark and humid place. Experiments were performed across several seasons, and thus include winter as well as summer (mostly foraging) bees, of unmatched ages and taken from different hives. These differences may explain the observed variability in learning rates in controls across days and experiments. However, they are unlikely to account for differences between groups as bees from the same hive were assigned to control and experimental groups on every experimental day.

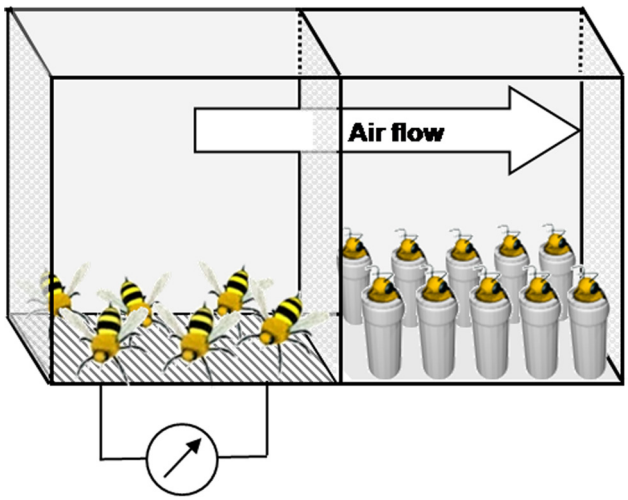

FIGURE 1 | Experimental design for exposure to the natural blend of the sting alarm pheromone (SAP). Before conditioning, harnessed bees from the *exposed* group were placed in one compartment (*right*) for 30 min, in the presence of unrestrained bees receiving electric shocks (6–9.5 V) each time they landed on the electric grid placed in their compartment (*left*). The alarm pheromones emitted were directed toward the harnessed bees by an air flow created by an exhaustion system (indicated by the arrow; *air flow*). For the *sham* group, no shock was applied to the grid.

EXPOSURE PROCEDURES

Two hours after feeding, animals were exposed either collectively to control compounds or to the natural alarm pheromone, or individually to IPA (the main component of SAP) or to solvent. In all cases, exposure lasted 30 min and was followed by a 30-min rest (unless stated otherwise) before conditioning experiments.

Exposure to the natural pheromone was performed by placing the restrained bees to be conditioned and 50 unrestrained bees in two compartments of a cage (11 cm × 12 cm × 8.5 cm), separated by a perforated wall allowing odorant diffusion (**Figure 1**). Electric shocks were delivered to the unrestrained bees through an electric grid connected to a generator whenever they touched the floor of the cage. The bees that received the electric shocks reacted by emitting SAP, which was acknowledgeable to the experimenter by the characteristic posture of the emitting bees (**Figure 1**; left compartment) and by the typical banana smell of the pheromone (Free, 1987; Núñez et al., 1998). The voltage was increased progressively from 6.5 V to 9.5 V during the 30-min period in order to avoid desensitization. The restrained bees were placed in the other compartment in front of an air extractor (*exposed* group), so that the pheromone released by the unrestrained bees was blown toward them (**Figure 1**; right compartment). As a control, another group of unrestrained bees was placed in the setup with the unrestrained bees in a separate compartment, but no shock was delivered to the unrestrained bees (*sham*). A third control group included bees that were harnessed but not placed in the setup (*untreated*).

A more controlled protocol was used, in order to avoid possible variations in the amount of SAP received by each individual bee in the cage. It made use of IPA, by adapting previously published procedures (Núñez et al., 1998; Balderrama et al., 2002). IPA, a main component of SAP, can trigger by itself many effects of exposure to the full pheromone blend (Boch et al., 1962; Collins and Blum, 1983). Each restrained bee was placed in an individual 35 mL glass vial containing a piece of filter paper (1.5 × 1.5 cm) soaked with 24% IPA (6 μL IPA + 19 μL mineral oil), unless specified otherwise. Control bees were handled the same way and exposed to mineral oil alone. In one experiment designed to test for the specificity of the effect of IPA, two additional controls included bees exposed to methyl salicylate or geraniol at the same concentration as IPA.

CONDITIONING

Bees were subjected to olfactory PER conditioning consisting of three trials, following a standardized protocol described elsewhere in detail (Bitterman et al., 1983). Briefly, each acquisition trial lasted 40 s; it included a familiarization phase of 13 s in the setup, followed by the forward-paired presentation of an odorant (the CS) and sucrose solution (the US). The presentations of the CS (1-nonanol, pure) and the US (sucrose, 50% w/w in water) lasted 4 s and 3 s, respectively, with a 1 s overlap. Pure odorants are generally used for bee conditioning in order to avoid any concentration decrease due to evaporation over the conditioning trials. In an additional experiment, other odorants (1-hexanol, citral, or nonanal) were used as CSs to make sure that the effect of IPA exposure was not CS-specific. All chemicals were from Sigma-Aldrich (Lyon, France). Before conditioning, all bees were tested for their PER in response to 50% sucrose. Those bees that failed to respond were discarded; bees that failed to respond to the US during the three conditioning trials were also discarded.

ASSESSMENT OF SENSITIVITY TO SUCROSE

In order to look for possible effects of IPA exposure on the sucrose responsiveness, unconditioned responses elicited by sucrose were assessed in bees exposed to IPA or mineral oil, using a protocol

described elsewhere (Scheiner et al., 1999). Those bees had not been conditioned previously, since providing the sucrose reward during conditioning would have modified their responsiveness in the test. Briefly, the bees were first allowed to drink water *ad libitum* in order to ensure that they would respond specifically to sucrose, and were then presented successively with six sucrose solutions of increasing concentrations (0.1, 0.3, 1, 3, 10, and 30%), which were applied on the antennae, interspersed with water stimulations to avoid sensitization. No responses to water were recorded during the experiment; this indicates that the recorded responses to the sucrose solutions were indeed elicited by the sucrose. For each animal, the presence or absence of a PER was recorded for each concentration, and its individual sucrose response score (SRS) was calculated as the number of stimuli eliciting a PER (e.g., SRS = 3 for an individual responding to 3, 10, and 30% sucrose but not to lower concentrations). Bees with a SRS of 0 (i.e., not responding to any concentration) were discarded as in the learning experiments (see above).

GENERALIZATION

In order to test for possible differences in olfactory discrimination, bees were prepared and conditioned as previously but were exposed to IPA, oil, or geraniol *after* conditioning, instead of before. Exposure started right after the last conditioning trials and lasted 30 min. After a 30-min rest (i.e., 1 h post-conditioning), bees were presented with the odorant used as the CS (1-nonanol) and two novel odorants (1-hexanol and nonanal) without reward, with the same timing as during conditioning. These two odorants were chosen given their high (nonanal) and low (1-hexanol) similarity to 1-nonanol (Guerrieri et al., 2005). The order in which the odors were presented was randomized across bees. After the test bees were checked for their PER in response to 50% sucrose; bees that failed to respond were discarded from the whole experiment.

STATISTICS

Multiple comparisons were performed using analysis of variance (ANOVA) as the critical conditions required for its application to dichotomous variables were met (at least 40 subjects per group, Lunney, 1970). Although the second criterion (equal sample sizes) was not met, the robustness of the significant effects detected by the ANOVAs was supported by all *post hoc* analyses (which confirmed all effects), thus suggesting that a requirement for equal group sizes was not critical in our study. *Post hoc* comparisons (pairwise) were performed using a Chi-square test on the absolute numbers of bees in each category. For the sucrose response analysis, the SRS classes 4–6 were pooled so as to have the required minimum of five individuals per category. In case of multiple comparisons, Bonferroni corrections were applied (wherever applied, the corrected alpha level – 0.025 or 0.0125 – is indicated in the text and in the figure legends). All statistics were run on the R software (R Development Core Team, 2009).

RESULTS

EXPOSURE TO ALARM PHEROMONES IMPAIRS LEARNING PERFORMANCE

We studied the effect of exposure to SAP on olfactory PER conditioning, by comparing the learning performances of bees from the *SAP-exposed* group to those of control bees (*sham* and *untreated* groups) (see Materials and Methods). During conditioning, each individual received three paired presentations of 1-nonanol (CS) with sucrose (US) (absolute conditioning). To rule out undesired effects of treatment on sucrose responsiveness and thus on the motivation to learn, all experiments used only bees that responded to the sucrose reward before and after conditioning, and in all three conditioning trials. As shown in **Figure 2**, bees from the three groups increased their responses to the odor during conditioning trials, thus showing learning of the odor-sucrose association. Accordingly, a two-way repeated-measure ANOVA (Treatment, Trials) revealed that overall bees increasingly responded to the CS across trials (*Trial:* $F = 245.2$, df = 2, 586, $p < 0.001$). However, learning success differed significantly between groups ($F = 3.97$, df = 2, 293, $p = 0.02$), as shown by the proportions of bees displaying a conditioned response to the CS after three conditioning trials. Indeed, SAP-exposed bees exhibited significantly fewer conditioned responses (52%) on the last conditioning trial than both control groups. These, in turn, showed very similar performances (*untreated*: 66%; *sham*: 68% $\chi^2 = 0.04$, df = 1, $p = 0.84$). This effect of exposure to SAP on learning was significant (*controls* vs. *exposed*: $\chi^2 = 5.56$, df = 1, $p = 0.02$, $\alpha = 0.025$).

THE EFFECT OF THE PHEROMONAL BLEND IS MIMICKED BY ITS ACTIVE COMPONENT ISOPENTYL ACETATE (IPA)

Sting alarm pheromone is a complex blend of about 40 components (Hunt, 2007), among which IPA is sufficient to elicit most of the responses triggered by the entire blend (Boch et al., 1962; Collins and Blum, 1983). Therefore, we tested whether exposure to IPA alone could have similar effects as exposure to the whole pheromone blend. For this, we used a procedure in which individual bees were constantly exposed to a determined amount of IPA, thus ensuring a continuous exposure to a constant amount of odorant.

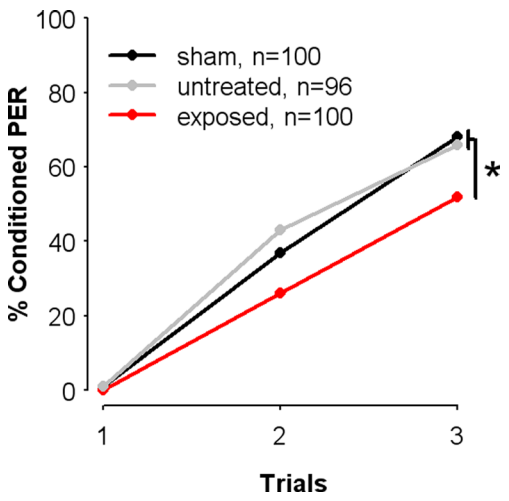

FIGURE 2 | Exposure to the natural blend of the sting alarm pheromone impairs olfactory learning. Learning performances measured as the percentages of conditioned proboscis extension responses (percentage conditioned PER) elicited by the CS, 1-nonanol (conditioned PER) during three conditioning trials. Learning was impaired in bees previously exposed to the SAP (*exposed*), as compared with control groups (*sham* and *untreated*). *$p < 0.025$.

Indeed, in the cage setup used in the previous experiment the SAP concentration could not be controlled precisely since the number of bees releasing it varied over time, and it decreased over the stimulation period as we observed that some of the shocked bees seemed to learn to avoid the grid. We thus asked whether IPA could impair appetitive learning in a similar way as the whole pheromone, and whether this effect depends on the dose of IPA to which the bees are exposed. Different groups of bees were exposed individually to different dilutions of IPA in mineral oil (4, 8, 24, and 40%) or to oil alone as a control. **Figure 3** shows the percentages of conditioned responses obtained in the last trial of the differently exposed groups. Exposure to IPA affected learning in a dose-dependent manner (*Dose*: $F = 4.51$, df = 4, 237, $p = 0.0016$). While lower concentrations (4 and 8%) did not induce a significant response decrease relatively to the control, higher concentrations resulted in lower conditioned responses (Oil/IPA 24%: $\chi^2 = 6.34$, $p = 0.0117$; Oil/IPA 40%: $\chi^2 = 10.67$, $p = 0.0011$, $\alpha = 0.0125$). Interestingly, this effect was comparable to that of SAP in the previous experiment, as shown by the similar relative decreases in performances in both conditions (24% IPA: −23%; SAP: −19%). Hence, exposure to a sufficient concentration of IPA allows reproducing the effect of exposure to natural SAP on learning, in a more controlled situation.

In order to test the duration of the effect of the 30-min exposure to IPA, we used the 24% concentration, which corresponds to the amount of IPA contained in 3–10 stings (Hunt et al., 2003), and for which a clear, significant decrease in conditioned responses was found with respect to the control in the third conditioning trial. We introduced different delays (1.5, 3.5, 5.5, 24, 48, and 72 h) between IPA exposure and olfactory conditioning. An independent group was used for each delay. A significant impairment of learning was observed for delays up to 24 h, but not for longer ones (48 and 72 h) (**Figure 4**). Thus, the modulation introduced by IPA induces a long-lasting learning impairment that mimics the effect of exposure to the whole pheromonal blend.

For all further experiments, we used the 24% IPA dilution, in order to further analyze the modulatory effect of SAP in precisely controlled conditions.

LEARNING IMPAIRMENT IS SPECIFICALLY CAUSED BY EXPOSURE TO IPA

Olfactory learning impairment might be the consequence of continuous exposure to any odorant rather than the response to an alarm signal. We thus exposed independent groups of bees to oil,

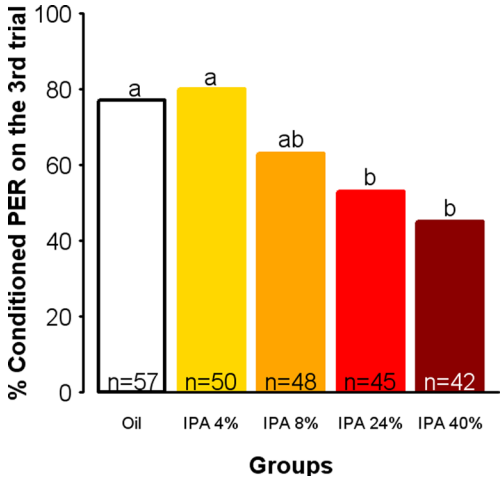

FIGURE 3 | Exposure to IPA impairs learning in a dose-dependent manner. Learning performances, as indicated by percentages of conditioned PER to the conditioned odor on the third trial (PER conditioning). Independent groups were exposed to mineral oil only or to IPA at a given concentration (4, 8, 24, or 40% w/w in oil). As compared with controls (*Oil*), groups of bees exposed to increasing IPA concentrations show an increasing learning impairment, which reaches the significance level for the higher doses. Different letters indicate significant differences compared with controls ($\alpha = 0.0125$).

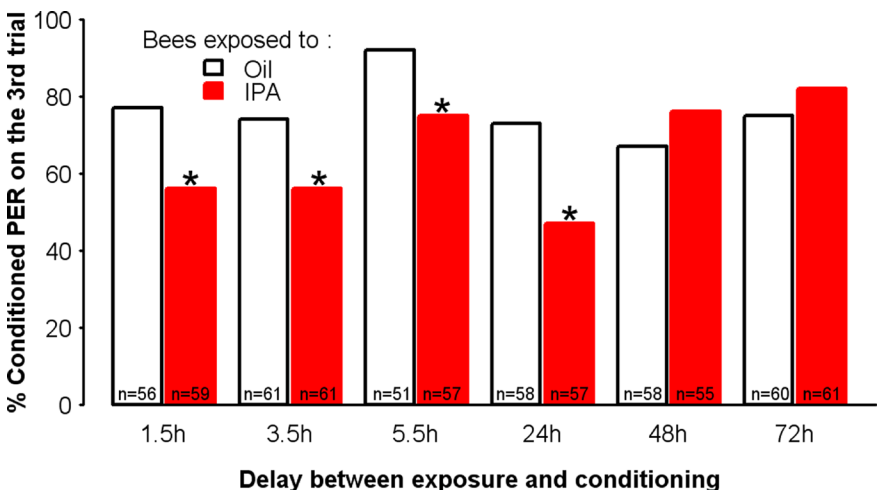

FIGURE 4 | IPA impairs learning up to 24 h after exposure. Learning performances, as indicated by percentages of conditioned PER to the conditioned odor on the third trial (PER conditioning), after a delay between exposure (either to IPA at the 24% concentration or to mineral oil) and olfactory conditioning that varied for each group. Compared with controls (*Oil*), learning was impaired for delays up to 24 h. By contrast, for delays longer than 24 h, no significant impairment of learning was found. *$p < 0.05$ (as compared with respective *Oil* control groups).

IPA, methyl salicylate or geraniol (all at the same concentration: 24%) for 30 min, and 30 min after exposure, we conditioned them in parallel, using the same protocol as above (with 1-nonanol as the CS). Methyl salicylate was chosen because it is a non-pheromonal compound with the same functional group (ester) as IPA. Geraniol, on the other hand, is the main component of the attractive Nasonov pheromone used to mark places of interests such as food sources or the hive entrance (Boch and Shearer, 1962; Free, 1987); it offers, therefore, the possibility of testing the effect of another pheromone signal with a different hedonic value from that of the SAP. The effect of geraniol exposure was analyzed relative to that obtained in the two other groups (oil-exposed and IPA-exposed), which were studied in parallel (**Figure 5A**). Similarly, the effect of methyl salicylate geraniol exposure was compared with that obtained in its corresponding two control groups (oil-exposed and IPA-exposed) (**Figure 5B**).

Neither methyl salicylate nor geraniol affected learning relatively to the control situation (geraniol/oil: $\chi^2 = 0.75$, df = 1, $p = 0.39$; methyl salicylate/oil: $\chi^2 = 0.43$, df = 1, $p = 0.51$). Besides, in both cases bees exposed to IPA showed learning performances that were significantly lower than those of bees exposed to methyl salicylate, geraniol, or mineral oil ($p < 0.025$ in all cases). Thus, the decrease in learning induced by IPA was specific to this compound and its alarm message given that geraniol did not induce any decrease in learning. Spurious factors related to olfactory processing such as saturation of odorant receptors as a consequence of exposure can be excluded based on these results.

LEARNING IMPAIRMENT INDUCED BY IPA EXPOSURE IS INDEPENDENT OF THE ODORANT USED AS CONDITIONED STIMULUS

If exposure to IPA results in a general decrease in appetitive learning, this effect should be observed regardless of the odorant used as the CS. We tested this hypothesis by conditioning independent groups of bees using different CSs, after exposure to IPA or to oil for 30 min. As expected, the decrease in learning induced by IPA exposure was independent of the odor used for conditioning. As shown in **Figure 6**, bees exposed to IPA could learn dissimilar odors such as 1-hexanol, citral, and nonanal but their final level of conditioned responses was always significantly lower than that of control bees exposed to mineral oil (nonanal: $\chi^2 = 4.46$, df = 1, $p = 0.035$, citral: $\chi^2 = 4.20$, df = 1, $p = 0.040$, 1 hexanol: $\chi^2 = 4.20$, df = 1, $p = 0.040$).

LEARNING IMPAIRMENT DUE TO EXPOSURE TO IPA IS NOT DUE TO A DIMINISHED RESPONSIVENESS TO SUCROSE

According to previous findings obtained by one of us (Balderrama et al., 2002), we found that exposure to IPA had an impact on responses to sugar. There were significantly more bees failing to display sugar-induced PER at least once during conditioning among those exposed to IPA (23.5%) than to oil (6.7%). Such bees were considered to lack the motivation required for optimal learning, and thus were systematically discarded from all experiments. Thus, undesired effects of IPA exposure on sucrose processing could, in principle, be discarded as bees kept in our experiments always responded to the 50% sucrose solution used as reward. However, IPA might diminish the subjective value of sucrose reward (Scheiner et al., 2004), thus inducing lower conditioning performances in IPA-exposed bees. To test this hypothesis, we measured the individual sensitivity to sucrose of both IPA-exposed and control bees, by determining their SRS (see Materials and Methods). This score is a standard measure of sucrose responsiveness in honeybees despite responding identically to the 50% sucrose solution (Scheiner et al.,

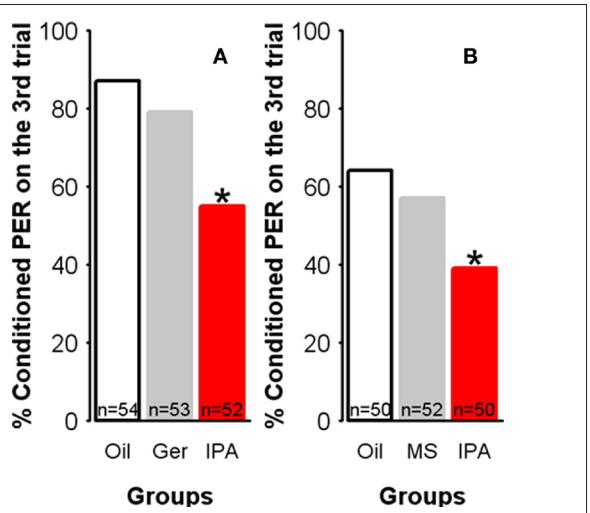

FIGURE 5 | Learning impairment is specific to IPA exposure. Learning performances, as indicated by the percentage of conditioned PER to the conditioned odor on the third trial (PER conditioning), for each group. **(A)** Compared with controls (*Oil*), learning remained unaffected after exposure to geraniol (*Ger*), a compound belonging to the attractive Nasonov pheromone or **(B)** to methyl salicylate (*MS*), a non-pheromonal ester. By contrast, bees exposed to IPA show significantly fewer conditioned responses. $*p < 0.025$ (as compared with respective control groups).

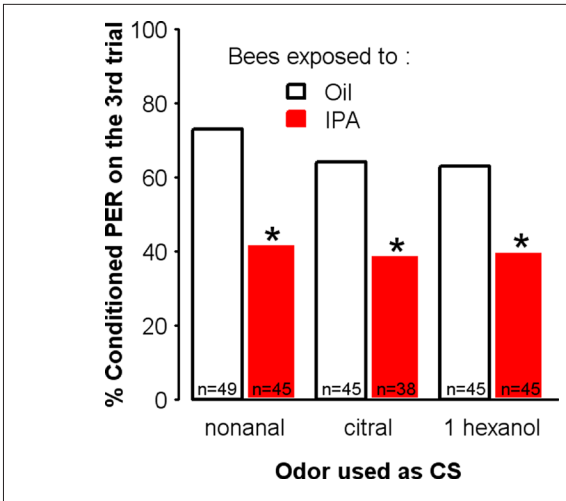

FIGURE 6 | IPA exposure impairs learning irrespective of the odorant used as the conditioned stimulus. Learning performances, as indicated by percentages of conditioned responses to the conditioned odor on the third trial (PER conditioning), for each group. Independent groups of bees were conditioned in a three-trial protocol, after exposure to either IPA (24% in mineral oil) or mineral oil alone. In each pair of groups, a different odorant (nonanal, citral, or 1-hexanol) was used as the CS during the three-trial conditioning. In all cases, control bees (*Oil*) learned significantly better than exposed bees (*IPA*). Thus, IPA exposure impairs subsequent associative olfactory learning irrespective of the odorant selected for conditioning. $*p < 0.05$ (as compared to respective *Oil* control groups).

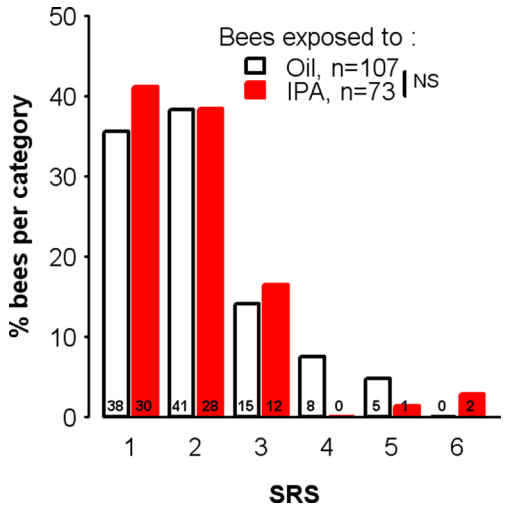

FIGURE 7 | Learning impairment after IPA exposure is not due to decreased sucrose responsiveness. Distribution of bees exposed to isopentyl acetate (*IPA*) or mineral oil (*Oil*) according to SRS (sucrose response score) values. Unconditioned responses elicited by sucrose were assessed, using 6 sucrose solutions of increasing concentrations (0.1, 0.3, 1, 3, 10, and 30%, in water). For each animal, the presence or absence of a PER was recorded for each concentration, and its individual SRS was calculated as the number of stimuli eliciting a PER (e.g., SRS = 3 for an individual responding to 3, 10, and 30% sucrose but not to lower concentrations). The number of bees with a given value in each group is indicated by the number on each bar. Among bees showing motivation to respond to sucrose, no difference was found between the two groups ($\chi^2 = 3.68$, df = 3, $p = 0.30$; bees with SRS values higher than three were grouped in a single category to allow the use of the chi-square test, see Materials and Methods). NS: non-significant.

2004; Rueppell et al., 2006; Roussel et al., 2009). We used this method to determine whether IPA-exposed and control bees differed in their sucrose sensitivity, independently of conditioning since we aimed to study the unconditioned response without prior experience of any sucrose reward. Bees were grouped in classes corresponding to their SRSs, ranging from 1 (low responsiveness, i.e., bees responding only to the highest sucrose concentration) to 6 (high responsiveness, i.e., bees responding to all six sucrose concentrations). Bees with a SRS of 0, i.e., not responding to any sucrose concentration, were discarded to focus the analysis on bees showing appetitive motivation as in the learning experiments.

The distribution of IPA-treated and control bees among the different SRS classes is shown in **Figure 7**. We found no difference between IPA-exposed and control bees in terms of their SRS ($\chi^2 = 3.68$, df = 3, $p = 0.30$). Thus, among bees showing motivation to respond to sucrose (i.e., those whose learning performance was analyzed), exposure to IPA impaired learning without affecting responsiveness to the US.

EXPOSURE TO IPA AFFECTS OLFACTORY GENERALIZATION

In order to ask whether IPA might affect olfactory processing, we inverted the sequence of treatments: we conditioned first the bees with 1-nonanol as the CS during three conditioning trials and, immediately after the last conditioning trial, we exposed them to IPA, geraniol, or mineral oil. After the 30-min resting period following exposure, we performed an olfactory generalization test in which bees were presented with the CS (1-nonanol) and with two novel odorants, nonanal and 1-hexanol. While 1-nonanol and nonanal are perceived by bees as similar, 1-nonanol and 1-hexanol, are perceived as dissimilar (Guerrieri et al., 2005). As expected since bees were not exposed to any substance before conditioning, acquisition rates were equivalent in all three groups (two-way ANOVA, *Trial:* $F = 227.2$, df = 2,350, $p < 0.001$, *Group:* $F = 0.26$, df = 2,175, $p = 0.77$). After exposure either to mineral oil, geraniol, or IPA, responses to the three odorants, 1-nonanol, nonanal, and 1-hexanol, differed between groups. While response levels to the CS were similar between groups ($\chi^2 = 1.40$, df = 2, $p = 0.49$), bees exposed to IPA displayed slightly but significantly lower levels of generalization (dissimilar odorant: $\chi^2 = 5.11$, df = 2, $p = 0.08$, similar odorant: $\chi^2 = 10.1$, df = 2, $p = 0.006$) (**Figure 8A**). In order to confirm this observation, we examined individual response profiles in the three groups. Bees were categorized as bees responding to the CS only, bees showing generalization toward the similar odorant, bees showing generalization to both the similar and dissimilar odorants, and bees responding to neither of the stimuli (**Figure 8B**). Overall, exposure significantly affected individual responses ($\chi^2 = 15$, df = 6, $p = 0.02$), with the most profound effect observed for specific responses (*CS only*): more than half (55%) of the bees in this category were IPA-exposed bees. Conversely, IPA-exposed bees showed full generalization (response to the three odorants) twice as less often (21%) as bees exposed to geraniol or oil (resp. 41 and 39%). These results exclude the possibility that IPA exposure impairs odorant perception and discrimination, because the consequences of such exposure were opposite to those that one would predict under such hypothesis: instead of becoming less responsive or less selective to the conditioned odorant, IPA-exposed bees remained responsive to that odorant but increased their response selectivity, thus decreasing odorant generalization.

DISCUSSION

Learning and memory performances rely on a variety of intrinsic (related to the animal's internal state or physiology) and environmental factors. Studies on the behavioral and neural bases of learning and memory tend to focus on the former but less on the latter as environmental factors are usually viewed as external to the biological machinery mediating individual plasticity. However, environmental factors and among them, social ones, may dramatically influence physiological processes, and therefore the nervous system responsible for learning and memory processes. In the honeybee, much attention has been given to the modulation of learning and/or memory by intrinsic factors. Using the protocol of PER conditioning, learning has been shown to depend on age (Ray and Ferneyhough, 1999; Behrends et al., 2007), caste (Sigg et al., 1997; Behrends et al., 2007) and motivational state (Scheiner et al., 2001a,b). However, surprisingly in the case of a highly social insect, only a few studies have shown influences of the environment on plastic processes (Farina et al., 2005; Arenas et al., 2009), and particularly of social signals from conspecifics (Vergoz et al., 2007a). Pheromones, the main class of intraspecific communication signals in social insects, have been known for years to modulate reflex responses to a variety of stimuli, but it was only recently that effects on learning were reported. The QMP could impair aversive

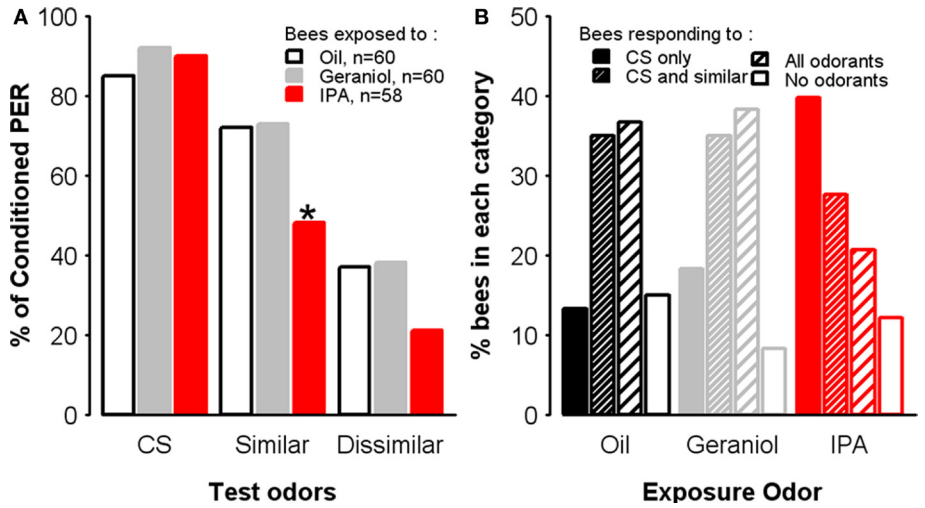

FIGURE 8 | Exposure to IPA affects olfactory generalization. Learning performances, as indicated by percentages of conditioned PER to the conditioned odor on the third conditioning trial, for each group. Independent groups of bees were exposed to oil, geraniol, or IPA, immediately after conditioning, and then tested 1 h after the end of conditioning. Generalization was assessed by measuring PER in response to unrewarded presentations of 1-nonanol (CS), nonanal (an odorant perceptually similar to 1-nonanol) and 1-hexanol (perceived as dissimilar to 1-nonanol). **(A)** Percentages of conditioned responses to the three odorants, for each group. Bees exposed to IPA respond less to the similar odor. $*p < 0.05$ (as compared with respective control groups). **(B)** Distribution of bees according to their individual response profiles. Specific responses to the CS are more frequent in bees exposed to IPA, while most control bees (*Oil* and *Geraniol*) show generalization responses.

learning (olfactory conditioning of the sting extension reflex, SER), but not appetitive learning (olfactory conditioning of PER) (Vergoz et al., 2007a). We thus wondered whether other pheromones might modulate appetitive learning. Our results are the first experimental evidence of a pheromonal modulation of appetitive learning.

The SAP triggers aggressive behavior in worker honeybees and decreases the probability to engage into foraging for food sources (Free, 1987; Hunt, 2007; Nieh, 2010). From this point of view, the negative modulation of appetitive learning by SAP and by its main component, IPA, when used at ecologically relevant amounts, makes sense. As appetitive learning is one of the main processes mediating foraging activities of honeybees (Giurfa, 2007), depressing foraging activities, and concomitantly appetitive learning, might be part of a strategy that helps attending better potential aversive signals in a defensive context signalized by SAP and/or IPA. In such a context, discriminating "friend from foe" is important for efficient defense of the colony (Breed, 1983); it relies on olfactory cues such as cuticular hydrocarbons (e.g., Breed and Stiller, 1992; Châline et al., 2005; Dani et al., 2005), comb wax components (D'Ettorre et al., 2006) or chemicals contained in the vertebrates' breath (Breed et al., 2004). Considering the higher sensitivity to SAP of young guards compared with older foraging bees (see Hunt, 2007) and the clear age-dependence for the effect of QMP on aversive learning (Vergoz et al., 2007b), age and/or caste may be a critical factor for the modulation of learning by IPA. Since we did not consider age differences here, this crucial point deserves further examination.

As SAP is known as a releaser pheromone, its effects (or those of IPA) have been studied on a short timescale (over minutes). Interestingly, here the learning impairment after a 30-min exposure lasted up to 24 h (but not longer), which suggests that at least some effects of releaser pheromones may last longer than usually considered. Changes in learning performances over hours may involve plastic changes in the brains of IPA-exposed bees, such as modifications of synaptic transmission and/or neural excitability in the olfactory pathway. In this respect, different brain neuropiles involved in learning might be affected over different time-courses. Consistent with this idea, exposure to IPA was shown to affect the expression of immediate-early genes in the antennal lobes (the primary olfactory centers) (Alaux and Robinson, 2007). However, exposure to a plant odorant (hexanal) yielded the same result in that same study, so that it cannot be attributed exclusively to IPA. This unspecific action may be due to the very short exposure time (1 min) used in that work (Alaux and Robinson, 2007). Here, using a longer exposure time, we verified that exposure to another plant odorant (methyl salicylate) or to another pheromonal compound (geraniol) does not affect olfactory learning, so that the impairment induced by IPA in our work is indeed specific. As geraniol is a main element of the attractive Nasonov pheromone (Boch et al., 1962; Free, 1987), we interpret the effect of IPA or SAP as a learning impairment related to their biological significance as alarm signals and potential stress factors. It would be interesting to test for a specific up-regulation of immediate-early genes in our experimental conditions, which could lead to altered learning performances over a period of hours. Consistent with the idea of a general learning impairment, this effect could be reproduced using different odorants as CSs. Interestingly, appetitive learning is also impaired in bees exposed to physiological stress, either immune (Mallon et al., 2003; Iqbal and Mueller, 2007) or metabolic (Farooqui, 2008; Amdam et al., 2010). Thus, impaired learning may be part of a general response to stress in the honeybee. It may simply reflect the unavailability of cognitive resources aimed at

solving an inappropriate problem (here appetitive learning) in a situation in which such resources should support responses in an aversive, defensive context.

Importantly, this effect cannot be interpreted as a mere consequence of impaired perception of the conditioned odorant (the CS), as bees exposed to IPA still discriminate the CS from a novel odorant in a generalization test (they do so even better than controls). Such a lower generalization combined with lower acquisition has been reported previously in aging forager bees, and interpreted as the result of a possible compensatory mechanism (Behrends et al., 2007). In our experimental context, an interesting hypothesis would be that SAP, or IPA, affects generalization levels by priming the olfactory system to focus specifically on signals that may be relevant in an aversive context indicated by the alarm pheromone. By contrast, exposure to an attractive pheromone like the Nasonov pheromone (or its main component, geraniol) did not alter generalization toward novel odorants. These results raise the interesting question of whether generalization differs between an aversive and an appetitive framework, irrespective of pheromonal exposure.

The decrease in learning performance was neither due to a decreased sensitivity to sucrose (the US). In a previous work, it was shown that bees exposed to IPA tend to respond less to sucrose (Balderrama et al., 2002). Our finding that more bees failed to respond to sucrose during conditioning after exposure to IPA confirms this result. However, in order to study the effects of IPA exposure on associative learning irrespective of a decrease in appetitive motivation, we discarded IPA-exposed bees that did not respond to the US. We thus kept bees showing consistent appetitive unconditioned responses, which enabled us to show that even in the case of an unaffected reward evaluation (as indicated by similar SRS values) acquisition is altered after exposure to IPA. It is clear, therefore, that the impairment of learning induced by IPA cannot be solely attributed to a deficit in reward perception or evaluation. All in all, the fact that processing of neither the CS nor the US was deteriorated in the bees used in our experiment suggests that exposure to IPA affects the ability to form the CS-US association itself, independently of effects on CS and/or US perception.

What could be the neurobiological substrate of such a response? If CS signaling is unaffected (and CS responses are even improved), and if US processing was unaffected in the bees selected for the experiments, how could IPA act on the association between CS and US in order to impair learning? Biogenic amines play key roles in the regulation of learning in insects (Giurfa, 2006), and QMP was shown to impair aversive learning by inhibiting dopamine signaling (Vergoz et al., 2007a). While dopamine has been mainly involved in aversive conditioning in insects, octopamine is crucial for appetitive learning (Schwaerzel et al., 2003; Riemensperger et al., 2005; Unoki et al., 2005; Vergoz et al., 2007b). In honeybees, both sucrose responsiveness and PER acquisition performance are enhanced (resp. decreased) after activation (resp. inhibition) of octopaminergic transmission (Hammer and Menzel, 1995; Menzel et al., 1999; Scheiner et al., 2002; Pankiw and Page, 2003). Consistent with this, levels of octopamine (and dopamine) are depressed in the brains of bees submitted to stressful treatments (Chen et al., 2008), as it has been proposed in other species (Chentsova et al., 2002). Still, other evidence shows that stress and/or pain can increase octopamine titers (Harris and Woodring, 1992; Möbius and Penzlin, 1993; Hirashima et al., 2007). These discrepancies may be linked to spatial (e.g., brain or hemolymph) and temporal variations in octopamine release. In any case, disruption of octopaminergic transmission is unlikely to be a unique and straightforward explanation for the effects of SAP exposure, as in the case of the response to oxidative stress (Farooqui, 2008). Indeed, the dissociation shown here between the impact of IPA exposure on sucrose responsiveness and learning supports this view. Dopamine might also be involved as it does not regulate exclusively aversive learning (Kim et al., 2007; Selcho et al., 2009). Considering the stable effect of exposure on learning observed here, expression changes in biogenic amine receptors in different brain neuropiles might also be involved, as dopamine receptors have been shown to be regulated after only 2 days of exposure to QMP (Beggs et al., 2007). Another possibility that deserves consideration is the fact that IPA exposure seems to activate the equivalent of an opioid system in honeybees (Balderrama et al., 2002). It has been suggested that such activation induces an analgesia-like state in honeybees, thus enhancing tolerance to potential noxious stimuli (Balderrama et al., 2002). Although this possibility seems interesting to consider, it is worth underlining that our bees are confronted with appetitive rather than with aversive conditioning after IPA exposure. An analgesia-like state, if any, would not be relevant in the case of a sucrose reinforcement experienced during olfactory learning. However, an alternative interpretation would posit that activation of a putative opioid-like system would be accompanied by learning impairment in *any* conditioning protocol, irrespective of its appetitive or aversive nature. Further experiments should help to understand the physiological bases of the neuromodulation of learning under stressful procedures.

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Role of tonic inhibition in associative reward conditioning in *Lymnaea*

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Changes in the strength of excitatory synaptic connections are known to underlie associative memory formation in the molluscan nervous system but less is known about the role of synaptic inhibition. Tonic or maintained synaptic inhibition has an important function in controlling the *Lymnaea* feeding system and is known to suppress feeding in the absence of food or in satiated animals. Tonic inhibition to the feeding network is provided by the N3t interneuron that has inhibitory monosynaptic connection with the central pattern generator interneuron, the N1M. Here we asked whether a reduction in the level of tonic inhibition provided by the N3t cell could play a role in reward conditioning? Semi-intact preparations made from hungry snails were conditioned using a previously developed one-trial chemical conditioning paradigm. We recorded electrical activity in a feeding motoneuron, the B3, at various time-points after conditioning. This allowed us to measure the frequency of spike activity in the N3t interneuron and monitor fictive feeding patterns that generate the rhythmic movements involved in food ingestion. We show that there is a reduction in N3t spiking at 1, 2, 3, and 4 h after conditioning but not at 10 and 30 min and the reduction in N3t firing inversely correlates with an increase in the conditioned fictive feeding response. Computer simulation of N3t–N1M interactions suggests that changes in N3t firing are sufficient to explain the increase in the fictive feeding activity produced by conditioning. A network model is presented that summarizes evidence suggesting that reward conditioning in *Lymnaea* is due to the combined effects of reduced tonic inhibition and enhanced excitatory synaptic connections between the CS pathway and feeding command neurons.

Keywords: reward classical conditioning, tonic inhibition, molluscan learning, modulation

INTRODUCTION

Modification in the strength of synaptic connections has been proposed to be the major mechanism of learning in both vertebrate and invertebrate systems (Milner et al., 1998; Kandel, 2001). Changes in the strength of excitatory synaptic connections have been the major focus of attention but there is increasing evidence that modification of inhibitory synaptic mechanisms are also involved in memory (e.g., Kojima et al., 1997; Hansel et al., 2001). In gastropod molluscs there are a number of examples of learning-induced changes in both inhibitory and excitatory synaptic connections. Indeed, interactions between these two types of changes have been shown to be important in both sensitization (Trudeau and Castellucci, 1993, *Aplysia* gill and siphon withdrawal reflex) and classical conditioning (Davis et al., 1983, *Pleurobranchaea* feeding; Crow and Tian, 2006, *Hermissenda* phototaxis).

In the present study, we focused on the role of inhibition in reward classical conditioning in the feeding system of the pond snail, *Lymnaea stagnalis*. This mollusc has been extensively used to study the neuronal and molecular basis of associative memory formation (Benjamin et al., 2000; Benjamin and Kemenes, 2009). A previously proposed mechanism for the increased feeding response to the CS after one-trial chemical conditioning relies on an enhanced excitatory synaptic response recorded in cerebral command-like neurons (cerebrobuccal interneurons, CBIs) that activate the feeding central pattern generator (CPG) (Kemenes et al., 2006). Now we examine an additional mechanism that might underlie the conditioned response that reduces the tonic inhibitory synaptic modulation of the CPG. This type of tonic or maintained inhibition is known to suppress feeding in quiescent animals in the absence of food or in satiated animals (Staras et al., 2003) but its modulation could also play a role in learning. We hypothesize that if the tonic inhibition is reduced by conditioning then it would make the feeding CPG and the CPG-driven motoneurons more easily activated by the CS. The inhibitory synaptic input that modulates feeding is known to originate from a CPG interneuron known as N3t. This N3t neuron has a monosynaptic inhibitory connection with N1M (**Figure 1**), a CPG interneuron whose required activation to produce a feeding rhythm depends on a reduction of N3t tonic inhibition (Staras et al., 2003). The reduction in the N3t inhibitory input leads to plateauing activity in the N1M and subsequent firing in the N2 and N3 CPG interneurons that fire in sequence (N1, N2, and N3) to drive a three phase feeding rhythm in the motoneurons. Under these circumstances the N3t fires phasically and becomes part of the CPG rhythm.

We use an *in vitro* version of the one-trial chemical conditioning procedure (Marra et al., 2006; Harris et al., 2010) to monitor changes in the frequency of N3t tonic activity in the first few hours after conditioning. We show that there is a reduction in the N3t

firing frequency at 1, 2, 3, and 4 h but not at 10 and 30 min after conditioning and this reduction correlates with an increase in the conditioned feeding response to the CS. Computer simulations based on a previously published model of the feeding network (Vavoulis et al., 2007) support the conclusion that the reduced level of tonic inhibition plays an important role in *Lymnaea* reward conditioning.

MATERIALS AND METHODS

IN VITRO CONDITIONING AND ELECTROPHYSIOLOGICAL RECORDING

Adult *L. stagnalis*, were raised in the breeding facilities at the University of Sussex. Animals were starved for 3 days and dissected to obtain a semi-intact preparation. This consisted of the entire CNS and chemo-sensory structures (i.e., lips and esophagus) as described by Straub et al. (2006) (**Figure 2**). The preparations were perfused with normal snail saline (NS) containing 50 mM NaCl, 1.6 mM KCl, 2 mM $MgCl_2$, 3.5 mM $CaCl_2$, 10 mM HEPES buffer in water. The semi-intact preparations were conditioned by perfusing the lips with 0.27 mM amyl acetate (CS) in NS for 2 min, immediately followed by 2 min of 0.27 mM amyl acetate applied to the lips together with 20 mM sucrose (US) perfused to both lips and esophagus. The conditioned response was tested by perfusing the lips with the CS for 2 min and measuring the electrophysiological response in the B3 motoneuron at various time points (10, 30 min, 1, 2, and 3 h) after the one-trial conditioning. The B3 motoneurons can be identified because of their large size and unique location on the surface of the buccal ganglia (Benjamin and Rose, 1979). B3 motoneurons were recorded using sharp electrodes (20–40 MΩ) filled with 4 M potassium acetate. NL 102 (Digitimer Ltd) and Axoclamp 2A (Axon Instrument, Molecular Device) amplifiers were used and data were acquired using a micro 1401 Mk II interface and analyzed using Spike 2 software (Cambridge Electronic Design, Cambridge, UK).

The B3 motoneurons were recorded because they act as a monitor of N3t firing (see Staras et al., 2003). Spikes in the N3t cells generate 1:1 monosynaptic EPSPs on the B3s (Rose and Benjamin, 1979; Elliott and Benjamin, 1985) and can therefore be used an indirect method for recording N3t firing rates (circuit shown in **Figure 1**). The N3t cells are small and difficult to record in every preparation making it more convenient to monitor N3t firing in the B3 motoneurons. The B3s were also used to measure fictive feeding responses to application of the CS. Fictive feeding consists of bursts of spikes in motoneurons like the B3s that correspond to feeding cycles in the intact animal (Rose and Benjamin, 1979). Fictive feeding responses were calculated as a difference score by subtracting the number of B3 bursts in the 2-min before application of the CS from the number occurring in the 2-min after application of the CS. Other types of analysis were also carried out, including measuring the effect of conditioning on the duration of fictive feeding bursts.

COMPUTATIONAL MODELING

A computational model was used to simulate the effects of varying N3t firing frequency on the probability of firing of the N1Ms. The analysis was based on previously published models of the feeding CPG interneurons of *L. stagnalis* (Vavoulis et al., 2007). The N1M model neuron was continuously stimulated by injecting a constant

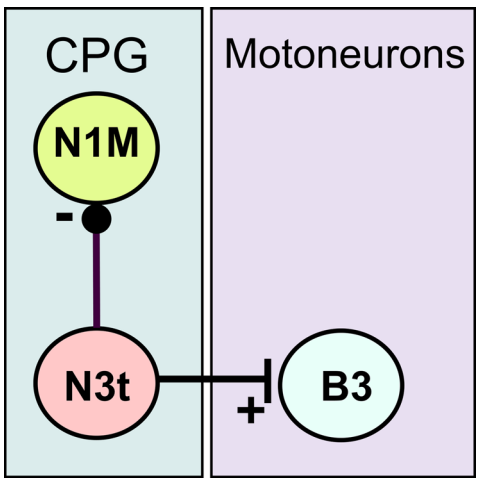

FIGURE 1 | Synaptic connectively of the N3t neuron. The N3t has monosynaptic inhibitory connection (black dot) with the CPG neuron, the N1M, and a monosynaptic excitatory connection (vertical bar) with the B3, a motoneuron that innervates the buccal mass. The B3 neuron is routinely recorded to monitor of N3t firing as each spike in the N3t is accompanied by 1:1 EPSPs in the B3 (Elliott and Benjamin, 1985). Tonic N3t firing inhibits activity in the N1M either completely or partially depending on the firing rate of the N3t (Staras et al., 2003).

excitatory current of 0.4 μA/cm^2 and the frequency of N3t firing was varied between 1 and 5 Hz. The N3t inhibitory inputs on N1M were stochastically simulated assuming that N3t spike generation is a random Poisson process and that each N3t spike induces an IPSC on N1M with maximal conductance 0.15 mS/cm^2, reversal potential −75 mV and synaptic activation described by an alpha function with a time constant equal to 40 ms. These values were chosen such that N3t-induced IPSPs on N1M are of similar amplitude and duration in both the model and biological neurons. The model was numerically solved using an exponential Euler integration method with constant time step equal to 0.005 ms. For each simulation only the first 1000 ms were analyzed.

DATA ANALYSIS

The frequency of N3t excitatory inputs recorded on the B3 motoneurons was measured at different time points from the beginning of the recording. The traces were visualized off-line using Spike2 and the frequency was sampled for at least 30 s of recording using a Spike2 script. The duration of the CS induced feeding cycles in the B3 motoneurons was similarly measured off-line. The control groups used are constituted of preparations presented with either CS alone or US alone, no statistical difference could be observed between these two types of control treatment (Student's *t*-test, $P > 0.05$; CS alone, $n = 6$; US alone, $n = 5$). The comparisons between groups have been carried out using either *t*-test or a one-way analysis of variance (1-way ANOVA) followed by Newman–Keuls test. Values of $P < 0.05$ are indicated with a single asterisk (*), values of $P < 0.01$ a double asterisk (**) and values of $P < 0.001$ with a triple asterisk (***). Correlations between data sets were studied using Pearson's correlation test. Statistical analyses were carried out using either Prism (Graphpad Software) or R (R-project) software.

RESULTS

N3t TONIC FIRING RATE IS DECREASED AFTER CONDITIONING

To test whether learning leads to a change in the firing rates of the N3t cell, the frequency of N3t synaptic inputs were recorded in the B3 motoneurons before and after a single pairing of the CS and US in the semi-intact preparation (**Figure 2**). **Figure 3A** is an example of intracellular recording from a B3 motoneuron showing N3t EPSP inputs in experimental and control preparations. The top trace shows the N3t-induced EPSPs observed before pairing and the middle trace 3 h after pairing. A clear reduction in the frequency of N3t inputs occurs. **Figure 3B** shows the more quantitative analysis where the N3t firing rates before training are compared with those at 10 min 1, 2, and 3 h after pairing ($n = 11$). The repeated measurement ANOVA indicates a source of significant difference [$F(4, 40) = 4.9$, $P < 0.01$] in the data and the *post hoc* analysis indicates a significant reduction in the N3t input frequency at 1, 2, and 3 h after pairing compared with before (Newman–Keuls *post hoc* $P < 0.05$). However, no statistical difference was observed between the N3t input frequency before and 10 min after pairing ($P > 0.05$).

A further set of more detailed experiments were carried out where N3t spontaneous firing rates were measured in an experimental paired group (CS + US) and compared with controls. B3 motoneurons were recorded in preparations at 30 min and 4 h after conditioning as well as at 10 min, 1, 2, and 3 h to provide a more complete time-course of learning-induced N3t firing changes. An example of an intracellular recording from a B3 in a control preparation is shown in **Figure 3A** (bottom trace). The frequency of the N3t inputs at 3 h in this control preparation is higher than the one observed in a paired preparation at the same time point after conditioning. The histogram in **Figure 3C** provides a statistical analysis of N3t firing in the paired and control preparations at different time points after conditioning. The data for each time point are obtained from a different group of preparations. The ANOVA showed source of difference [$F(1, 122) = 6.1$, $P < 0.0001$] between the groups. Further comparison between paired and control groups at each time point were made using a Newman–Keuls test. Comparisons of data from paired and control preparations at a particular time-point showed a significant difference at 1, 2, 3, and 4 h (1 h paired vs 1 h control $P < 0.01$, $n = 15$, $n = 11$, respectively; 2 h paired vs 2 h control $P < 0.05$, $n = 14$, $n = 11$, respectively; 3 h paired vs 3 h control $P < 0.05$, $n = 14$, $n = 11$, respectively; 4 h Paired vs 4 h control $P < 0.01$, $n = 11$, $n = 12$, respectively) but not at 10 and 30 min (10 min paired vs 10 min control $P > 0.05$, $n = 9$, $n = 11$, respectively; 30 min paired vs 30 min control $P > 0.05$, $n = 7$, $n = 8$, respectively). The mean level of N3t firing was maintained in control preparations over the 1–4 h period of the experiment (**Figure 3C**) indicating that the reduction in the frequency observed in the paired groups over the same time period was not due to preparation "run down."

APPLICATION OF THE CS DOES NOT CHANGE THE FREQUENCY OF N3t FIRING

The frequency of N3t firing was measured for 30 s prior to CS application and compared with firing for 30 seconds during the CS application in trained preparations. There was no statistical difference between the two scores (Student's *t*-test, $P > 0.05$) (**Figure 4**) indicating that application of the CS does not influence the level of firing of the N3ts in conditioned preparations.

INVERSE CORRELATION BETWEEN FICTIVE FEEDING RESPONSE AND N3t FREQUENCY AFTER CONDITIONING DEPENDS ON ACTIVATION OF THE CS PATHWAY

The N3t frequency before CS application was compared with the conditioned fictive feeding response measured as a difference score (see Materials and Methods). Plotting the N3t input frequency against of fictive feeding cycles revealed a significant inverse correlation ($r = -0.7$, $P < 0.05$, $n = 10$) at the 4-h time point (**Figure 5A**). Interestingly, the number of background fictive feeding cycles observed in the 2-min *preceding* the CS test is not correlated with the N3t frequency ($r = -0.1$, $P > 0.05$, $n = 10$) (**Figure 5B**). This suggests that the conditioned fictive feeding response depends not only on changes in the N3t firing rate but also on additional changes in the CS pathway.

DELAYED REDUCTION IN CYCLE DURATION AFTER CONDITIONING

A comparison of CS-induced fictive feeding cycles at different time points after conditioning indicated that their mean duration was significantly reduced at 1, 2, and 4 h compared

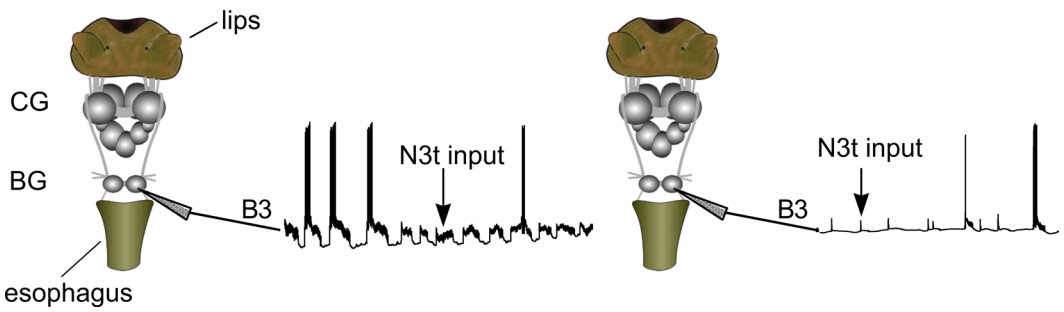

FIGURE 2 | The semi-intact preparation used to monitor the effects of conditioning on N3t activity. The preparation includes the whole CNS and the chemosensory sensory structures involved in the feeding response (lips and esophagus). The B3 is located in the buccal ganglia (BG) and other parts of feeding circuitry are located in the cerebral ganglia (CG). The monosynaptic EPSP inputs of N3t on the B3 motoneurons were monitored at 3 h before and after conditioning. A reduction in the frequency of the post-synaptic input can be observed after conditioning.

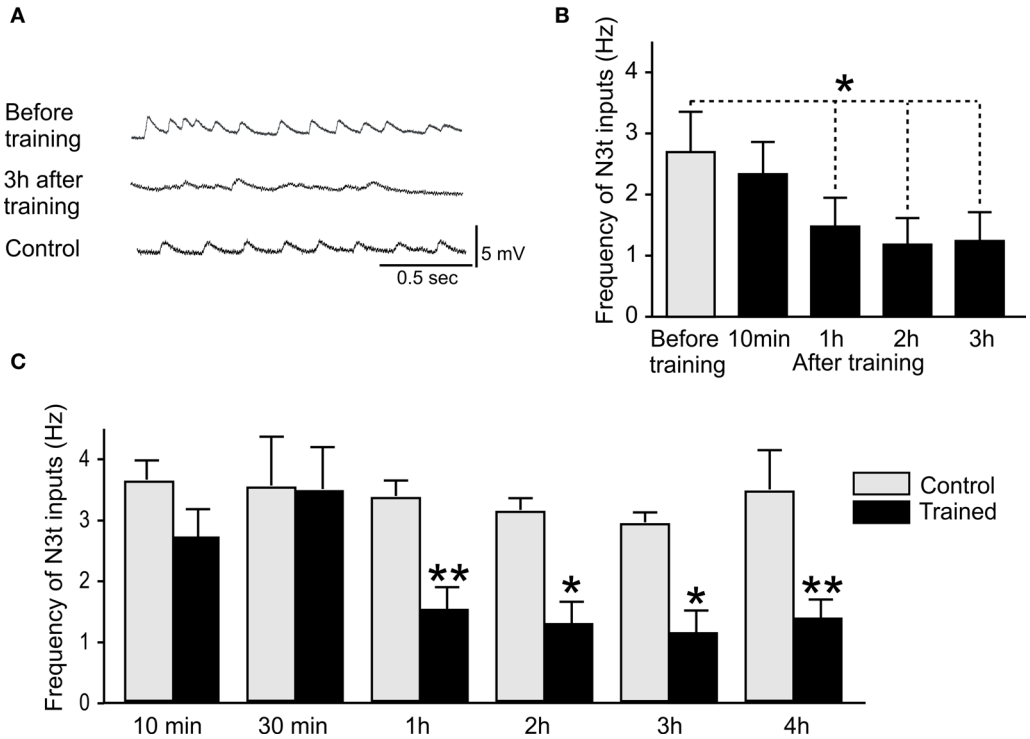

FIGURE 3 | Reduction in N3t frequency following single-trial conditioning. **(A)** Intracellular recordings showing examples of N3t synaptic inputs on B3 motoneurons. The top trace shows the N3t-driven inputs before pairing, the middle trace, a B3 motoneuron 3 h after pairing. The frequency of the N3t inputs is lower than before pairing. The bottom trace is an intracellular recording of a B3 motoneuron from a control preparation at the 3-h time point. The frequency of the N3t inputs in control preparations is higher than in the experimental preparation at the same time point, and similar to the frequency before pairing. **(B)** The N3t frequency is compared at various time points before and after pairing. At 1, 2, and 3 h after pairing the paired group are significantly lower than before pairing, but there is no statistical difference at 10 min after pairing (see text for detailed statistical analysis). **(C)** Comparison of the N3t firing frequency in paired and control groups of preparations at different time points after conditioning. There are significant difference between paired and control groups at 1, 2, 3, and 4 h conditioning but not at 10 and 30 min (see text for statistics).

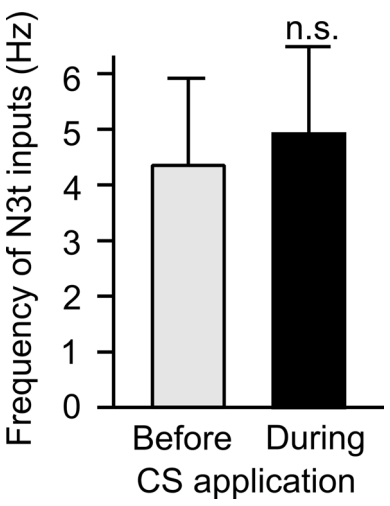

FIGURE 4 | CS application does not change the frequency of the N3t inputs on the B3. The frequency of N3-driven EPSPs on the B3 was measured immediately before and during CS application following pairing. The data from eight preparations tested at different time points after pairing were combined, no statistically significant difference can be observed using a two-tailed paired *t*-test ($P > 0.05$).

with 10 min (**Figure 6**). An initial ANOVA showed a source of difference [$F(3, 63) = 5.85$, $P < 0.001$] between the groups and a *post hoc* test showed a significant difference between the 10-min time point ($n = 11$) compared with each of the later time points (Newman–Keuls *post hoc* test 10 min vs 1 h $P < 0.01$, $n = 14$; 10 min vs 3 h $P < 0.001$, $n = 28$; 10 min vs 4 h $P < 0.01$, $n = 13$).

COMPUTATIONAL MODELING SUGGESTS THAT REDUCING N3t FIRING INCREASES THE PERCENTAGE OF PREPARATIONS SHOWING N1M FIRING

To further investigate the effect of reducing the N3t activity on the generation of a feeding cycle, we performed computer simulations based on a previously published computational model of the relevant feeding CPG interneurons (Vavoulis et al., 2007). The computer model allowed us to manipulate the frequency of N3t and test whether lowering N3t firing frequency was sufficient to induce activation of the N1M from a previously sub-threshold stimulus. A constant depolarizing current was injected into the model N1M at different mean firing frequencies of N3t. The model predicts that the percentage of preparations showing N1M bursting is highest when the N3t is firing at its lowest rate (1 Hz) and rapidly decreases as the firing rate is progressively increased to

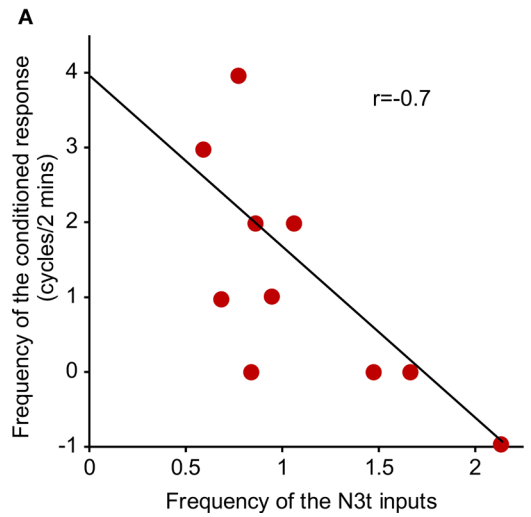

FIGURE 5 | Inverse correlation between N3t firing rate and frequency of fictive feeding response. (A) The N3t frequency before CS application was compared with the conditioned fictive feeding response measured as a difference score. There is a significant inverse correlation ($r = -0.7$, $P < 0.05$, $n = 10$) at the 4-h time point. **(B)** The number of background fictive feeding cycles observed in the 2-min *preceding* the CS test is not correlated with the N3t frequency ($r = -0.1$, $P > 0.05$, $n = 10$).

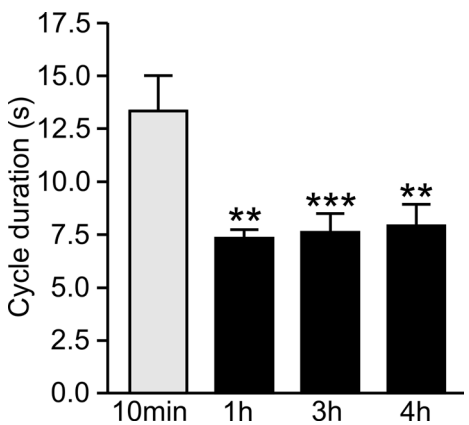

FIGURE 6 | Delayed decrease in fictive feeding cycle duration after conditioning. The duration of fictive feeding cycles at 1, 2, and 3 h after pairing were compared with the duration at 10 min after pairing. There is a statistically significant reduction at the 1, 2, and 3 h time points compared with 10 min (see text for statistics) but no other significant difference between the groups.

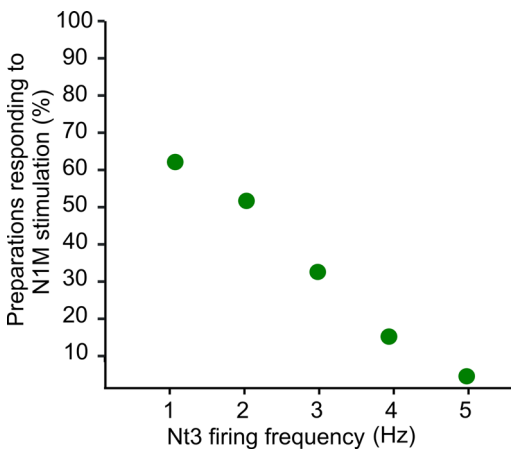

FIGURE 7 | Computer simulation of N3t firing frequency and preparation responsiveness. A constant depolarizing current was injected into the model N1M at different firing frequencies of the N3t. Whether the N1M ("preparation") responded by generating a burst of action potentials to the depolarizing stimulus depended on the firing rate of the N3t. The preparations were more likely to respond when N3t is firing at its lowest rate (1 Hz) and responsiveness rapidly decreased as the firing rate is progressively increased to 5 Hz. Spike activity in the N1M in the biological network is a reliable indicator of activation of the whole CPG network and the feeding ingestive rhythm.

5 Hz (**Figure 7**). Generation of a burst of spikes in the N1M is a reasonable assay of "preparation responsiveness" since in the biological system spike activity in the N1M is always accompanied by rhythmic spike activity in the rest of the CPG network (Elliott and Benjamin, 1985) and this drives rhythmic fictive feeding activity in motoneurons like the B3.

DISCUSSION

The main result from these experiments is that the frequency of tonic N3t firing is reduced following single-trial *in vitro* chemical conditioning. The reduction level was observed at 1 h after conditioning and up to 4 h but did not occur at the earlier time points of 10 and 30 min. Importantly, the frequency of the N3t input is inversely correlated with the strength of the conditioned response, i.e., the conditioned response measured as changes in the fictive feeding response to the CS is stronger in preparations displaying a lower level of inhibition. Application of the CS did not change the frequency of N3t firing so conditioning reduces

the "background" level of N3t firing. The N3t cells monosynaptically inhibit the most important CPG interneuron, the N1M (see **Figure 1**), and it is this inhibitory synaptic connection that mediates the inhibitory effects of the N3t firing on the rhythmic activity underlying fictive feeding activity (shown in Staras et al., 2003). We suggest a permissive role for the learning-induced reduction in background inhibition, lowering the threshold for activation of the feeding CPG by the CS. This can be considered as part of the memory trace for reward conditioning. It cannot be important for the earliest phase of memory formation ("short-term memory") because no changes in N3t firing rate were recorded in the first hour after conditioning. A second type of change produced by conditioning was the reduction in the duration of the fictive feeding cycles at 1, 2, and 3 h after pairing compared with 10 min. From previous work (Staras et al., 2003) we know that duration of motoneuron cycles is correlated inversely with the frequency of feedback inhibition from the N3ts to the N1Ms so bursts are longer in duration when inhibitory feedback is reduced. After conditioning, reduction in N3t firing rate occurs at 1, 2, 3, and 4 h but not at 10 min (**Figure 3**) so this can account for burst duration being greater at 10 min than the later time-points.

It is important to note that the results from the present experiments were obtained in preparations made from hungry snails. Earlier work by Staras et al. (2003) in naïve snails showed that firing rates in the N3t cells and their consequent inhibitory modulation of feeding was dependent on their level of hunger and satiety. Hungry snails had significantly lower levels of maintained N3t firing (inhibition) than preparations made from satiated snails. However the reductions in N3t firing due to learning were greater (1–2 Hz) than the maximum produced by hunger alone (2–3 Hz). This is an important result because it indicates that the effects of learning are greater than motivation factors such as hunger and satiety.

Our results do not give any information on the mechanisms underlying the change in N3t activity. For instance, whether they involve changes in the intrinsic properties of the neuron and/or synaptic inputs from other interneurons in the feeding network. However, the results do indicate that the likely location of the learning-induced changes is in the buccal ganglia. The N3t cells and their target B3 and N1M neurons are located in this part of the feeding system with their anatomy and synaptic connectivity restricted to these paired ganglia (Elliott and Benjamin, 1985). Previous work showed that higher-order modulatory neurons in the cerebral ganglia provide another site for electrical changes following one-trial chemical conditioning (Straub et al., 2004; Kemenes et al., 2006). The cerebral mechanism produces an enhanced excitatory synaptic response in the CBIs due to a Ca^{2+}-dependent pre-synaptic facilitation of the CS to CBI pathway by the modulatory Cerebral Giant Cells (Kemenes et al., 2006). However, this excitatory mechanism was first observed at 16–24 h after conditioning and so may not interact temporally with the inhibitory mechanism described in the present paper which so far has only been observed from 1 to 4 h after conditioning. However, there must be some independent change in the CS pathway in the 1–4 h time period because the correlation between N3t firing and the conditioned fictive feeding response requires the application of the CS and cannot be due solely to a reduction in background N3t firing.

Interactions between excitatory and inhibitory synaptic changes induced by classical conditioning have been described in several invertebrate systems (Davis et al., 1983; Crow and Tian, 2006). The results from aversive classical conditioning of feeding *Pleurobranchaea* are of particular interest because they involve learning-induced changes in background synaptic inputs in the paracerebral (phasic type) command cells in naïve animals. Conditioning changes the balance of "spontaneous" inhibitory and excitatory synaptic inputs to the command neurons so that the preparations show a decreased level of excitatory inputs and an increased level of inhibitory inputs on application of the CS (food or touch), reducing the ability of the paracerebral cells to activate feeding. Davis et al. (1983) also examined the effects of hunger and satiety on the response to food stimuli and found

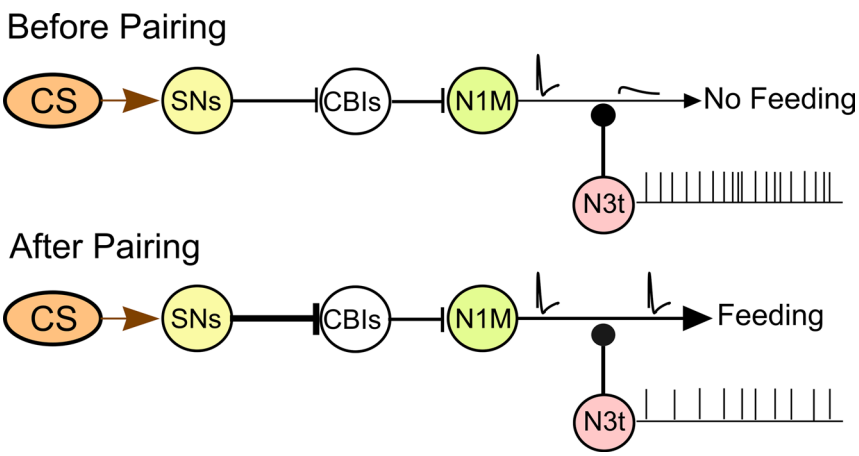

FIGURE 8 | Circuit model for the CS-induced activation of the feeding CPG. The diagram summarizes the current knowledge of the pairing-induced cellular changes in the CNS. The CS pathway does not produce a behavioral response before pairing and only attenuated (blocked) spikes are conducted to the distal axonal regions of the N1M. Following pairing the CS triggers a feeding response. A strengthening of the excitatory pathway is proposed between the CS sensory pathway and the CBIs. Conditioning reduces N3t inhibition of the N1M bringing it closer to the threshold potential for firing with full sized spikes present in the N1M distal axon (see text for further discussion).

that satiated animals responded in the same way as aversively conditioned animals, i.e., the balance of inhibition/excitation was changed in favor of inhibition. However, unlike *Lymnaea* where training produced greater changes in background inhibition than hunger and satiety, the effects of satiety appear to be similar (or greater) in strength to those due to learning.

We present a circuit-level model of the *Lymnaea* feeding pathway before and after one-trial chemical conditioning (**Figure 8**) based on previously published data on the feeding network and its chemosensory inputs (Straub et al., 2004, 2006; Kemenes et al. 2006). The CS pathway has no effect on feeding before pairing. Following pairing the CS triggers a feeding response. The feeding response is generated through the sensory neuron (SN), CBI, N1M pathway. Evidence exists that the excitatory SN to CBI synapse is enhanced after conditioning (Straub et al., 2004). Conditioning also reduces spike activity in the N3t interneuron therefore reducing the inhibition of the N1M making it more likely to respond to the CS. Once the N1M starts firing it drives activity in the rest of the CPG network (Staras et al., 2003). The model is consistent with the results of computer simulations presented in this study. The simulations suggest that changes in N3t firing frequency, within the experimentally observed range, are sufficient to change the probability of activation of the N1M. We hypothesize that the location of the N3t to N1M synapse is on the axonal region of the N1M. Small changes in membrane potential have been shown to affect the propagation of the action potential between two different sections of a neurite in both vertebrates and invertebrates (Evans et al., 2003; Debanne, 2004) and we suggest that this might be occurring in the N1M cell so that attenuated spikes in the distal N1M axon before pairing would be propagated as full spikes after conditioning. The model emphasizes that chemical reward conditioning is likely to involve changes in synaptic strength at a number of different sites in the network.

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Roles of aminergic neurons in formation and recall of associative memory in crickets

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We review recent progress in the study of roles of octopaminergic (OA-ergic) and dopaminergic (DA-ergic) signaling in insect classical conditioning, focusing on our studies on crickets. Studies on olfactory learning in honey bees and fruit-flies have suggested that OA-ergic and DA-ergic neurons convey reinforcing signals of appetitive unconditioned stimulus (US) and aversive US, respectively. Our work suggested that this is applicable to olfactory, visual pattern, and color learning in crickets, indicating that this feature is ubiquitous in learning of various sensory stimuli. We also showed that aversive memory decayed much faster than did appetitive memory, and we proposed that this feature is common in insects and humans. Our study also suggested that activation of OA- or DA-ergic neurons is needed for appetitive or aversive memory recall, respectively. To account for this finding, we proposed a model in which it is assumed that two types of synaptic connections are strengthened by conditioning and are activated during memory recall, one type being connections from neurons representing conditioned stimulus (CS) to neurons inducing conditioned response and the other being connections from neurons representing CS to OA- or DA-ergic neurons representing appetitive or aversive US, respectively. The former is called stimulus–response (S–R) connection and the latter is called stimulus–stimulus (S–S) connection by theorists studying classical conditioning in vertebrates. Results of our studies using a second-order conditioning procedure supported our model. We propose that insect classical conditioning involves the formation of S–S connection and its activation for memory recall, which are often called cognitive processes.

Keywords: octopamine, dopamine, classical conditioning, memory recall, olfactory learning, visual learning, crickets

INTRODUCTION

Biogenic amines regulate various functions of central nervous systems in vertebrates and invertebrates (Blenau and Baumann, 2001). In vertebrates, dopamine (DA) pathways are involved in the coordination of motor behavior, motivation, addiction, and reward-based learning of a wide range of sensory stimuli (Schultz, 1998, 2006). In insects, DA appears to play roles in regulating motor behavior (Blenau and Baumann, 2001) and arousal (Andretic et al., 2005), and octopamine (OA), the invertebrate counterpart of noradrenaline, plays roles in desensitizing sensory inputs and regulating various forms of behavior (Roeder, 1999), including aggression (Stevenson et al., 2005; Hoyer et al., 2008; Zhou et al., 2008) and sleep (Crocker et al., 2010). In this article, we review recent advances in studies on the roles of OA-ergic and DA-ergic neurons in classical conditioning in insects, focusing on findings from our behavioral and pharmacological studies on crickets *Gryllus bimaculatus*.

ROLES OF AMINERGIC NEURONS IN FORMATION OF OLFACTORY MEMORY IN HONEY BEES AND FRUIT-FLIES

Insects are useful animal models for the study of cellular and molecular mechanisms underlying learning and memory (Giurfa, 2003; Heisenberg, 2003; Davis, 2005; Menzel and Giurfa, 2006; Menzel et al., 2006; Keene and Waddell, 2007). This is mainly because insect brains consist of a relatively small number ($<10^6$) of neurons and the organization of "microbrains" (Mizunami et al., 1999, 2004) is relatively simple, and thus their functions are highly susceptive to genetic, pharmacological, and other means of experimental manipulation. One of the pioneering studies on the roles of aminergic neurons in insect learning was performed in honey bees by Hammer (1993). A hungry honey bee extends its proboscis in response to sucrose stimulation applied to its antennae, proboscis, or tarsi. The proboscis extension response can be conditioned by pairing an odor applied to the antennae (conditioned stimulus, CS) with sucrose stimulation (unconditioned stimulus, US) (Kuwabara, 1957; Erber et al., 1980). Hammer (1993) observed that pairing of an odor with intracellular stimulation of the VUMmx1 neuron, a putative octopamine immunoreactive neuron (Kreissl et al., 1994) that exhibited responses to sucrose stimulation, induced a conditioning effect. Hence, he concluded that this neuron mediates the reinforcing property of sucrose reward in olfactory conditioning. Later, Hammer and Menzel (1998) showed that local injection of octopamine into the antennal lobes and the calyces of the mushroom bodies, termination areas of the VUMmx1 neuron, substituted the sucrose US in olfactory conditioning; the antennal lobes are primary olfactory centers and the mushroom bodies are higher-order olfactory and multi-sensory association centers (Erber et al., 1980; Heisenberg et al., 1985; Mizunami et al., 1998a,b; Okada et al., 1999; Heisenberg, 2003; Davis, 2005; Menzel and Giurfa, 2006). In addition, Farooqui et al. (2003) showed that RNA interference of OA receptors or pharmacological blockade of OA receptors by mianserin in the antennal lobe impaired olfactory conditioning.

Another pioneering study was performed in the fruit-fly *Drosophila* by Schwaerzel et al. (2003). Fruit-flies can be conditioned to choose an odor associated with sucrose, or avoid an odor associated with electric shock (Dudai et al., 1976; Tully and Quinn, 1985). Schwaerzel et al. (2003) demonstrated that transgenic flies defective in OA or DA synthesis exhibited defects in appetitive olfactory learning with sucrose reward or aversive olfactory learning with electric shock, respectively. Subsequent studies in fruit-flies confirmed that OA- or DA-ergic neurons convey signals for sucrose reward or electric shock punishment, respectively, in olfactory conditioning in larval (Schroll et al., 2006; Honjo and Furukubo-Tokunaga, 2009; Selcho et al., 2009) and adult flies (Riemensperger et al., 2005; Tomchik and Davis, 2009; Gervasi et al., 2010), although a few exceptions have also been found (Kim et al., 2007, Sitaraman et al., 2008). Notable findings in these studies are that photoactivation of OA-ergic or DA-ergic neurons paired with an odor stimulation successfully induced an appetitive or aversive conditioning effect, respectively, in larval flies (Schroll et al., 2006) and that photoactivation of a class of DA-ergic brain neurons also induced an aversive conditioning effect in adult flies (Claridge-Chang et al., 2009). Roles of DA in conveying aversive US have also been demonstrated in honey bees in olfactory conditioning of the sting extension reflex, in which an odor was paired with electric shock punishment (Vergoz et al., 2007).

PROCEDURES FOR OLFACTORY AND VISUAL PATTERN CONDITIONING IN CRICKETS

We studied the roles of OA-ergic and DA-ergic signaling in classical conditioning in crickets *Gryllus bimaculatus*. We have demonstrated that crickets have excellent olfactory learning capabilities: for example, they can learn (1) to associate an odor with reward by a single operant or classical conditioning trial (Matsumoto and Mizunami, 2000, 2002a), (2) to form a lifetime olfactory memory (Matsumoto and Mizunami, 2002b), (3) to memorize seven pairs of odors at the same time (Matsumoto and Mizunami, 2006), and (4) to associate one odor with reward and another odor with punishment in one visual context and to associate the opposing in another visual context (Matsumoto and Mizunami, 2004). Their high learning capabilities may reflect their omnivorous foraging habit, i.e., they test many potential food items to assess whether they are edible or not. Capacity for forming visual place memory has also been demonstrated in crickets (Wessnitzer et al., 2008). Moreover, we have shown that crickets are suitable materials for the study of molecular mechanisms underlying learning and memory by using pharmacological manipulation (Matsumoto et al., 2006, 2009) and RNA interference (Takahashi et al., 2009).

We used a "classical conditioning and operant testing" procedure, which is based on a high capability of crickets to transfer memory formed in a classical conditioning situation to an operant testing situation (Matsumoto and Mizunami, 2002a; Unoki et al., 2005, 2006). For appetitive olfactory conditioning, crickets were individually placed in a beaker, and one of two odors (e.g., banana and apple odors) was presented to their antennae and then water reward was presented to their mouth (**Figure 1A**). For aversive olfactory conditioning, one of these two odors was presented to the antennae before presenting 2 M sodium chloride solution to the mouth. In the odor preference test, animals were allowed to freely choose between two odor sources. Each odor source consisted of a container containing a filter paper soaked with a solution of odor essence. The time that the animals touched the gauze net covering the top of each container with their mouths or palpi was measured for evaluating relative odor preference of the animals (**Figure 1B**). For visual pattern conditioning, either of a white-center and black-surround pattern or a black-center and white-surround pattern was paired with water reward or sodium chloride punishment (**Figure 1C**). In the preference test, the time that the animals touched each of the two patterns was measured for evaluating relative preference (**Figure 1D**).

These procedures were highly effective for achieving conditioning. In the case of appetitive olfactory conditioning, for example, one conditioning trial was sufficient to establish conditioning, with its memory lasting for several hours (mid-term memory; see **Figure 4**; Matsumoto and Mizunami, 2002a; Unoki et al., 2005). Two appetitive conditioning trials with a 5-min interval induce memory that lasts for at least 1 day (see **Figure 4**), which matches protein-synthesis-dependent long-term memory because it is blocked by injection of a protein-synthesis inhibitor, cycloheximide, into the hemolymph before conditioning (Matsumoto et al., 2003).

ROLES OF AMINERGIC NEURONS IN FORMATION OF OLFACTORY MEMORY IN CRICKETS

We studied the effect of OA and DA receptor antagonists on appetitive and aversive olfactory conditioning in crickets (Unoki et al., 2005). Crickets injected with epinastine or mianserin, antagonists of insect OA receptors (Roeder et al., 1998; Degen et al., 2000b), into the hemolymph before conditioning exhibited a complete impairment of appetitive conditioning to associate an odor with water reward (**Figure 2A**); the preference for the rewarded odor after conditioning did not significantly differ from that before conditioning. On the other hand, these animals exhibited no impairment of aversive learning with saline punishment (**Figure 2B**). The latter observation shows that OA receptor antagonists do not impair sensory function, motor function, or motivation necessary for learning, and we thus conclude that OA is specifically involved in conveying water reward. We also found that fluphenazine, chlorpromazine, or spiperone, antagonists of insect DA receptors (Degen et al., 2000a; Mustard et al., 2003), completely impaired aversive learning with sodium chloride punishment (**Figure 2C**) but did not affect appetitive learning with water reward (**Figure 2D**). The latter finding indicates that DA receptor antagonists do not impair the sensory function, motor function, or motivation necessary for learning, and thus we conclude that DA is specifically involved in conveying sodium chloride punishment. It should be cautioned that the specificity of antagonists used in our studies is not necessarily perfect (see Discussion in Unoki et al., 2005). However, two different kinds of OA receptor antagonists impaired appetitive learning but not aversive learning and three different kinds of DA receptor antagonists impaired aversive learning but not appetitive learning, suggesting that the impairments were due to the blockade of OA or DA receptors. Thus, we concluded that OA- or DA-ergic neurons convey information about appetitive or aversive US, respectively, for olfactory conditioning in crickets (Unoki et al., 2005).

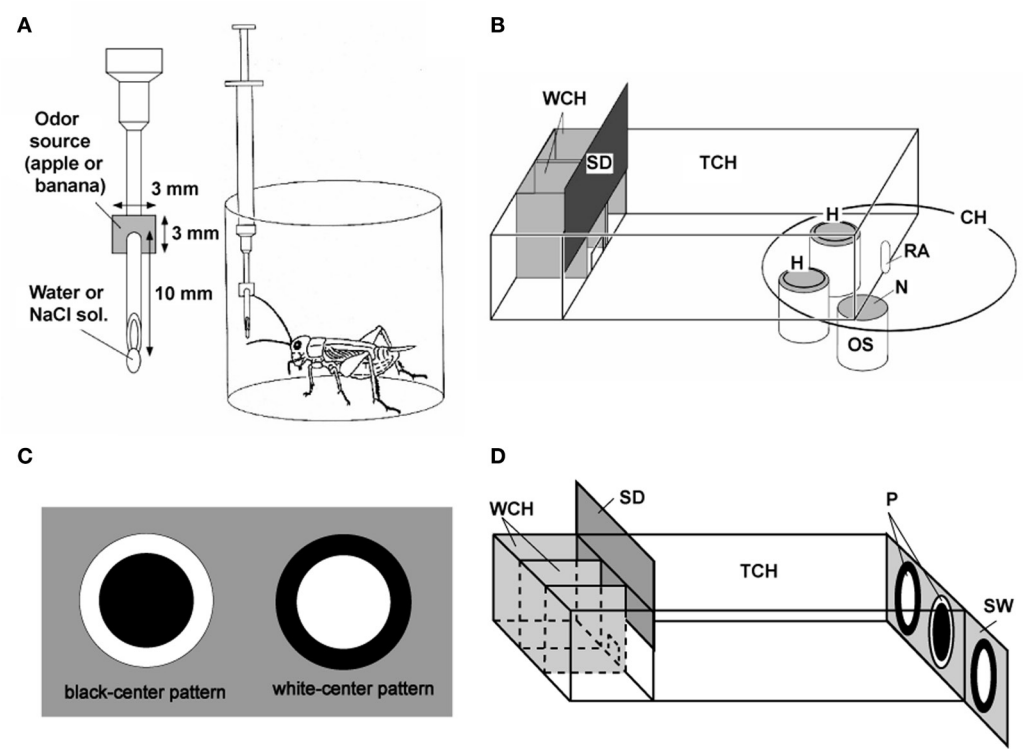

FIGURE 1 | Procedures for olfactory and visual pattern conditioning in crickets. (A) Procedures for olfactory conditioning. One of two odors (e.g., banana and apple odors) was used as CS, and water or 20% sodium chloride solution was used as US. A syringe containing water or sodium chloride solution was used for conditioning. A filter paper soaked with banana or apple essence was attached to the needle of the syringe. The filter paper was approached to the cricket's antennae so as to present an odor, and then water or sodium chloride was presented to the mouth for appetitive or aversive conditioning, respectively. **(B)** Apparatus for the odor preference test. On the floor of the test chamber (TCH), there were two holes (H) connecting the chamber with odor sources (OS). Each odor source consisted of a container containing a filter paper soaked with 3 μl solution of banana or apple essence, covered with fine gauze net (N). Three containers were mounted on a rotative container holder (CH) and two of three odor sources could be presented at the same time. A cricket was placed in the waiting chamber (WCH) for 4 min for acclimation and then allowed to enter the test chamber to visit odor sources, by opening a sliding door (SD). Two minutes later, the relative positions of the banana and apple sources were changed. The preference test lasted for 4 min. RA: rotating axle. **(C)** Visual patterns used for conditioning. A black-center and white-surround pattern (black-center pattern) or a white-center and black-surround pattern (white-center pattern) was used as CS and water or sodium chloride solution was used as US. A pattern was attached to the needle of the syringe. The pattern was presented above the cricket's head and then water or sodium chloride was presented to the mouth for appetitive or aversive conditioning, respectively. **(D)** Apparatus for the pattern preference test. Two white-center patterns and one black-center pattern (P) were presented on a gray sliding wall (SW) at the end of the test chamber, and two of the three patterns could be presented at the same time. After 4-min acclimation in the waiting chamber, the cricket was allowed to enter the test chamber and to visit visual patterns. Two minutes later, the relative positions of the patterns were changed by sliding the wall, and the choices of the cricket are noted during the next 2 min. Modified from Matsumoto and Mizunami (2002a) and Unoki et al. (2006).

ROLES OF AMINERGIC NEURONS IN FORMATION OF VISUAL PATTERN MEMORY AND COLOR MEMORY

We next studied the effect of OA and DA receptor antagonists on appetitive and aversive conditioning of visual pattern (Unoki et al., 2006) and color (Nakatani et al., 2009). Crickets injected with epinastine or mianserin, OA receptor antagonists, into the hemolymph exhibited a complete impairment of appetitive learning to associate a visual pattern with water reward, but aversive learning to associate a visual pattern with sodium chloride punishment was unaffected (**Figure 3A**). In contrast, fluphenazine, chlorpromazine, or spiperone, DA receptor antagonists, completely impaired aversive learning but not appetitive learning (**Figure 3B**). We also found the same for color learning: OA receptor antagonists impaired appetitive color learning with water reward without affecting aversive color learning with sodium chloride punishment. In contrast, DA receptor antagonists impaired aversive color learning without affecting appetitive color learning (Nakatani et al., 2009). These findings indicate that the roles of OA-ergic and DA-ergic neurons in conveying information about appetitive and aversive US, respectively, are ubiquitous in learning of odor, visual pattern and color stimuli, suggesting that these neurons serve as the general reward or punishment system for insect learning.

In mammals, midbrain DA-ergic neurons play major roles in a wide range of visual, auditory, and somatosensory stimuli and thus are considered to serve as a general reward system (Schultz, 1998, 2006). It appears that the roles of aminergic neurons in conveying reinforcement signals are conserved across different phyla, but the kind of reinforcement signal that each biogenic amine mediates is different: DA mediates appetitive reinforcement in mammals but mediates aversive reinforcement in insects. Future studies on the roles of DA in learning in phylogenetically ancient species may clarify how different roles of DA in positive or negative reinforcement

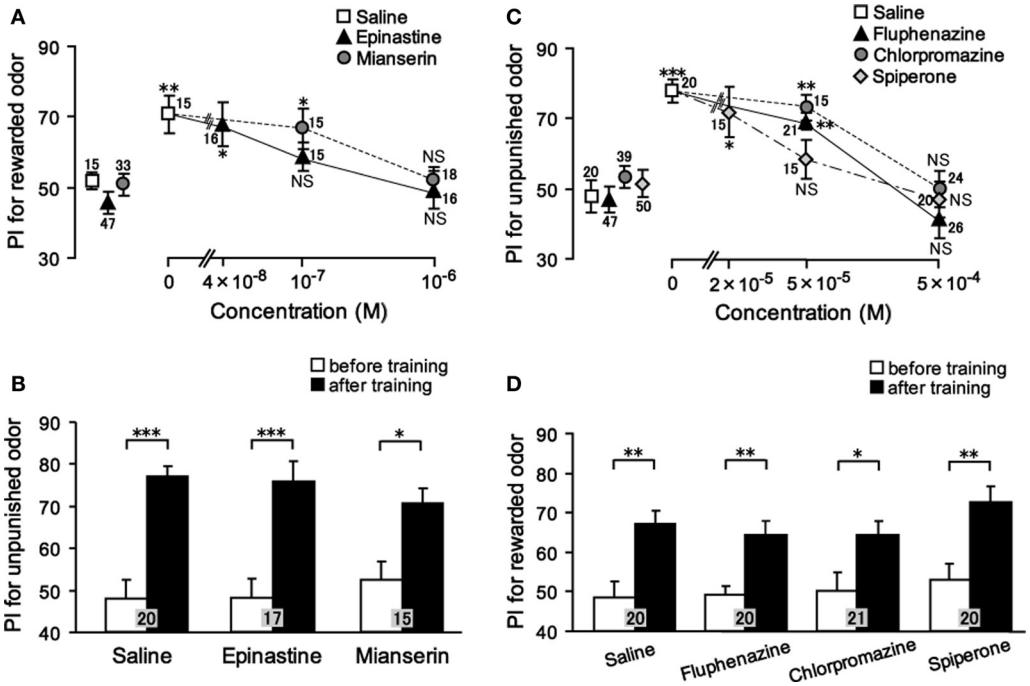

FIGURE 2 | Effects of OA or DA receptor antagonists on appetitive and aversive olfactory conditioning. (A) Dose-dependent effects of OA receptor antagonists on appetitive olfactory conditioning. Six groups of crickets were injected with 3 μl saline (white squares) or saline containing 0.04, 0.1, or 1 μM epinastine (black triangles) or 0.1 or 1 μM mianserin (gray circles). **(B)** Effects of OA receptor antagonists on aversive olfactory conditioning. Three groups of crickets were injected with 3 μl saline or saline containing 1 μM epinastine or 1 μM mianserin 30 min before 6-trial aversive conditioning. **(C)** Dose-dependent effects of DA receptor antagonists on aversive olfactory conditioning. Eight groups of crickets were injected with 3 μl saline (white squares) or saline containing 50 or 500 μM fluphenazine (black triangles), 50 or 500 μM chlorpromazine (gray circles) or 20, 50, or 500 μM spiperone (white diamonds). **(D)** Effects of DA receptor antagonists on appetitive olfactory conditioning. Four groups of crickets were injected with 3 μl saline or saline containing 500 μM fluphenazine, 500 μM chlorpromazine or 500 μM spiperone 30 min before 2-trial aversive conditioning. Relative odor preferences were measured as preference indexes for rewarded odor **(A,D)** or unpunished control odor **(B,C)** before (data points at the left in **A**, **C**; white bars in **B**, **D**) and at 30 min after conditioning (data points at the right in **A**, **C**; black bars in **B**, **D**) and are shown with mean ± SEM. The number of animals is shown at each data point. The results of statistical comparison before and after conditioning are shown as asterisks (Wilcoxon, WCX test, $p < 0.05$; $^{*}p < 0.01$; $^{*}p < 0.001$, NS $p > 0.05$). Modified from Unoki et al. (2005).

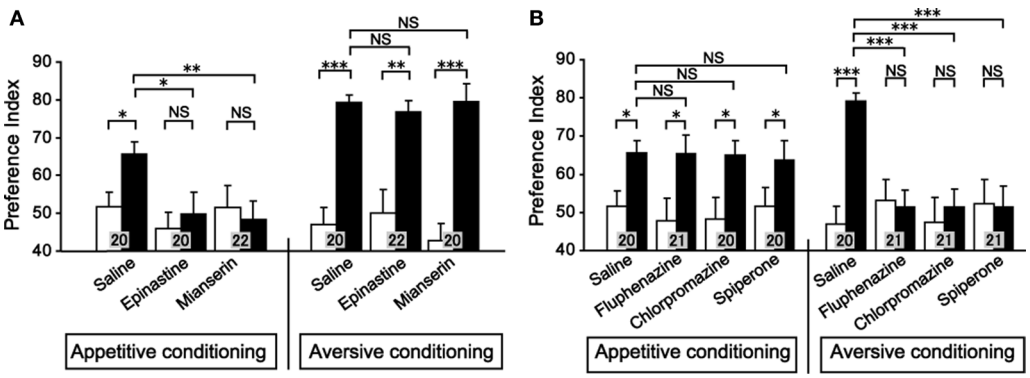

FIGURE 3 | Effects of OA or DA receptor antagonists on appetitive and aversive visual pattern conditioning. (A) Effects of OA receptor antagonists. Six groups of crickets were each injected with 3 μl saline or saline containing 1 μM epinastine or 1 μM mianserin at 30 min before 8-trial appetitive (left) or before 12-trial aversive conditioning (right). **(B)** Effects of DA receptor antagonists. Six groups of crickets were each injected with 3 μl saline containing 500 μM fluphenazine, 500 μM chlorpromazine, or 500 μM spiperone at 30 min before 8-trial appetitive (left) or before 12-trial aversive conditioning (right). Preference indexes for rewarded visual pattern (in the case of appetitive conditioning) and those of unpunished visual pattern (in the case of aversive conditioning) before (white bars) and at 30 min after conditioning (black bars) are shown with mean ± SEM. The number of animals is shown at each data point. The results of statistical comparison before and after conditioning (WCX test) and between experimental and saline-injected control groups (M–W test) are shown as asterisks ($p < 0.05$; $p < 0.01$; $p < 0.001$, NS $p > 0.05$). Modified from Unoki et al. (2006).

have emerged during the course of evolution. Neurotransmitters meditating negative reinforcement in mammals are less known, but roles of noradrenaline, serotonin, or DA in some forms of aversive learning have been suggested (Daw et al., 2002; Harley, 2004; Wise, 2004; Schultz, 2006).

DIFFERENT DYNAMICS OF APPETITIVE MEMORY AND AVERSIVE MEMORY

In the work described above, we noticed that the time courses of appetitive memory and aversive memory fundamentally differ (Unoki et al., 2005, 2006; Nakatani et al., 2009). In **Figure 4**, the time course of memory after 1-trial, 2-trial, and 6-trial appetitive olfactory conditioning and that after 2-trial and 6-trial aversive olfactory conditioning are shown. A group subjected to 1-trial appetitive conditioning exhibited a significant level of retention at 30-min after conditioning: the preference for rewarded odor was significantly greater than that before conditioning. The memory did not significantly decay from 30 min to 1 h after conditioning, but it was completely diminished at 1 day after conditioning. Two-trial and 6-trial groups exhibited a significant level of 30-min retention, with no significant decay of retention from 30 min to 1 day after conditioning. Two-trial aversive conditioning group exhibited a significant level of 30-min retention. However, the memory decayed to a non-significant level at 1 h after conditioning. Six-trial aversive conditioning group exhibited a significant decay of retention from 30 min to 1 h after training. Thereafter, a significant level of retention was maintained with no significant decay from 1 h to 1 day after conditioning. In short, aversive olfactory memory exhibited a prominent decay from 30 min to 1 h after conditioning, whereas appetitive olfactory memory exhibited little decay from 30 min to 1 day after conditioning.

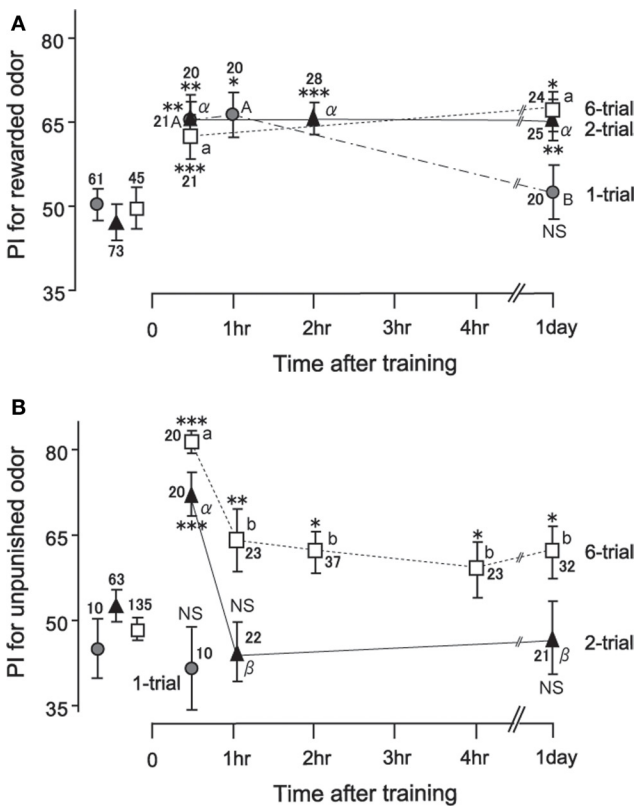

FIGURE 4 | Time course of memory retention after appetitive (A) and aversive **(B)** conditioning. Seventeen groups of crickets were subjected to 1-trial (gray circles), 2-trial (black triangles), or 6-trial (open squares) appetitive or aversive conditioning trials with an ITI of 5 min. Preference indexes (PIs) for rewarded odor **(A)** or those for unpunished control odor **(B)** before (data points at the left) and at various times after conditioning are shown with mean ± SEM. The number of animals is shown at each data point. The results of statistical comparisons before and after conditioning are shown as asterisks (WCX test; $p < 0.05$; $p < 0.01$; $p < 0.001$; NS $p > 0.05$) and those at different times after conditioning are shown as letters (M–W test, different letter indicating at least $p < 0.05$). Modified from Unoki et al. (2005), with data on one-trial aversive conditioning provided by A. Hatano.

We found similar distinction of dynamics between appetitive memory and aversive memory for visual pattern conditioning (Unoki et al., 2006) and color conditioning (Nakatani et al., 2009) in crickets. It was obvious that the number of conditioning trials, and hence the levels of initial acquisition, also influenced memory dynamics, but effects of these factors did not account for the difference in memory dynamics observed after appetitive and aversive learning: reward memory was sustained even when the level of 30-min retention was low, and punishment memory exhibited a characteristic decay even when the level of 30-min retention was high.

Comparisons with studies in other species of insects showed that our finding that aversive memory is less durable than appetitive memory is not specific to the type of US we used (water as appetitive US and sodium chloride as aversive US) or the species used (crickets). In fruit-flies, it has been reported that punishment memory after conditioning of an odor with electric shock punishment decays much faster than reward memory after conditioning of an odor with sucrose reward, regardless of intensity of electric shock and the concentration of the sucrose solution (Tempel et al., 1983). A recent study also suggested that aversive olfactory memory with quinine or saline punishment is less durable than appetitive olfactory memory with sucrose reward in fruit-fly larvae (Honjo and Furukubo-Tokunaga, 2009). Thus, dynamics of punishment memory and reward memory differ for different intensities or kinds of unconditioned stimulus (US; water or sugar as reward and saline, quinine, or electric shock as punishment), for different species of insects (crickets and fruit-flies), and for different conditioning paradigms (individual conditioning in crickets and group conditioning in fruit-flies).

By examining the literature in human psychology, we have proposed that these findings in insects are comparable to findings in humans (Nakatani et al., 2009). Many studies in educational psychology have proposed that punishment is highly effective for immediately suppressing behavior of children at school or home, but the effect tends to be short-lived compared to the effect of reward (Peine and Howarth, 1975; Gershoff, 2002; Driscoll, 2005). However, to our knowledge, no convincing evidence supporting the argument that the dynamics of reward and punishment memory fundamentally differ has been obtained in rigorously controlled animal experiments (Walters and Crusec, 1977), except for several studies on insects discussed above. Obviously, our proposal that different dynamics after punishment and reward learning is conserved across phyla is highly speculative, and more studies on vertebrates are clearly needed to evaluate the validity of this idea.

If different dynamics of punishment and reward memory is conserved across different phyla, what is the possible adaptive significance for it? We have proposed that it is related to a different significance of reward and punishment leaning for survival in a changing environment (Nakatani et al., 2009). Since the environment is constantly changing, stimuli that once served as predictors of punishment may change to predict reward or vice versa. Consider that an inedible food item in one season may become profitable in the next season. In this case, long-term retention of avoidance of stimuli that once predicted aversive stimuli is not necessarily beneficial, because it reduces the opportunity to obtain useful resources in the future. Stimuli that once predicted reward may also be changed to predict punishment, but long-term retention of preference for once-rewarded stimuli has no such cost, because animals can re-learn to avoid such stimuli when they encounter the stimuli again. Thus, we have proposed that different adaptive significance of durability of memory between reward and punishment learning is the basis of different dynamics of reward and punishment memories (Nakatani et al., 2009).

PARTICIPATION OF OCTOPAMINERGIC AND DOPAMINERGIC NEURONS IN APPETITIVE AND AVERSIVE MEMORY RECALL

We then studied the effect of an OA or DA receptor antagonist on appetitive or aversive memory recall (retrieval). The results suggested that intact OA-ergic or DA-ergic signaling is necessary for recall of appetitive or aversive memory, respectively, after olfactory learning and visual pattern learning (Mizunami et al., 2009). Crickets were subjected to appetitive or aversive olfactory conditioning and were injected with an OA or DA receptor antagonist before retention test. Injection of epinastine or mianserin, OA receptor antagonists, completely impaired appetitive olfactory memory recall but had no effect on aversive olfactory memory recall (**Figure 5A**). On the other hand, injection of fluphenazine, chlorpromazine, or spiperone, DA receptor antagonists, completely impaired aversive memory recall but had no effect on appetitive memory recall (**Figure 5B**). This is in accordance with observations in honey bees that disruption of OA-ergic transmission in the antennal lobe, the primary olfactory center, by an OA receptor antagonist (mianserin) or by RNA interference of the OA receptor gene disrupted appetitive olfactory memory recall (Farooqui et al., 2003), although the results of the study by Farooqui et al. were not conclusive as we have discussed before (Mizunami et al., 2009). We also found that OA and DA receptor antagonists impaired appetitive and aversive memory recall, respectively, in visual pattern conditioning (Mizunami et al., 2009). Therefore, we concluded that intact synaptic transmission from OA- and DA-ergic neurons is needed for the recall of appetitive memory and aversive memory, respectively, in both olfactory and visual pattern learning.

We noticed that our findings are not consistent with conventional neural models of insect classical conditioning. **Figure 6A** depicts perhaps the best model proposed to account for the roles of extrinsic and intrinsic neurons of mushroom bodies in olfactory conditioning in the fruit-fly *Drosophila* (Schwaerzel et al., 2003). This model assumes that (1) "CS" neurons (intrinsic neurons of the mushroom body, called Kenyon cells) that convey signals about a CS make synaptic connections with dendrites of "CR" neurons (efferent (output) neurons of the mushroom body lobe), activation of which leads to a CR (conditioned response)

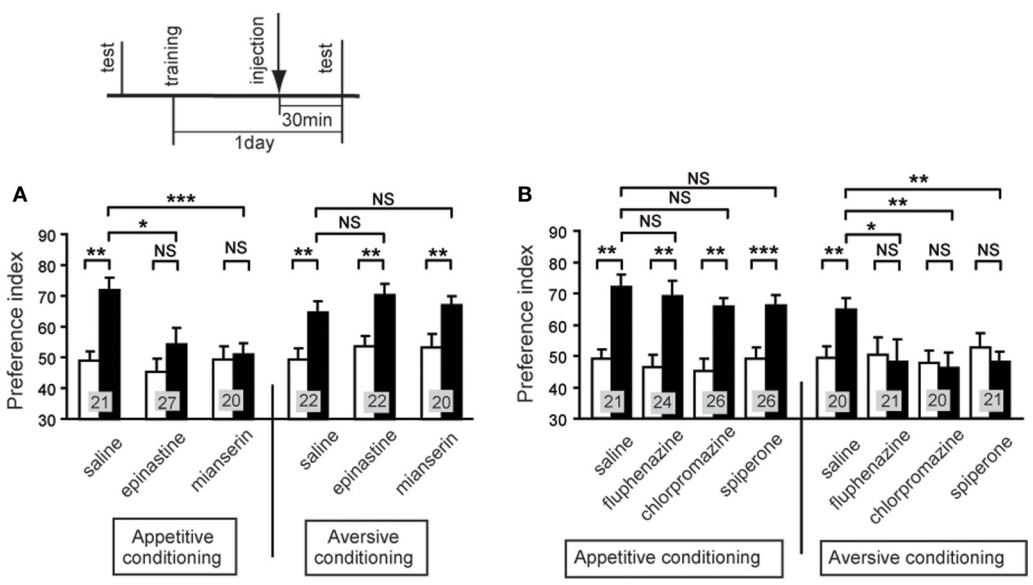

FIGURE 5 | Octopamine and dopamine receptor antagonists impair appetitive and aversive olfactory memory recall, respectively. Effects of OA **(A)** or DA **(B)** receptor antagonists on olfactory memory recall. Twelve groups of crickets were each subjected to 2-trial appetitive (left) or 6-trial aversive (right) olfactory conditioning trials. On the next day, each group was injected with 3 μl of saline or saline containing 1 μM epinastine, 1 μM mianserin, 500 μM fluphenazine, 500 μM chlorpromazine or 500 μM spiperone at 30 min before the final test (upper diagram). Preference indexes for rewarded odor (in the case of appetitive conditioning) or unpunished control odor (in the case of aversive conditioning) before (white bars) and 1 day after (black bars) conditioning are shown with mean + SEM. The number of crickets is shown at each data point. The results of statistical comparison before and after conditioning (WCX test) and between experimental and saline-injected control groups (M–W test) are shown as asterisks ($p < 0.05$; $p < 0.01$; $p < 0.001$, NS $p > 0.05$). Modified from Mizunami et al. (2009).

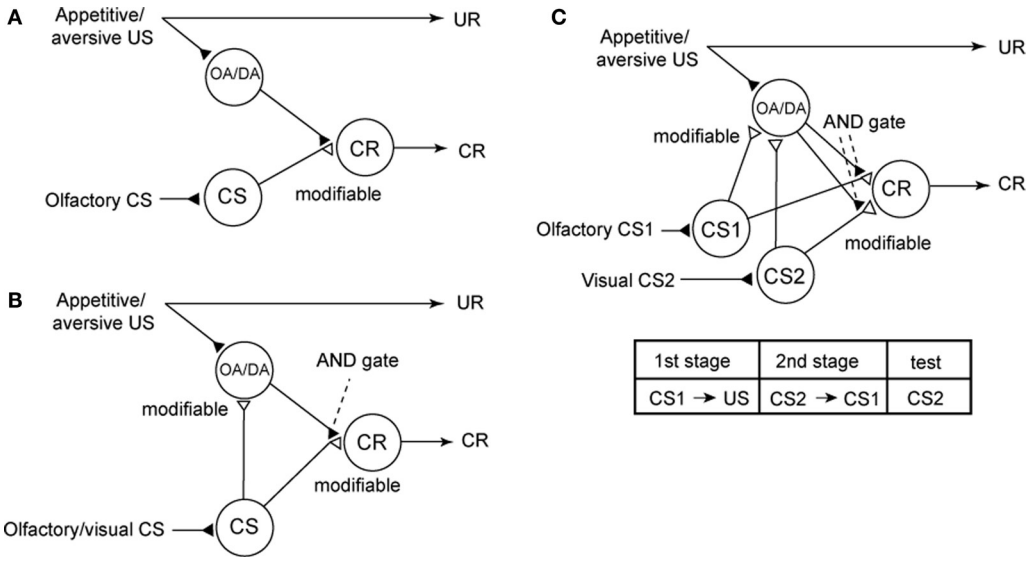

FIGURE 6 | Conventional and new models of classical conditioning in insects. (A) A model proposed to account for the roles of intrinsic and extrinsic neurons of the mushroom body in olfactory conditioning in fruit-flies (Schwaerzel et al., 2003). OA-ergic or DA-ergic neurons ("OA/DA" neurons) convey signals for appetitive or aversive US, respectively. "CS" neurons, which convey signals for CS, make synaptic connections with "CR" neurons that induce the conditioned response (CR), the efficacy of the connection being strengthened by conditioning. "OA/DA" neurons make synaptic connections with axon terminals of "CS" neurons. **(B)** A new model of classical conditioning, termed Mizunami–Unoki model. The model assumes that efficacy of synaptic transmission from "CS" neurons to "OA/DA" neurons is strengthened by conditioning and that coincident activation of "OA/DA" neurons and "CS" neurons is needed to activate "CR" neurons to lead to a CR (AND gate). **(C)** Mizunami–Unoki model to account for second-order conditioning, in which an odor (CS1) is paired with water or sodium chloride solution and a visual pattern (CS2) is paired with the odor (CS1), as indicated in the inset. The model predicts that pairing of CS1 and US at the first conditioning stage results in enhancement of synapses from "CS1" neurons to "OA/DA" neurons, and activation of the synapses (by CS1) at the second conditioning stage leads to simultaneous activation of "OA/DA" and "CS2" neurons, and this leads to enhancement of synaptic transmission from "CS2" neurons to "OA/DA" neurons and to "CR" neurons. Modified from Mizunami et al. (2009).

that mimics UR (unconditioned response), but these synaptic connections are silent or very weak before conditioning, (2) OA- or DA-ergic efferent neurons projecting to the lobes ("OA/DA" neurons), which convey signals for appetitive or aversive US, respectively, make synaptic connections with axon terminals of "CS" neurons, and (3) the efficacy of the synaptic transmission from "CS" neurons to "CR" neurons that induces a conditioned response (CS–CR or S–R connection) is strengthened by coincident activation of "CS" neurons and "OA/DA" neurons during conditioning (assuming Kandelian synaptic plasticity; see Abrams and Kandel, 1988). In short, this model assumes that presentation of a CS after conditioning activates the CS–CR or S–R connection to induce a CR. Thus, this model is characterized as an S–R model (**Figure 7A**), following terminology in studies on classical conditioning in higher vertebrates (Rescorla, 1988; Pickens and Holland, 2004; Holland, 2008). It can be pointed out that the S–R model accounts for most forms of classical conditioning in invertebrates, including classical conditioning of gill withdrawal reflex in the mollusk *Aplysia,* where pairing of a gentle tactile stimulus to the siphon (CS) and a strong tactile stimulus to the gill (US) results in an enhancement of the efficacy of synaptic transmission from siphon sensory neuron to gill motor neuron (Abrams and Kandel, 1988; Kandel, 2001; Roberts and Glanzman, 2003), which is characterized as an S–R connection. The model by Schwaerzel et al. (2003), however, is inconsistent with our findings because it predicts that activation of OA- or DA-ergic neurons is not required for appetitive or aversive memory recall, respectively.

We have proposed a new model (**Figure 6B**), with minimal modifications of the model proposed by Schwaerzel et al. (2003). We have assumed that (1) activation of "OA/DA" neurons and resulting release of OA or DA are needed to "gate" the sensori-motor pathway from the "CS" neurons to "CR" neurons after conditioning and (2) synaptic connection from "CS" neurons to "OA/DA" neurons representing US is strengthened by coincident activation of "CS" neurons and "OA/DA" neurons by pairing of a CS with a US (assuming Hebbian synaptic plasticity). The latter connection is termed a CS–US or S–S connection (**Figure 7B**; Rescorla, 1988; Pickens and Holland, 2004), In short, our model assumes that two kinds of memory traces are formed by conditioning and that activation of both memory traces is needed for memory recall. This model corresponds to a hybrid of the S–R and S–S models (for explanation of the S–S model, see legends of **Figure 7**).

An alternative possibility to explain our findings is that different sets of "OA/DA" neurons govern reinforcement and memory retrieval processes, respectively. This is achieved by modifying the model shown in **Figure 6A** by assuming other "OA/DA" neurons in neural pathways downstream of the "CR" neurons. This model, however, failed to account for our results with second-order conditioning described below.

EVALUATION OF OUR MODEL BY USING A SECOND-ORDER CONDITIONING PROCEDURE

The critical assumption of our model is that the pathway from neurons representing CS to OA/DA neurons representing appetitive or aversive US (S–S connection) is strengthened by conditioning. We evaluated

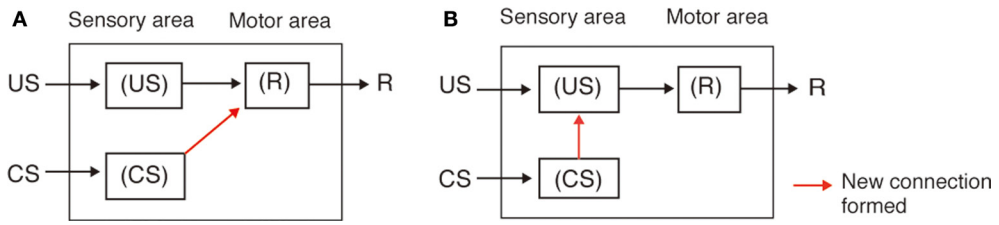

FIGURE 7 | S–R and S–S theories to account of classical conditioning. Two theories, i.e., the stimulus–response (S–R) association theory and the stimulus–stimulus (S–S) association theory, have been proposed to account for classical conditioning in higher vertebrates including humans (Rescorla, 1988). In the S–R theory **(A)**, classical conditioning is viewed as the strengthening of a new reflex pathway for the CS to evoke a conditioned response (CR) (i.e., a pathway from neurons that code for the CS to neurons whose activities lead to behavioral response), as a result of pairing of the CS with a US (Rescorla, 1988; Pickens and Holland, 2004; Holland, 2008). According to this view, an initially insignificant event, CS, is incorporated into the reflex system under the control of a more biologically significant stimulus, US, whenever those two events occur in close temporal contiguity. This view accounts for some forms of classical conditioning in higher vertebrates (Rescorla, 1988; Pickens and Holland, 2004). Many other forms of classical conditioning in higher vertebrates, however, have been suggested to involve the strengthening of S–S connection, i.e., connection from neurons representing CS to those representing US **(B)**. According to this view (S–S theory), an association is formed between internal representation of the CS and that of the US are strengthened (i.e., a connection from neurons that code for the CS to neurons that code for the US is strengthened), and the growth of this association permits the CS to activate a representation of the US in the absence of the US itself. This anticipatory activation of the US representation produces the CR. This view is referred to as *cognitive account* of classical conditioning, since it assumes the formation of internal representation of the relationship between external sensory events (i.e., contingent occurrence of the CS and US) (Rescorla, 1988; Pickens and Holland, 2004; Holland, 2008). Notice that these theories address the question of what kinds of connections are strengthened by learning, not the question of how or by which mechanism such connections are formed.

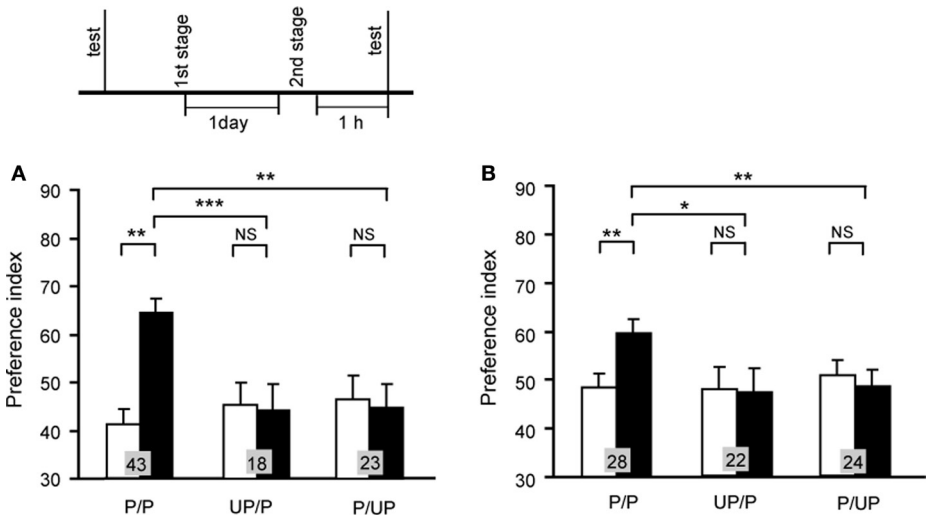

FIGURE 8 | Appetitive (A) and aversive **(B)** second-order conditioning. Two groups of animals were each subjected to appetitive **(A)** or aversive **(B)** second-order conditioning trials (P/P groups). Four control groups were each subjected to unpaired presentations in the first (UP/P groups) or second (P/UP groups) stage in appetitive **(A)** or aversive **(B)** second-order conditioning. Animals received 4 first-stage trials and then 4 second-stage trials for appetitive second-order conditioning and 6 first-stage trials and then 4 second-stage trials for aversive second-order conditioning. Preference indexes for the CS2 (in the case of appetitive second-order conditioning) or control pattern (in the case of aversive second-order conditioning) before (white bars) and after (black bars) conditioning are shown with mean + SEM. The results of statistical comparison before and after conditioning (WCX test) and between experimental and saline-injected control groups (M–W test) are shown as asterisks ($p < 0.05$; $p < 0.01$; $p < 0.001$, NS $p > 0.05$). Modified from Mizunami et al. (2009).

this assumption by using a second-order conditioning procedure (Mizunami et al., 2009). Second-order conditioning (**Figure 6C**) is a procedure for testing whether a CS can acquire the reinforcing property of a US, by pairing a CS (CS1) with a US and then pairing another CS (CS2) with CS1 (Rescorla, 1988). Our model predicts that blockade of OA or DA receptors (on axon terminals of "CS" neurons) during the initial CS1–US pairing stage does not impair the enhancement of synapses from "CS" neurons to "OA/DA" neurons. This is because blockade of OA or DA receptors should not affect normal activities of "CS" neurons and "OA/DA" neurons (**Figure 6C**). Hence, CS1 should act as a reinforcer in the second CS1–CS2 pairing stage, although CS1 *per se* does not induce a CR (notice that impairments of first-order conditioning by DA/DA receptor antagonists are shown in **Figures 2 and 3**). Therefore, our model predicts that blockade of OA or DA receptors during the first CS1-US pairing stage does not impair second-order conditioning, but the same treatment during the second CS2–CS1 pairing stage or during the final retention test impairs second-order conditioning.

We first studied whether second-order conditioning can be achieved in crickets (**Figure 8**; Mizunami et al., 2009). We used an olfactory stimulus as CS1 and a visual pattern as CS2. For appetitive

or aversive conditioning, an odor (CS1) was paired with water or sodium chloride solution, respectively, and then a visual pattern (CS2) was paired with an odor (CS1). A group of animals that was subjected to appetitive second-order conditioning trials exhibited significantly increased preference for the CS2 (**Figure 8A**). In contrast, control groups that were each subjected to unpaired presentations of stimuli at the first or second conditioning stage exhibited no significantly increased preference for the CS2 (**Figure 8A**), thus indicating that the increased preference for the CS2 in the experimental group is truly the result of second-order conditioning. Similarly, we showed that second-order aversive conditioning could be achieved (**Figure 8B**).

We then studied the effect of OA or DA receptor antagonist on appetitive or aversive second-order conditioning (**Figure 9**). A group of animals injected with epinastine before the first conditioning stage of appetitive second-order conditioning exhibited a significantly increased preference for the CS2, thus indicating that blockade of OA receptor during the first stage does not impair appetitive second-order conditioning (**Figure 9A**). In contrast, the group injected with epinastine before the second stage (**Figure 9B**) or before final test (**Figure 9C**) for appetitive second-order conditioning exhibited no significantly increased preference for the CS2, indicating a complete impairment of second-order conditioning. Similarly, blockade of DA receptor antagonist at the first stage did

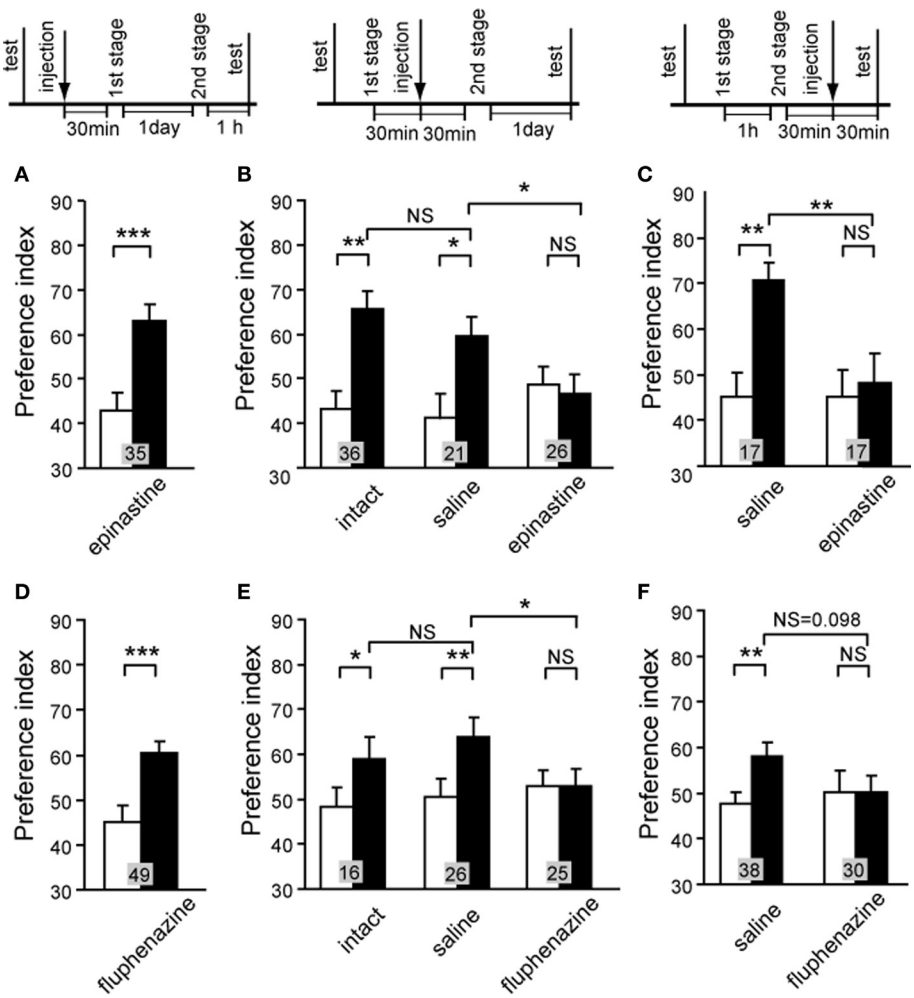

FIGURE 9 | Octopamine and dopamine receptor antagonists impair appetitive and aversive second-order conditioning. (A–C) Three groups of animals were each injected with 3 μl of saline containing 1 μM epinastine at 30 min before the first conditioning stage **(A)**, before the second conditioning stage **(B)** or before the final test **(C)** in appetitive second-order conditioning. One control group received no injection (**B**, intact), and two other groups were each injected with saline at 30 min before the second conditioning stage (**B**, saline) or before the final test (**C**, saline). **(D–F)** Three groups were each injected with 3 μl of saline containing 500 μM fluphenazine at 30 min before the first conditioning stage **(D)**, before the second conditioning stage **(E)** or before the final test **(F)** in aversive second-order conditioning. Animals received 4 first-stage trials and then 4 second-stage trials for appetitive second-order conditioning and 6 first-stage trials and then 4 second-stage trials for aversive second-order conditioning. One control group received no injection (**E**, intact), and two other groups were each injected with saline at 30 min before the second conditioning stage (**E**, saline) or before the final test (**F**, saline). Preference indexes for the CS2 (in the case of appetitive second-order conditioning) or the control pattern (in the case of aversive second-order conditioning) before (white bars) and after (black bars) conditioning are shown with mean + SEM. The results of statistical comparison before and after conditioning (WCX test) and between experimental and saline-injected control groups (M–W test) are shown as asterisks ($p < 0.05$; $p < 0.01$; $p < 0.001$, NS $p > 0.05$). Modified from Mizunami et al. (2009).

not impair aversive second-order conditioning, but that at the second stage or final test impaired aversive second-order conditioning (**Figures 9D–F**).

The results were in full accordance with our model. In addition, the results were inconsistent with an alternative model in which different sets of OA/DA neurons participate in reinforcement and memory recall, because the model predicts that blockade of OA/DA receptors at any stage of conditioning impairs second-order conditioning.

Our finding that OA-ergic signaling and DA-ergic signaling are needed for appetitive and aversive memory recall, respectively, is in accordance with some previous findings in honey bees and fruit-flies but not with other findings, as we have discussed elsewhere (Mizunami et al., 2009). Thus, we proposed that activations of OA- or DA-ergic neurons are needed for memory recall in some forms of classical conditioning in insects but not in other forms (Mizunami et al., 2009). The critical factors for determining the requirement of OA- or DA-ergic signaling in appetitive/aversive memory recall remain to be clarified.

FUTURE PERSPECTIVES

Many questions concerning the roles of OA- or DA-ergic neurons in insect learning remain to be addressed. Firstly, although there has been a substantial progress in identifying DA- and OA-ergic neurons involved in appetitive and aversive learning in honey bees (Hammer, 1993) and fruit-flies (Schroll et al., 2006; Claridge-Chang et al., 2009), more studies need to be performed to clarify the morphology and physiology of DA or OA-ergic neurons participating in insect learning. In crickets, it has been suggested that the mushroom bodies are involved in olfactory learning (in *Acheta domesticus*: Scotto-Lomassese et al., 2003), and thus OA- and DA-ergic neurons projecting to the mushroom bodies need to be anatomically and physiologically characterized. Detailed immunohistochemical studies for OA and DA will provide the first step for this. Secondly, we have proposed that different dynamics of appetitive and aversive memory are the result of different dynamics of biochemical processes after activation of OA and DA receptors, respectively (Nakatani et al., 2009). To examine this hypothesis, physiology, and biochemistry of neurons participating in association of CS and US, most probably Kenyon cells of the mushroom bodies, need to be characterized. Thirdly, our successful demonstration of cross-modal second-order conditioning suggests that OA- or DA-ergic neurons participating in olfactory learning also participate in visual pattern learning (Mizunami et al., 2009), and this suggestion needs to be examined by electrophysiologically characterizing responses of OA- and DA-ergic neurons during olfactory learning and visual learning. Fourthly, mammalian midbrain DA-ergic neurons have been suggested to convey prediction error for rewarding outcomes, namely, they encode the error between expected and actual rewarding outcomes (Schultz, 1998, 2006), and it is important to clarify whether or not OA-ergic (resp. DA-ergic) neurons in insects work in the same way or not in appetitive (resp. aversive) learning, respectively. Further examination of these questions should contribute to clarification of phylogenetically conserved principles of brain systems underlying learning and memory among vertebrates and invertebrates, as well as each specific functional specialization in each group.

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Histone H3 acetylation is asymmetrically induced upon learning in identified neurons of the food aversion network in the mollusk *Helix lucorum*

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Regulation of gene expression is an essential step during long-term memory formation. Recently, the involvement of DNA-binding transcription factors and chromatin remodeling in synaptic plasticity have been intensively studied. The process of learning was shown to be associated with chromatin remodeling through histone modifications such as acetylation and phosphorylation. We have previously shown that the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) regulatory cascade plays a key role in the food aversion conditioning in the mollusk *Helix*. Specifically, command neurons of withdrawal behavior exhibit a learning-dependent asymmetry (left–right) in MAPK/ERK activation. Here, we expanded our molecular studies by focusing on a potential MAPK/ERK target – histone H3. We studied whether there is a learning-induced MAPK/ERK-dependent acetylation of histone H3 in command neurons RPa(2/3) and LPa(2/3) of the right and left parietal ganglia and whether it is asymmetrical. We found a significant learning-dependent increase in histone H3 acetylation in RPa(2/3) neurons but not in LPa(2/3) neurons. Such an increase in right command neurons depended on MAPK/ERK activation and correlated with a lateralized avoidance movement to the right visible 48 h after training. The molecular changes found in a selective set of neurons could thus represent a lateralized memory process, which may lead to consistent turning in one direction when avoiding a food that has been paired with an aversive stimulus.

Keywords: learning, epigenetics, histone H3 acetylation, MAPK/ERK, neuronal networks, lateralization, *Helix*, chromatin remodeling

INTRODUCTION

Long-term memory formation requires gene expression regulation, which occurs through the chromatin remodeling and regulation of DNA-binding transcription factors (TFs; Reul and Chandramohan, 2007). Histone modifications such as acetylation, phosphorylation, and DNA methylation lead to chromatin remodeling upon learning (Wood et al., 2006; Sweatt, 2009).

Histone acetylation is associated with activation of transcription (Peterson and Laniel, 2004). The amount of histone acetylation is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Importantly, defects in long-term memory dependent on acetylation are compensated by injection of HDAC inhibitors (Alarson et al., 2004; Korzus et al., 2004; Wood et al., 2006; Fischer et al., 2007; Abel and Zukin, 2008).

Prior investigations have demonstrated that histone phosphorylation, followed by acetylation, may be induced via the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase)-dependent pathway (Levenson et al., 2004; Chwang et al., 2006; Sweatt, 2009) during long-term memory formation. This regulatory cascade has been intensively studied in the last decade. The MAPK/ERK pathway plays a fundamental role in adaptive processes both in vertebrates and invertebrates. Its activation pattern determines cellular survival or apoptosis, effectiveness of pre-existing synapses or growth of new synaptic connections (Kaplan and Miller, 2000; Thomas and Huganir, 2004). It is also an essential step during long-term memory formation (Martin et al., 1997; Atkins et al., 1998; Crow et al., 2001; Sananbenesi et al., 2003; Sharma and Carew, 2004; Feld et al., 2005; Ribeiro et al., 2005).

Mollusks have played a key role in these studies due to the relative simplicity of their central nervous system (CNS) and their stereotyped behavior, which exhibits nevertheless different levels of plasticity (Kandel, 2001). For many years we have been using the terrestrial mollusk *Helix lucorum* and its food aversion conditional reflex to investigate long-term memory formation (Grinkevich, 1994; Grinkevich and Vasil'ev, 2000; Grinkevich et al., 2003, 2007, 2008). Several forms of conditioned avoidance reflex have been reported for this snail (Stepanov et al., 1988; Grinkevich and Vasil'ev, 2000; Balaban, 2002). In one paradigm this mollusk can be trained to avoid a piece of food (the conditioned stimulus, CS; e.g., carrot) if it is appropriately paired with an electric shock (the unconditioned stimulus, US). Neuronal networks underlying feeding behavior and withdrawal in *Helix* have been determined and neural correlates of withdrawal behavior have been described in detail (Balaban, 2002).

Abbreviations: Ac-H3-histone, acetylated-H3-histone; DMSO, dimethyl sulfoxide; HATs, histone acetyltransferases; HDACs, histone deacetylases; MAPK/ERK, mitogen-activated protein kinase/extracellular signal-regulated kinase; NaB, sodium butyrate; PD98059, upstream ERK kinase MEK1 inhibitor; TFs, transcription factors.

We have previously demonstrated that MAPK/ERK, as well as its downstream targets, such as TFs controlling gene expression via CRE, SRE, and AP-1 elements, are involved in the regulation of food aversion learning in adult *Helix*. Moreover MAPK/ERK activation is serotonin-dependent (Grinkevich and Vasil'ev, 2000; Grinkevich et al., 2003, 2007, 2008). In contrast to adults, juvenile *Helix* snails, which possess immature mechanisms of sensitization and undeveloped conditioned avoidance responses, do not exhibit MAPK/ERK activation in the CNS after training (Grinkevich et al., 2008).These snails differ from the adults in the spectrum of TFs that bind to regulatory elements SRE and AP-1 (Grinkevich and Vasil'ev, 2000; Grinkevich et al., 2003). In addition, we demonstrated that a significant MAPK/ERK-dependent increase in histone H3 acetylation occurs in adult animals after learning, whereas no increase in histone H3 acetylation was observed in juveniles. The injection of sodium butyrate, an inhibitor of HDAC, prior to training led to induction in histone H3 acetylation and significantly ameliorated long-term memory formation in juvenile snails.

Recently, we have studied molecular processes underlying learning in command neurons RPa(2/3) and LPa(2/3) controlling withdrawal behavior of adult snails. Such neurons constitute the plastic link of food aversion reflex and might be responsible for unilateral right [RPa(2/3)] or left [LPa(2/3)] turning when withdrawal or escape responses are initiated. Balaban (1979) reported that RPa(2/3) and LPa(2/3) neurons are responsible for producing contractions of ipsilateral body walls so that they may not be involved in the production of bilateral movements of the foot, which are mediated by ipsilateral populations of motor neurons. We focused on left and right command neurons and showed that serotonin-dependent MAPK/ERK activation is involved in the formation of the withdrawal reflex; moreover we found that following learning, there is an asymmetry of MAPK/ERK activation in the left and right command neurons, which could result in the lateralization of molecular memory processes (Kharchenko et al., 2010). Specifically, we found that after food aversion learning phospho-ERK levels increased significantly in RPa(2/3) command neurons but no increase was found in LPa(2/3) command neurons. We concluded that learning involves synchronous and asymmetric serotonin-dependent MAPK/ERK activation and that such an asymmetry may reflect lateralization of memory processes in the mollusk brain. Here we expanded our molecular analyses of command neurons in the framework of food aversion learning in *Helix*, and focused on histone H3 acetylation, a process so far unexplored in this experimental context. We aimed at understanding whether histone H3 acetylation is induced in RPa(2/3) and LPa(2/3) command neurons after learning and whether it is MAPK/ERK-dependent. We analyzed if, consistently with MAPK/ERK activation observed in our previous work (Kharchenko et al., 2010), learning-dependent induction of histone H3 acetylation is also asymmetrical between the left and right command neurons. Our results show that food aversion learning in *Helix* induces a significant learning-dependent increase in histone H3 acetylation in command neurons of the right parietal ganglion RPa(2/3) but not in the symmetrical command neurons of the left parietal ganglion LPa(2/3). Moreover histone H3 acetylation in command neurons RPa(2/3) was MAPK/ERK-dependent. We suggest that these unilateral molecular changes in command neurons during learning lead to consistent turning in one direction when avoiding a food stimulus that has been paired with an aversive stimulus.

MATERIALS AND METHODS

CONDITIONED REFLEX FORMATION

Experiments were carried out on adult (20–25 g) snails *H. lucorum*. Animals were trained to associate a piece of carrot as the CS with an electric shock as the US. Conditioned food aversion is established in this protocol, following the procedure established by Balaban (2002). Specifically, a piece of carrot was placed at a distance of 1 cm from the head of a snail freely moving on a metal plate (serving as one of stimulating electrodes). When the snail began to eat the carrot, another stimulating electrode was manually placed on the snail's head, and an electric shock (DC, 5 mA, 0.5 s) was applied. Food and the shock US were presented to the midline. If the snail did not contact the carrot during 2 min, a piece of carrot was placed close to its mouth, and the electric shock was applied. Thus, all trained snails received equal amount of CS and US stimulation. The training procedure consisted of eight CS–US pairings applied at 15 min interval (four treatments per day). Animals were deprived of food during 3 days before the experiments. Naive animals were used as control group.

CENTRAL NERVOUS SYSTEM

Prior to the isolation of the CNS, animals were anesthetized with ice-cold saline supplemented by the injection of isotonic solution of $MgCl_2$. In the case of animals that were previously trained, the subesophageal complex of ganglia was quickly removed from the head 10 min after training and placed into a camera containing saline solution (80 mM NaCl; 4 mM KCl; 7 mM $CaCl_2$; 5 mM $MgCl_2$; 5 mM TRIS–HCl; pH = 7,8). In order to quantify H3 histone in specific subsets of neurons, the ganglia were delicately opened under microscope using cutters and tweezers. Identified neurons or groups of neurons were then quickly dissected and suctioned into a pipette, and transferred to the extraction buffer. Command neurons RPa2 and RPa3 or LPa2 and LPa3 from three individual animals were combined for analysis. All procedures were performed at 4°C.

DRUGS AND INJECTION PROCEDURE

The MEK1 inhibitor PD98059 (Cell Signaling) was freshly dissolved in dimethyl sulfoxide (DMSO) at the concentration of 20 mM. Then 6 μl of PD98059 or vehicle were injected into the *cephalopedal sinus* 30 min prior to conditioning. The total volume of adult *Helix* hemolymph was estimated at 3 ml resulting in an approximate 500-fold dilution of the drug in hemolymph and a final concentration of PD98059 in hemolymph of around 40 μM.

HISTONE EXTRACTION AND IMMUNOBLOTTING

To identify histone acetylation status, CNS were homogenized in extraction buffer: 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM, 0.2 mM PMSF, 1% protease inhibitor cocktail (Sigma), 0.1 mM Na_3VO_4, and 1% Igepal CA-630. Histones were extracted according to Levenson et al. (2004). All procedures were performed on ice. Tissue homogenates were centrifuged at 7,700×*g* for 5 min

(4°C). The pellet was resuspended in 1 ml of 0.4 N H_2SO_4 (30 min histone extraction) and was centrifuged at 14,000×*g* for 10 min (4°C). The supernatant was transferred to a fresh tube, and proteins were precipitated with the addition of 250 μl of 100% trichloroacetic acid containing 4 mg/ml deoxycholic acid (Na_+ salt, Helicon) for 30 min and then centrifuged at 14,000×*g* for 30 min (4°C). The supernatant was discarded, and the protein pellet was washed with 1 ml of acidified acetone (0.1% HCl) followed by 1 ml of acetone for 5 min each. Protein precipitates were collected by centrifugation (14,000×*g*, 5 min, 4°C) and were then resuspended in 10 mM Tris (pH 8) and stored at −80°C. Protein concentration was measured by Bradford assay. Samples were boiled with loading buffer and equal amount of protein was loaded into the 14% SDS-PAGE. Protein markers were from Fermentas (Lithuania). Separated proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell). Ponceau S staining was used to check transfer quality. Membranes were incubated in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 5% non-fat dry milk for 1 h at 4°C to block non-specific binding. Following this blots were incubated with primary Acetylated-H3-Histone antibodies (4°C overnight) and with secondary antibodies conjugated with HRP (horseradish peroxidase) for 1 h. Immunolabeling was detected by enhanced chemoluminescence using ECL system (standard protocol and components from Amersham Pharmacia Biotech). Subsequently, blots were stripped (glycine-HCl, pH 2.8, two times for 20 min each at 55°C), saturated 1 h in 5% non-fat dry milk and incubated with antibodies against total form of histone H3. After exposure of membranes, films were scanned and amount of protein was quantified using Gel Pro Anal computer program.

The amount of acetylated histone H3 was normalized to total histone H3 whose level remains stable with respect of learning. To visualize H3-histone acetylation polyclonal antibodies against Acetylated Lysine 14-H3-histone (Upstate Biotechnology, Millipore Corporation) were used. Polyclonal antibodies against total histone H3 (Upstate Biotechnology, Millipore Corporation) were used for analysis of H3 content. Antibodies against Acetylated-H3-histone and total histone H3 were diluted 1:1,000 and secondary antibodies (Amersham) were diluted 1:1,500–1:2,500.

DATA ANALYSIS

For statistical analyses we used ANOVA followed by Fisher's and Tukey's tests for *post hoc* comparisons. Binomial tests were used for comparing laterality of behavior. Significance of results was accepted at $p \leq 0.05$. Results are presented as mean ± SEM. All analyses were carried out with SPSS statistical package.

RESULTS

HISTONE H3 ACETYLATION IN COMMAND NEURONS CONTROLLING WITHDRAWAL BEHAVIOR UPON FOOD AVERSION LEARNING

To study the involvement of histone H3 acetylation in conditioned food aversion in *Helix*, we quantified histone H3 acetylation in identified command neurons (premotor withdrawal interneurons) of the food aversion network following learning. These neurons constitute the main plastic element in the network controlling withdrawal behavior of *Helix* upon electric shock stimulation and are involved, therefore, in US processing (Balaban, 2002). For this purpose, we designed a micro variant of western blot analysis, which allowed us to detect proteins purified from single neurons. We analyzed and compared histone H3 acetylation in the left (L) and right (R) command neurons of the parietal ganglia (Pa). Specifically, we analyzed LPa2 and LPa3 [LPa(2/3)], RPa2 and RPa3 [RPa(2/3)] command neurons. These are giant neurons (about 250 microns) symmetrically located in the left and right parietal ganglia, respectively, which can be easily visualized and isolated (**Figure 1**). As a control, we analyzed neurons belonging to the D-group, which do not participate in the food aversion network and are located on the right parietal ganglia (Maksimova and Balaban, 1983).

Groups of three snails were conditioned and were then sacrificed 15 min after training. Command neurons of the right and the left parietal ganglia were separately combined (RPa2 and RPa3 together, and LPa2 and LPa3 together) and a comparative analysis of H3 acetylation involving D-group neurons was performed. Three groups of animals were analyzed: control naïve animals pretreated with the vehicle, trained animals pretreated with the vehicle and trained animals pretreated with the MEK inhibitor PD98059 (40 μM) dissolved in vehicle.

Fifteen minutes after training, acetylation of histone H3 increased in command neurons RPa(2/3) of the right parietal ganglion (**Figure 2**). Specifically, the increase in histone H3 acetylation was detected in RPa(2/3) command neurons (ANOVA: $F1,11 = 6.034$, $p < 0.032$, learning vs control). In contrast, no difference in histone H3 acetylation was found in command neurons of the left parietal ganglion LPa(2/3) ($F1,9 = 0.07$, $p = 0.8$, learning vs control). Thus, after food aversion learning, induction of histone H3 acetylation takes place only in the right parietal ganglion. D-group neurons, which do not belong to the network

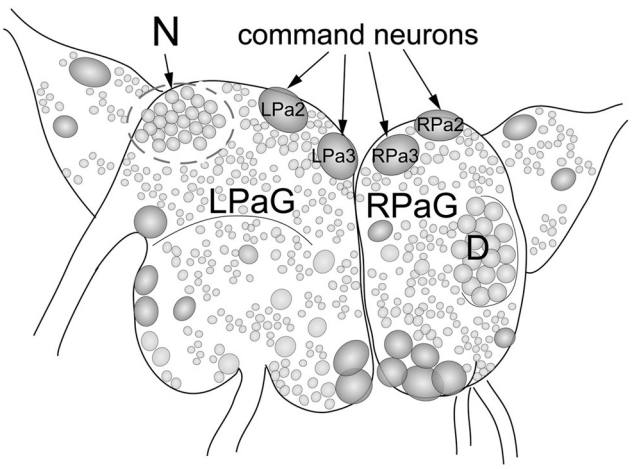

FIGURE 1 | Location of large identified neurons and neuronal clusters in the CNS of *Helix lucorum*. The figure shows the left and right parietal ganglia (LPaG, RPaG). Numbers designate individual identified neurons: giant neurons (2 and 3) symmetrically located in the left and right parietal ganglia, correspond to the giant interneurons (command neurons) of withdrawal behavior LPa2, LPa3 [LPa(2/3)] and RPa2, RPa3 [RPa(2/3)]. Outline areas indicate the region containing neurons belonging to the D-group and the N-group. Command neurons LPa(2/3), RPa(2/3), and neurons of the D-group were used for experiments.

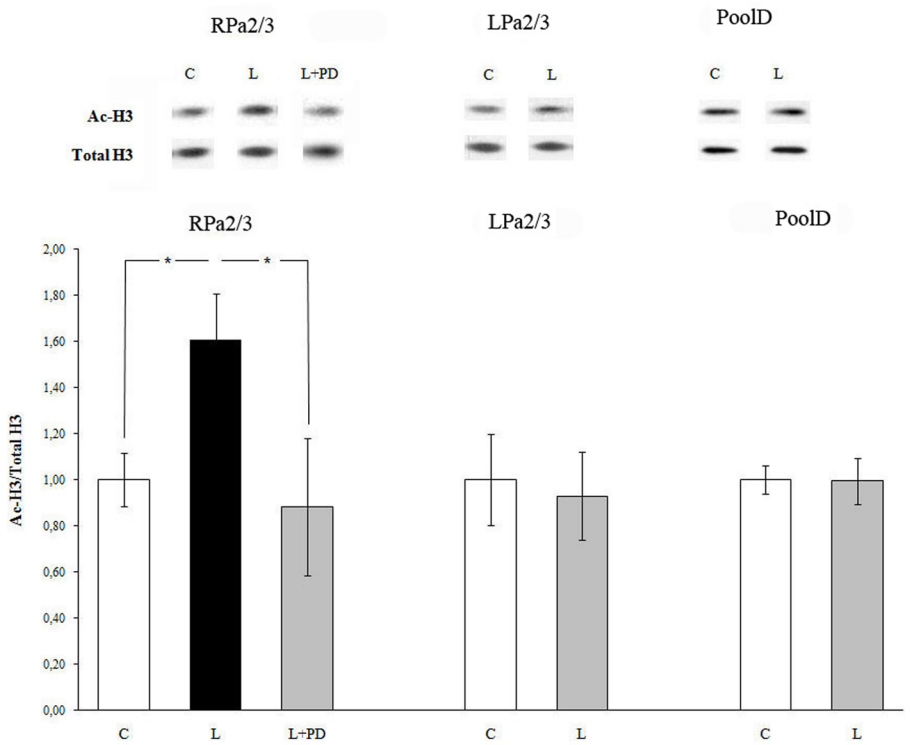

FIGURE 2 | Food aversion learning induces histone H3 acetylation in identified neurons of the CNS of *Helix lucorum*. Increased amount of H3 histone acetylation was detected 15 min after learning in command neurons of withdrawal behavior RPa(2/3) in comparison to naïve controls. The selective MEK inhibitor PD98059 abolished ERK activation. C, control naïve animals pretreated with the vehicle; L, trained animals pretreated with the vehicle; L + PD, trained animals pretreated with the MEK inhibitor PD98059 (40 μM). Data shown are mean ± SEM normalized ratios of ac-H3. Number of independent experiments: command neurons RPa 2/3 (C, $n = 6$; L, $n = 7$; L + PD, $n = 3$), LPa 2/3 (C, $n = 6$; L, $n = 5$); *$p < 0.04$; D-group (C, $n = 4$; L, $n = 4$). Upper panel – representative western blot.

controlling withdrawal behavior of *Helix* and which were thus used as a within-subject control, did not exhibit significant changes in histone H3 acetylation ($F1,6 = 0.13$, $p = 0.73$; **Figure 2**). Their total level of histone H3 did not change after training. To test whether the increase in histone H3 acetylation in RPa(2/3) neurons was MAPK/ERK-dependent, we injected animals with the MEK kinase inhibitor PD98059 30 min prior to training. We compared control vehicle-injected, trained vehicle-injected, and trained PD98059-injected animals. **Figure 2** shows that PD98059 injection inhibited the increase in histone H3 acetylation induced by learning in RPa(2/3) command neurons ($F2,13 = 4.01$, $p < 0.04$). As expected (see above), trained animals pretreated with vehicle exhibited a significantly higher level of histone H3 acetylation than control, untrained animals; $p < 0.03$ (*post hoc* Fisher test), thus confirming the asymmetric effect of training on histone H3 acetylation as a consequence of conditioning (see above). Similarly, a comparison between trained, vehicle-injected animals and trained, PD98059-injected animals was also significant (*post hoc* Fisher test: $p < 0.04$) as the latter did not exhibit a significant increase of H3 acetylation in RPa(2/3) neurons. Consequently, there was no difference between vehicle-injected untrained animals and trained, PD98059-injected animals (*post hoc* Fisher test: $p = 0.67$) in histone H3 acetylation in RPa(2/3) neurons. These results show that the increase in histone H3 acetylation detected in RPa(2/3) neurons is learning- and MAPK/ERK-dependent.

LATERALIZATION OF AVOIDANCE MOVEMENT DURING FOOD AVERSION REFLEX FORMATION IN *HELIX LUCORUM*

Studies performed by Salimova et al. (1984) showed that the capacity of mollusks to turn to the right or to the left sides differ in their latent periods. Asymmetric movements of mollusks could be related to different activities of the serotoninergic and dopaminergic systems underlying left-hand and right-hand movement (Salimova et al., 1984). In particular, they could be related to RPa(2/3) and LPa(2/3) neurons which are thought to be involved in the control of ipsi (unilateral) but not bilateral movements (Balaban, 1979). Given the asymmetry in terms of learning-dependent molecular processes between left and right unilateral command neurons, we reasoned that such asymmetry may result in snails learning to move away in an asymmetric way (i.e., to the right or to the left) from the piece of carrot they avoid. We thus analyzed whether *Helix* snails have a preferred direction of turning upon and after food avoidance learning. During training we did not observe any lateralization of avoidance movement ($p > 0.2$ Binomial test, $n = 18$). No direction preference was observed while testing the animals 24 h after training, either ($p > 0.2$ Binominal test, $n = 18$). But, interestingly 48 h after learning all animals demonstrated lateralization of avoidance movement direction ($p < 0.001$ Binominal test). All of the eighteen snails moved to the right while avoiding carrot (**Figure 3**). These results indicate that behavioral lateralization is established only after consolidation of the conditional reflex. Taken together our

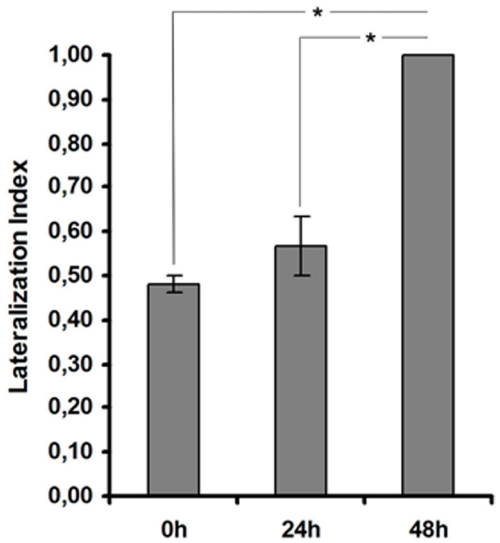

FIGURE 3 | Lateralization of avoidance movement during food aversion reflex formation in *Helix lucorum*. Forty-eight hours after training, *Helix* demonstrates lateralization of avoidance movement. An index to quantify lateralization of avoidance movement was calculated using $R/(L + R)$, where R represents the number of animals, which moved to the right while avoiding carrot, and L the number of animals, which moved to the left while avoiding carrot. $n = 18$ (three series, six animals in every series) for all groups. $^*p < 0.001$. Errors bars = SEM.

data demonstrate a correlation between a lateralized increase in histone H3 acetylation in RPa(2/3) neurons and a lateralized avoidance to the right 48 h after learning.

DISCUSSION

Our work shows that histone H3 acetylation is selectively increased in identified neurons of the CNS of *H. lucorum* upon food aversion learning. Such an increase was found in the command neurons of the right parietal ganglion RPa(2/3) but not in the symmetrical neurons of the left parietal ganglion LPa(2/3). The D-group neurons, which do not belong to the food aversion network, did not show an increase in histone H3 acetylation. Injection of the MAPK/ERK pathway inhibitor PD98059 prior to training prevented learning-dependent histone H3 acetylation in RPa(2/3) neurons, thus showing that acetylation is related to MAPK/ERK activity. We have previously shown that blocking MAPK/ERK activity via pretreatment with PD98059 impairs food avoidance learning in *Helix* (Grinkevich et al., 2008).

Our experiments suggest that changes in histone H3 acetylation in command neurons of withdrawal behavior are required for learning and are regulated by MAPK/ERK. Our data support findings obtained in other animals, showing the important role of acetylation during long-term memory formation (Kandel, 2001; Guan et al., 2002; Levenson and Sweatt, 2006).

Recently MAPK/ERK was reported to be involved in the regulation of histone acetylation in a number of studies carried out in vertebrates (Levenson et al., 2004; Chwang et al., 2006, 2007; Sweatt, 2009). It is supposed that MAPK/ERK-dependent acetylation of histones could be mediated by the CREB-binding protein (CBP), a known MAPK/ERK target and transcription activator, which possesses endogenous HATs activity (Alarson et al., 2004; Korzus et al., 2004; Wood et al., 2005). MAPK/ERK-dependent acetylation is mediated by RSK and MSK protein-kinases (Chwang et al., 2007). HDCAs play an important role in the regulation of histone acetylation. Injection of HDACs inhibitors was shown to improve long-term memory formation both in wild-type animals and mutants with dysfunctional CBP (Alarson et al., 2004; Korzus et al., 2004; Wood et al., 2005). Moreover, in the last years, the possibility of memory amelioration through HDACs inhibition even in animals with neurodegeneration has been suggested (Fischer et al., 2007; Abel and Zukin, 2008).

In addition, the central role of MAPK/ERK-dependent histone H3 acetylation during food aversion learning is supported by our research on juvenile snails. Juvenile animals, which possess immature mechanisms of long-term plasticity of avoidance behavior, in contrast to adults do not exhibit changes in histone H3 acetylation upon conditioning. This result is related with our previous findings, which demonstrated both a lack of MAPK/ERK activation and a difference in the spectrum of TFs binding DNA regulatory elements SRE and AP-1 the juvenile animals (Grinkevich et al., 2003, 2008). Thereby dysfunction of MAPK/ERK activation during training may result in a deficit in histone H3 acetylation in juvenile snails. Taken together, our data confirm the essential role of MAPK/ERK-dependent histone H3 acetylation in food aversion learning in *Helix*.

We suggest that sensory stimulation does not have a significant effect on H3 acetylation as after learning we observed an increase in H3 acetylation in RPa(2/3) neurons only, although left and right command neurons from parietal ganglia have common sensory fields (Balaban, 2002). Also, histone H3 acetylation induced by learning is due to sensitization underlying the formation of conditioned food aversion in *Helix*. It should be noted that similar biochemical alterations occur at the cellular level during the formation of both sensitization and conditioned defensive responses. These effects only differ in their magnitude and duration (Abrams et al., 1991; Grinkevich, 1994; Antonov et al., 2001). Moreover, our recent findings (Kharchenko et al., 2010) support the idea of a significant role of sensitization in the molecular processes underlying withdrawal reflex formation. We have shown asymmetrical activation of MAPK/ERK in RPa(2/3) neurons not only after learning but also after incubation in serotonin, the neurotransmitter which mediates the effect of the US and stimulates sensitization.

It has been previously shown that all command neurons, RPa(2/3) and LPa(2/3), trigger the withdrawal responses and are involved in habituation, sensitization, and aversive conditioning (Balaban, 2002). Command neurons of the right and left parietal ganglia constitute the plastic link of food aversion reflex and might be responsible for unilateral right [RPa(2/3)] or left [LPa(2/3)] turning when withdrawal or escape responses are initiated. Morphological and functional differences have been described for RPa(2/3) and LPa(2/3) neurons. Firstly, every command neuron has its own specific non-habituating area of the receptive field. RPa(2/3) and LPa(2/3) neurons have specific receptive fields, which are predominantly located ipsilaterally on the poda. Furthermore, there is a difference in the organization of the motor fields of these neurons (Bravarenko et al., 1982). Balaban (1979) reported that RPa(2/3) and LPa(2/3) neurons are responsible for producing contractions of ipsilateral body walls. These contractions may be related to the

presence of inhibitory synaptic connections from command neurons onto ipsilateral neurons of pedal ganglion, participating in the locomotory control. Activation of the command neurons in the right parietal ganglion may result in the contraction of the ipsilateral muscles via pedal neurons, which in turn may lead to the movement to the right (Ierusalimsky and Zakharov, 1994; Ierusalimsky et al., 1994; Zakharov et al., 1995).

Due to their ipsilaterality, command neurons of parietal ganglia may not be involved in the production of bilateral movements of the foot, which are mediated by other populations of motor neurons. Secondly, serotonin (5-HT) has opposite effects on acetylcholine (Ach)-dependent responses of LPa3 and RPa3; 5-HT increases Ach-dependent responses in RPa3 while it decreases them in LPa3. This fact is connected with differences in the Ca-systems of these neurons (Dyatlov, 1988). Moreover, the amount of brain specific proteins differs between command neurons of the right and the left parietal ganglia after avoidance learning (Shtark et al., 1982; Grinkevich, 1994). Additionally, a recent transcriptomic analysis from single neurons of *Aplysia* showed a significant heterogeneity of gene expression in neurons that seemed to be functionally similar (Moroz et al., 2006). Thus, our data suggest that command neurons located in the right and left parietal ganglia of *Helix* play different roles in food aversion learning. In particular we suggest that the molecular processes occurring in an increased way in right command neurons RPa(2/3), and which may reflect a lateralized memory upon food aversion learning, are related with unilateral turning to the right, visible 48 h after training. In other words, the unilateral command properties of these neurons would provide the substrate to generate a lateralized behavior established upon food aversion learning via increased and lateralized molecular processes.

Evidence for lateralization of the invertebrate nervous system has been recently reported for nematodes and insects. In the nematode *C. elegans* asymmetric expression of olfactory and taste receptors was shown in symmetrically located cells (AWCL/AWCR and ASEL/ASER), despite the fact that cells are morphologically and anatomically identical. Mutants symmetrically expressing these receptors have impaired odor and taste recognition (Hobert et al., 2002). Unilateral activation experiments indicate that the asymmetry extends to the level of behavioral output: ASEL lengthens bouts of forward locomotion (runs) whereas ASER promotes direction changes (turns) (Suzuki et al., 2008). In insects, anatomical and functional asymmetries of the nervous system have also been described in the fruit fly *Drosophila melanogaster*. Flies presenting an asymmetrical brain structure ("asymmetrical body") establish long-term memory after aversive conditioning (odor–shock associations), while those with symmetrical brains do not (Pascual et al., 2004). Honeybees (*Apis mellifera*) learn to associate odor delivered to their antennae and sugar reward delivered to antennae and proboscis (Giurfa, 2007) and different olfactory retention performances have been reported depending on which antenna, left or right is used (Letzkus et al., 2006). Using the same protocol, Rogers and Vallortigara (2008) showed a lateral shift of olfactory recall from the right to the left antennae 6–8 h after training, so that memory can now be recalled mainly when the left antenna is in use. Visual learning in bees consisting of color–sucrose associations (Giurfa, 2004) is also lateralized as bees learn a color stimulus better with their right eye (Letzkus et al., 2008). Our investigation shows that locomotion is also lateralized in snails after training. While no preference for a given movement direction was observed during training and 24 h after it, all tested animals moved to right while exhibiting avoidance of carrot 48 h after training. Behavioral lateralization occurs only after the final consolidation of the conditional reflex (48 h after learning). Whether such a movement lateralization is a cause or a consequence of the lateralization in MAPK/ERK activation and H3 acetylation, as observed in command neurons of the right parietal ganglion, remains an open question.

Long-term memory formation in *Helix* is associated with selective activation of the MAPK/ERK pathway and action on downstream targets, such as histone H3, in command neurons located in the right parietal ganglion. The asymmetry in MAPK/ERK activation and histone acetylation between right and left command neurons controlling withdrawal behavior suggests lateralization of a long-term memory trace in mollusk. The main question is why should the memory trace be asymmetrical in *Helix*? One possible explanation might be related to the developmental processes that build up a gastropod. Gastropods are different from their primitive mollusk ancestors in having an enlarged head and visceral mass, in most cases a logarithmically spiraled shell, and a visceral mass that has undergone a 180 rotation during development (torsion). This results in an asymmetrical development with the majority of growth occurring on the left or right side. On the other hand, as in *C. elegans* (Hobert et al., 2002) this asymmetry might be determined by the difference of the intracellular regulatory systems and TFs, specific for every cell. Therefore, the asymmetry would be genetically determined and learning and memory formation would build up on a pre-existing lateralized substrate, thereby leading to an increase in lateralization.

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Long-term habituation of the gill-withdrawal reflex in *Aplysia* requires gene transcription, calcineurin and L-type voltage-gated calcium channels

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Although habituation is possibly the simplest form of learning, we still do not fully understand the neurobiological basis of habituation in any organism. To advance the goal of a comprehensive understanding of habituation, we have studied long-term habituation (LTH) of the gill-withdrawal reflex (GWR) in the marine snail *Aplysia californica*. Previously, we showed that habituation of the GWR in a reduced preparation lasts for up to 12 h, and depends on protein synthesis, as well as activation of protein phosphatases 1 and 2A and postsynaptic glutamate receptors. Here, we have used the reduced preparation to further analyze the mechanisms of LTH in *Aplysia*. We found that LTH of the GWR depends on RNA synthesis because it was blocked by both the irreversible transcriptional inhibitor actinomycin-D and the reversible transcriptional inhibitor, 5,6-dichlorobenzimidazole riboside (DRB). In addition, LTH requires activation of protein phosphatase 2B (calcineurin), because it was disrupted by ascomycin. Finally, LTH was blocked by nitrendipine, which indicates that activation of L-type voltage-gated Ca^{2+} channels is required for this form of learning. Together with our previous results, the present results indicate that exclusively presynaptic mechanisms, although possibly sufficient for short-term habituation, are insufficient for LTH. Rather, LTH must involve postsynaptic, as well as presynaptic, mechanisms.

Keywords: behavior, non-associative learning, long-term memory, habituation, long-term depression, mollusk

INTRODUCTION

Habituation is a response decrement to the repeated application of a given stimulus that cannot be attributed to sensory adaptation, or sensory or motor fatigue (Thompson and Spencer, 1966; Rankin et al., 2009). Although apparently ubiquitous throughout the animal kingdom, and commonly regarded as the simplest form of learning, habituation remains poorly understood with respect to its underlying neuronal mechanisms, particularly in vertebrates. Some progress has been made, however, toward an understanding of the neural basis of habituation in simpler invertebrate organisms, particularly the crayfish (Krasne and Teshiba, 1995), the mollusk *Aplysia* (Glanzman, 2009), and the nematode *Caenorhabditis elegans* (Giles and Rankin, 2009). Nonetheless, even in these relatively simple organisms, there are large gaps in our understanding of the cell biology of habituation.

Habituation can exhibit both short- and long-term forms. In *Aplysia* the siphon-elicited gill- and siphon-withdrawal reflex can undergo habituation that persists for several weeks (Carew et al., 1972). This form of long-term habituation (LTH) is characterized by long-term synaptic depression of the sensorimotor pathway that mediates the withdrawal reflex (Carew and Kandel, 1973; Castellucci et al., 1978), as well as by retraction of the presynaptic terminals and branches of siphon sensory neurons (Bailey and Chen, 1983, 1988a). However, the signaling pathways whose activation during LTH triggers these long-term cellular changes remain fairly obscure. In a previous study (Ezzeddine and Glanzman, 2003) we used a reduced preparation to study the signaling pathways involved in LTH of the gill-withdrawal reflex (GWR) in *Aplysia*. We found that LTH of the GWR depends on protein synthesis, as well as activation of protein phosphatases 1 and 2A and postsynaptic glutamate receptors, specifically, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors. Here, we used our reduced preparation to test the roles of RNA synthesis, calcineurin activity and L-type Ca^{2+} channel activation in LTH in *Aplysia*.

MATERIALS AND METHODS

We have previously described the Materials and Methods in detail (Ezzeddine and Glanzman, 2003). Briefly, adult *Aplysia californica* (75–150 g) were obtained from a local supplier (Alacrity Marine, Redondo Beach, CA, USA) and housed for ≥24 h prior to the start of the experiments. During an experiment the animal was initially anesthetized with isotonic $MgCl_2$, and then the mantle shelf, gill, siphon, and tail were dissected away from the rest of the body, together with the CNS (minus the buccal ganglia), which was left connected to the siphon and gill via the siphon and branchial nerves, respectively. The abdominal artery was cannulated with polyethylene tubing (0.024 in OD, 0.011 in ID, Intramedic, Parsippany, NJ, USA), and this was connected to a peristaltic pump. During experiments the abdominal artery was perfused (rate = 1.5 ml/hr) with aerated normal artificial seawater (ASW, 15°C) via the cannula. The cannula was also used to selectively administer drugs to

the abdominal ganglion. Following cannulation of the abdominal artery, the preparation was pinned to the Sylgard-lined bottom of a Lucite experimental chamber. The siphon was left unpinned. The afferent vein of the gill was cannulated with polyethylene tubing and perfused with chilled, aerated ASW. The cannula in the afferent vein was secured with a surgical silk suture, and the suture was connected to a force transducer (Model 1040 or 1030, ADInstruments, Grand Junction, CO, USA), which was used to measure the GWR. Habituating and test stimuli were delivered to the siphon via pairs of Teflon-insulated platinum wires (0.005 mm in diameter, #773000, AM Systems, Carlsborg, WA, USA). One wire was inserted into each side of the siphon, and a ground wire was placed in the bath. The intensity of the stimuli (500 ms trains [25 Hz] of 10 ms current pulses) was set for each preparation to be just suprathreshold for reliably eliciting gill withdrawal, and remained fixed throughout an experiment. One side of the siphon (Trained) received both the test and habituating stimulation, whereas the other side (Untrained) received only the test stimulation.

After the intensity of the stimuli had been determined for the Trained and Untrained sides, the preparation was rested for 60–90 min. At the end of the rest period a single stimulus was delivered to the Trained side of the siphon. Preparations that did not respond to this initial stimulus with a visible gill contraction were discarded. Furthermore, data from a preparation were accepted only if the posttest response of the Untrained side was at least 80% of the pretest response.

All drugs were purchased from Sigma Aldrich (St. Louis, MO, USA). The drugs were initially dissolved in DMSO, and then diluted to their final concentrations in artificial seawater (ASW). (The final concentration of DMSO in the infusate was 0.1–0.2%.) The drugs were directly delivered to the abdominal ganglion via the cannula in the abdominal artery. The irreversible transcriptional inhibitor actinomycin-D was infused into the abdominal ganglion for 1 h prior to the pretests, and then washed out. All other drugs were infused into the abdominal ganglion starting immediately after the pretests, and then washed out of the abdominal ganglion with ASW prior to the posttests. In control preparations ASW, containing the same concentration of DMSO as the infusate used in the matched experimental preparations, was infused into the abdominal artery, and then washed out with normal ASW prior to the posttest. The experimental preparations and the control preparations used to test the effect of an experimental drug on habituation were from animals that were all collected at the same time.

The side of the siphon that was chosen to be the Trained side was alternated systematically between left and right sides. The Trained side initially received a pretest stimulus, and 5 min later a pretest stimulus was delivered to the other side of the siphon (Untrained side). 1 h after the pretest stimulus to the Untrained side, the Trained side received habituation training, which consisted of five blocks of stimuli (interblock interval = 90 min). During each block 30 stimuli (ISI = 30 or 60 s) were delivered to the Trained side. Following a 2 h rest period after habituation training, the Trained and Untrained sides each received a single posttest stimulus. The two posttest stimuli were separated by 5 min.

The peak amplitude (maximum–minimum) of each gill contraction was determined using a digital data acquisition system (MacLab 4 s/2e or PowerLab 8 s, ADInstruments, Colorado Springs, CO, USA). The minimum was the base line response recorded when the gill was in a relaxed state 1 s prior to stimulation. The pretest value of the GWR was set to be equal to 100% for the Trained and Untrained sides, and subsequent GWRs were normalized to the pretest value. All responses in the Results are expressed as the mean normalized response ± SEM. Non-parametric statistical tests were used for all comparisons. Wilcoxon tests were used for paired comparisons, and Mann–Whitney tests were used for unpaired comparisons unless otherwise indicated. All significance values reported represent two-tailed levels of significance.

RESULTS

LONG-TERM HABITUATION DEPENDS ON RNA SYNTHESIS

To test whether LTH of the GWR requires transcription, actinomycin-D (40 μM in ASW with 0.2% DMSO) was infused into the abdominal ganglion. Actinomycin-D's effects are irreversible; therefore, the drug was applied for 1 h, and then washed out with ASW immediately before the start of the pretests. The actinomycin-D treatment ($n = 6$) blocked LTH (**Figure 1A**). The difference between the pretest and posttest withdrawal in response to stimulation of the Trained side was not significant (mean normalized posttest response = 99.3 ± 12.5%, $p > 0.6$). Furthermore, the posttest GWR evoked by stimulation of the Untrained siphon side was not significantly different from the pretest GWR (mean normalized posttest response = 111.2 ± 9.5%, $p > 0.1$). Finally, there was no statistical difference between the Trained and Untrained posttest GWRs ($p > 0.1$).

In the control experiments ($n = 6$) ASW containing 0.2% DMSO was infused into the abdominal ganglion 1 h before the pretests, and washed out immediately before the start of the experiment. The training produced LTH of the GWR to Trained side stimulation (mean normalized posttest response = 47.3 ± 10.2%, $p < 0.04$ for the comparison with the Trained pretest response) (**Figure 1B**). By contrast, the posttest GWR to stimulation of the Untrained side of the siphon did not differ significantly from the pretest GWR (mean normalized response = 108 ± 8.8%, $p > 0.4$). Also, the posttest response evoked by Trained side stimulation was significantly less than that evoked by Untrained side stimulation ($p < 0.003$). In addition, there was a significant difference between the Trained posttest GWRs of the actinomycin-D-treated and control groups ($p < 0.003$). The Untrained posttest responses of the two groups, however, did not differ significantly ($p > 0.8$). Importantly, despite the fact that actinomycin-D treatment blocked LTH, Trained-site stimulation nonetheless produced normal short-term habituation (STH) of the GWR in the actinomycin-treated preparations. (Compare the Trained responses to the Block 1 habituating stimuli in **Figures 1A,B**).

To control for non-specific effects of actinomycin-D, we tested the effect of a reversible transcriptional inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Sehgal and Tamm, 1978; Yamaguchi et al., 1998; Apergis-Schoute et al., 2005), on LTH. DRB (150 μM in ASW with 0.2% DMSO) was present in the abdominal ganglion throughout the training period. The presence of DRB during habituation training blocked LTH ($n = 9$, **Figure 2A**). There was no significant change in the Trained GWR (mean normalized postest response = 115.1 ± 10.9%, $p > 0.2$ for the comparison with the pretest response). The GWR to stimulation of the Untrained

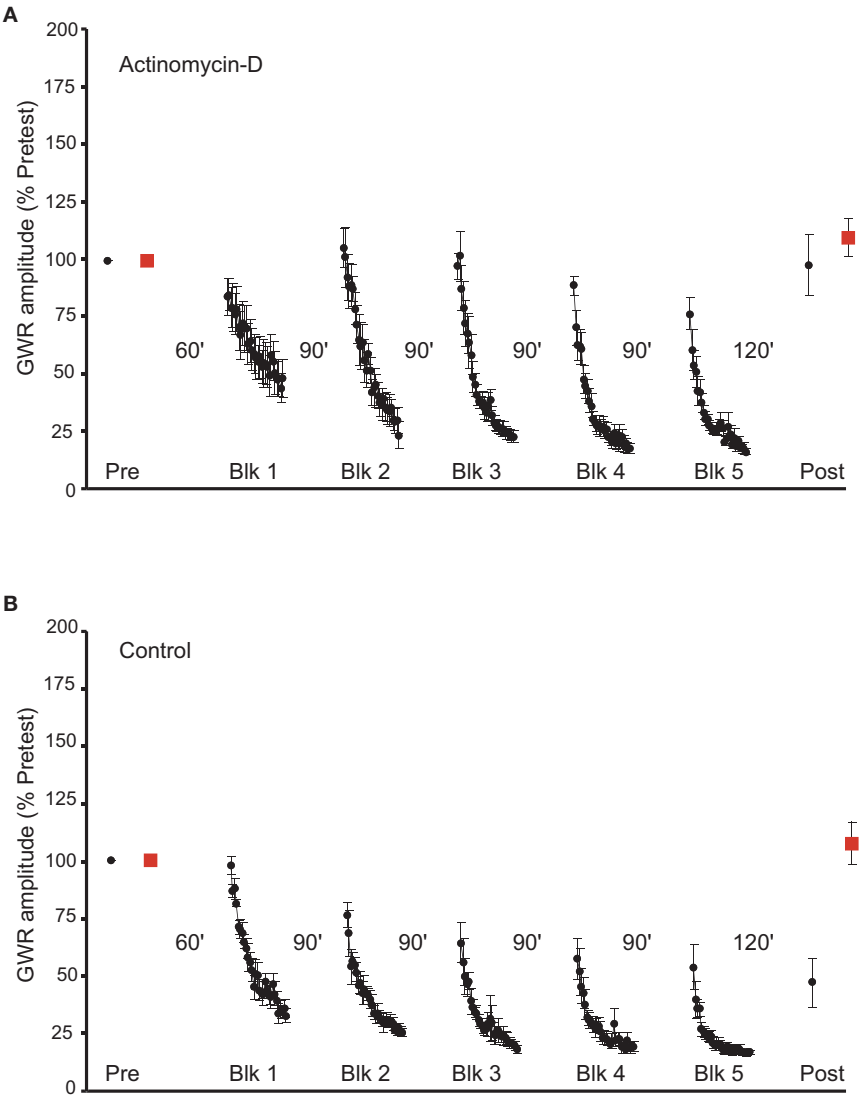

FIGURE 1 | Effect of actinomycin-D treatment on LTH. (A) Experiments in which preparations were treated with the irreversible transcriptional inhibitor actinomycin-D prior to habituation training ($n = 6$). The drug blocked the induction of LTH. **(B)** Data for control experiments ($n = 6$). Here, the abdominal ganglion was perfused with normal ASW plus DMSO for the time corresponding to the period of actinomycin-D treatment. The habituation training produced significant LTH of the GWR to stimulation of the Trained side of the siphon. In this and the following figures the solid circles represent the responses to Trained side stimulation, whereas the solid squares represents the responses to Untrained side stimulation.

side also did not change (mean normalized Untrained posttest response = 122.6 ± 19.1%, $p > 0.7$ for the comparison with the pretest response). In addition, the Trained and Untrained posttests did not differ ($p > 0.8$). Control experiments, in which ASW with 0.2% DMSO was infused into the abdominal artery, were performed using the same protocol as the DRB experiments. The habituation training produced significant LTH of the Trained GWR in the control experiments ($n = 9$, **Figure 2B**). There was a significant decrease in posttest response to Trained side stimulation (mean normalized posttest response = 75.6 ± 8.8% $p < 0.04$ for the comparison with the pretest response). However, there was a significant increase in the GWR to stimulation of the Untrained side (mean normalized posttest response = 118.4 ± 7.5%, $p < 0.03$ for the comparison with the Untrained pretest response). Furthermore, the posttest Untrained GWR was significantly greater than the posttest Trained GWR ($p < 0.004$). Finally, the posttest GWRs to Trained side stimulation differed significantly in the DRB-treated and control preparations ($p < 0.02$).

The effect of the habituation training on the GWR to Untrained side stimulation in the control experiments suggests that the training produced some generalized sensitization, as well as pathway-specific LTH, in these experiments. In our earlier study (Ezzeddine and Glanzman, 2003) we also observed that the GWR to Untrained side stimulation sometimes exhibited sensitization after habituation training, and that there was often sensitization of the GWR to Trained side stimulation during training in the presence of drugs

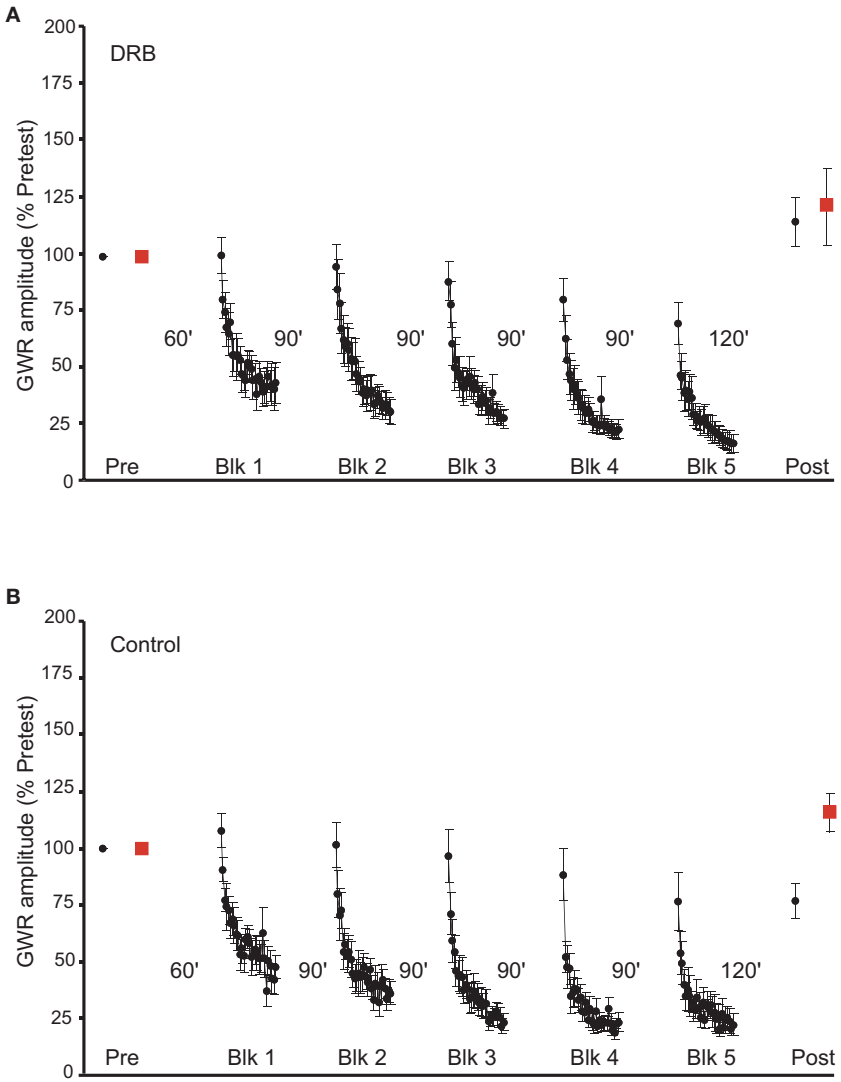

FIGURE 2 | Effect of DRB treatment on LTH. (A) Data from experiments ($n = 9$) in which preparations were treated with the reversible transcriptional inhibitor DRB during habituation training. DRB infusion disrupted LTH. **(B)** Data for the control experiments ($n = 9$). Here, training was carried out in the presence of ASW plus DMSO, which produced LTH on the Trained side and sensitization on the Untrained side.

that blocked LTH. We believe, as discussed in that paper, that these phenomena reflect the fact that the habituating stimuli can activate both habituating and sensitizing neuronal processes, as originally proposed by Groves and Thompson (1970). According to Groves and Thompson, the ultimate behavioral outcome, whether habituation, sensitization, or no change, is determined by the relative strengths of these two countervailing processes. It should be noted, however, that both the sensitization of the GWR on the Untrained side, and the sensitization of the reflex on the Trained side during habituation training in the presence of inhibitors of LTH (e.g., Blocks 2 and 3, **Figure 1A**), were highly variable and not always observed.

LONG-TERM HABITUATION REQUIRES CALCINEURIN ACTIVITY

We previously found that LTH depends on activation of NMDA-type receptors in abdominal ganglion. Activation of postsynaptic NMDA receptors would be expected to produce a rise in intracellular Ca^{2+} in neurons of the GWR circuit. The Ca^{2+}/calmodulin-dependent phosphatase calcineurin has been shown to be involved in both long-term synaptic depression (Mulkey et al., 1994) and memory extinction (Baumgartel et al., 2008) in mammals. Given that long-term depression (LTD) of the sensorimotor synapse (Lin and Glanzman, 1996) is a candidate mechanism for LTH in *Aplysia* (see Ezzeddine and Glanzman, 2003; Glanzman, 2009), we wished to know whether LTH was regulated by calcineurin activity. Accordingly, we tested the effect of ascomycin (10 μM in ASW with 0.2% DMSO), a selective calcineurin inhibitor (Sierra-Paredes and Sierra-Marcuño, 2008), on LTH. Ascomycin application during habituation training blocked LTH ($n = 7$, **Figure 3A**). The difference between the pretest and posttest responses for Trained side stimulation was not significant (mean normalized posttest response = 104.2 ± 14%, $p > 0.9$). However, as was the case for the control experiments in **Figure 2B**, the posttest response for

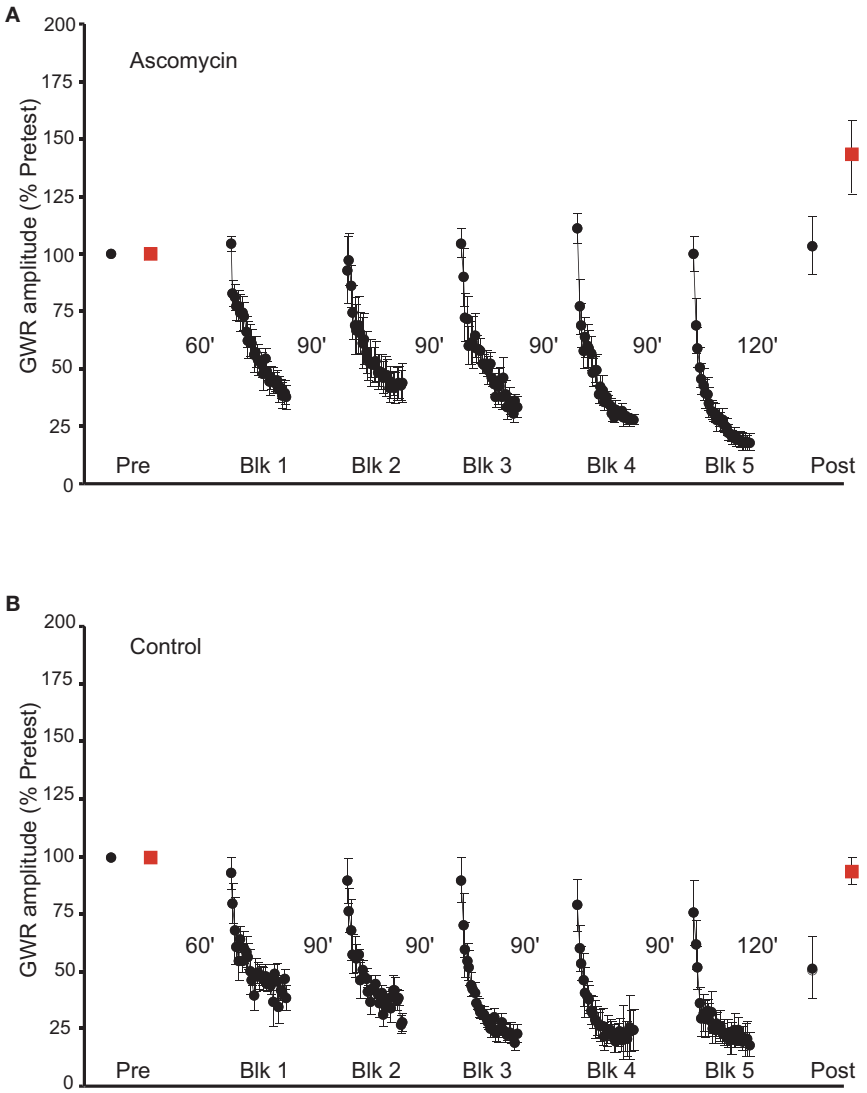

FIGURE 3 | Effect of inhibition of calcineurin on LTH. (A) Data from experiments ($n = 7$) in which the abdominal ganglion was infused with ascomycin during habituation training. Inhibition of calcineurin blocked LTH. **(B)** Control data from experiments ($n = 5$) in which a control drug, rapamycin, which does not inhibit protein phosphatases, was present in the abdominal ganglion during training. Training in the presence of rapamycin produced LTH of the GWR to Trained side stimulation.

the Untrained side exhibited significant enhancement (mean normalized posttest response = 143.2 ± 15.7%, $p < 0.05$ for the comparison with the pretest response). The difference between the posttest responses for the Trained and the Untrained sides, however, was not significant ($p > 0.1$). Control experiments ($n = 5$) were performed with rapamycin (10 μM in ASW with 0.2% DMSO) present in the cannula during training (**Figure 3B**). Rapamycin has the same binding site on calcineurin as ascomycin, but rapamycin does not inhibit dephosphorylation by calcineurin (Schreiber and Crabtree, 1992). In contrast to the effect of ascomycin, rapamycin did not affect LTH. Thus, habituation training resulted in a significantly reduced GWR for the Trained side (mean normalized posttest response = 51.4 ± 13%, $p < 0.03$ [*t*-test used here for the comparison with the pretest response]). The difference between the pretest and posttests responses for Untrained side, however, was not significant (mean normalized posttest GWR = 93.4 ± 6.7%, $p > 0.3$). As would be expected from these results, the posttest responses for the Trained and Untrained sides differed significantly in the experiments involving rapamycin treatment ($p < 0.03$). Finally, the posttest GWR to Trained side stimulation was significantly less in the rapamycin-treated preparations than in the ascomycin-treated preparations ($p < 0.05$). Notice that, although blockade of calcineurin activity with ascomycin disrupted LTH, it did not affect STH (**Figure 3A**).

ACTIVATION OF L-TYPE VOLTAGE-GATED CA^{2+} CHANNELS APPEARS TO BE NECESSARY FOR LTH

We previously found that activation of AMPA receptors, as well as of NMDA receptors, contributes to LTH of the GWR (Ezzeddine and Glanzman, 2003). It is therefore possible that

depolarization-induced activation of L-type voltage-gated Ca^{2+} channels (LVGCCs) is upstream of calcineurin activity during LTH. To test this possibility, we examined the effect of blockade of LVGCCs on LTH. We infused nitrendipine (100 μM in ASW with 0.14% DMSO), an antagonist of LVGCCs (Bolshakov and Siegelbaum, 1994), into the abdominal ganglion during habituation training ($n = 6$). Nitrendipine treatment blocked LTH to stimulation of the Trained side of the siphon (mean normalized posttest response = 116.8 ± 13%, $p > 0.3$ for the comparison with the pretest response) (**Figure 4A**). In these experiments habituation training did not alter the GWR to stimulation of the Untrained side; the mean normalized Untrained posttest response (157.8 ± 39.4%, was not significantly different from the mean normalized Untrained pretest response ($p > 0.4$), although the variability in the Untrained responses was admittedly large. Furthermore, the GWR evoked by posttest stimulation of the Trained side was not significantly different from that evoked by posttest stimulation of the Untrained side ($p > 0.9$). In control experiments ($n = 8$), performed at the same time as the nitrendipine experiments, the abdominal ganglion was infused ASW plus 0.14% DMSO. Here, habituation training produced significant LTH of the GWR to stimulation of the Trained side (mean normalized posttest response = 55.1 ± 12.4%, $p < 0.02$ for the comparison with the pretest response) (**Figure 4B**). There was no significant change in the GWR to stimulation of the Untrained side (mean Untrained normalized posttest response = 124.4 ± 15.3%, $p > 0.1$ for the comparison with the Untrained pretest response). Furthermore, the Trained side posttest GWR and the Untrained side posttest GWR differed significantly ($p < 0.004$). Finally, the difference between the posttest response to the Trained side stimulation in the nitrendipine group and that in the control group was highly significant ($p < 0.005$). These results support the idea that LVGCC activity is essential for LTH, although such activity appears not to be necessary for STH (**Figure 4A**).

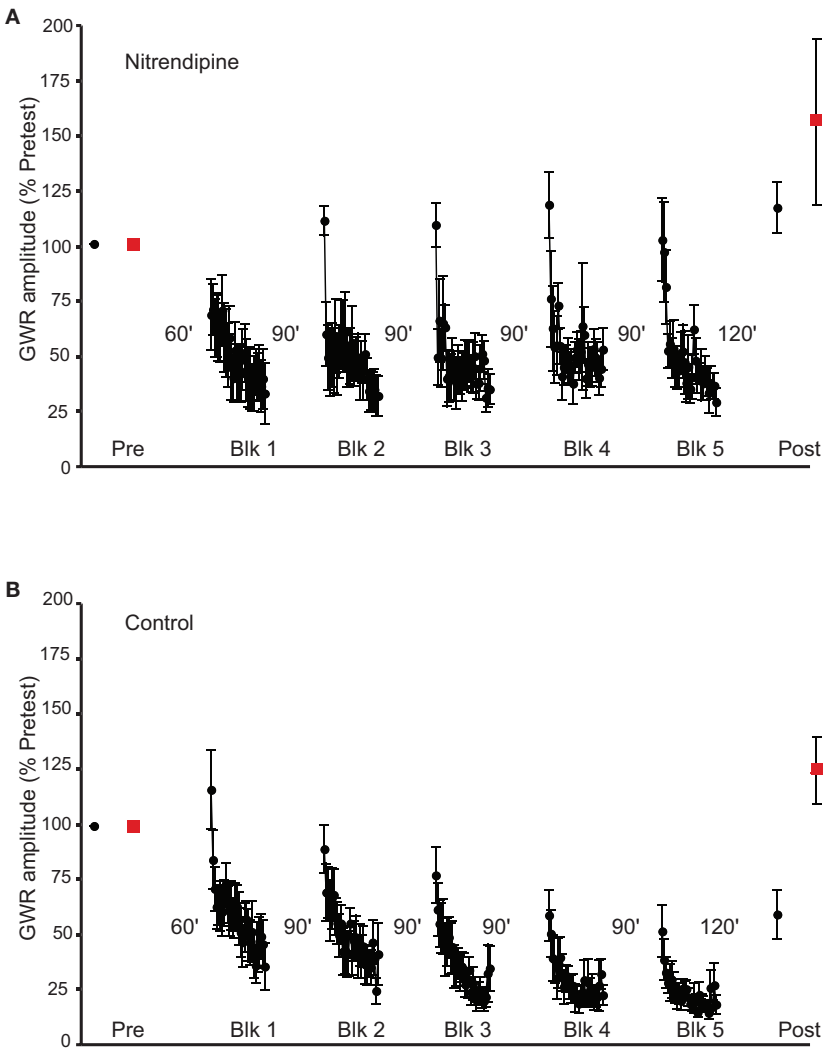

FIGURE 4 | Effect of inhibiting LVGCCs on LTH. (A) Data from experiments ($n = 6$) in which nitrendipine was present during habituation training. The drug blocked the induction of LTH. **(B)** Data from control experiments ($n = 8$) in which training occurred in the presence of ASW plus DMSO. The training resulted in LTH of the GWR.

DISCUSSION

ROLE OF GENE TRANSCRIPTION IN LTH IN *APLYSIA*

Memory in *Aplysia* has been divided into three stages: short-term, intermediate-term and long-term (Goelet et al., 1986; Sutton and Carew, 2002). These three stages are distinguished by both their temporal and mechanistic properties. Temporally – although their time courses overlap somewhat – short-term memory (STM) lasts from seconds to ~30 min; intermediate-term memory (ITM) lasts from ~30 min to 3 h; and long-term memory lasts for >10 h. Mechanistically, STM depends exclusively on posttranslational changes; ITM requires protein synthesis, but not gene transcription; and LTM requires both translation and transcription. However, most of the previous work characterizing the mechanisms underlying the different phases of memory in *Aplysia* has concerned only one type of memory – sensitization of the withdrawal reflex. Although an early report demonstrated that a neural correlate of STH of the GWR did not require protein synthesis (Schwartz et al., 1971), there has been little attempt until recently to determine whether there are different phases of memory for habituation that correspond mechanistically to those for sensitization. We previously showed that the habituation of the GWR that persisted for 1–6 h following a 5 h period of spaced training required protein synthesis (Ezzeddine and Glanzman, 2003). But in that study we did not test whether this form of habituation memory also required gene transcription. Here, we have shown that the habituation resulting from a similar training protocol requires transcription, because it was blocked by actinomycin-D and DRB. Therefore, this form of habituation fits the definition of LTM as it has commonly been defined in *Aplysia* (Goelet et al., 1986). It will be interesting in the future to determine whether there is a training protocol that produces an intermediate form of habituation memory, one that requires translation, but not transcription.

HOMOSYNAPTIC DEPRESSION CANNOT ACCOUNT FOR LTH IN *APLYSIA*

It has long been believed that the mechanism of habituation of the gill- and siphon-withdrawal reflex is due exclusively to presynaptic changes. This mechanism, homosynaptic depression (HSD), is thought to involve decreased transmitter release from presynaptic terminals due to repeated activation of sensory neurons (Castellucci and Kandel, 1974; Armitage and Siegelbaum, 1998). Currently, the mechanism underlying HSD in *Aplysia* is controversial. It was originally proposed that HSD was caused by depletion of the readily releasable pool of presynaptic vesicles (Gingrich and Byrne, 1985; Hochner et al., 1986; Bailey and Chen, 1988b). More recent evidence, however, indicates that HSD results from all-or-none silencing of presynaptic release sites (Gover and Abrams, 2009).

Although HSD may indeed be a major mechanism of STH, the data from this and our previous study (Ezzeddine and Glanzman, 2003) argue strongly against the notion that LTH can be explained by exclusively presynaptic changes. First, HSD does not depend on glutamate receptor activity (Armitage and Siegelbaum, 1998), whereas LTH does (Ezzeddine and Glanzman, 2003). Second, LTH requires activation of LVGCCs (present study), which is most likely caused by AMPA receptor-mediated postsynaptic depolarization (see Ezzeddine and Glanzman, 2003). Studies by Bailey and Chen (1983, 1988a) indicate that LTH is accompanied by presynaptic morphological changes, including fewer vesicles in sensory neuron-associated active zones, and a decrease in the number of varicosities per sensory neuron. These morphological data, together with our data, support the conclusion that LTH involves both pre- and postsynaptic long-term cellular changes. If correct, this conclusion raises the intriguing possibility that the pre- and postsynaptic changes are coordinated by transsynaptic signals (see Glanzman, 2009, for further discussion).

ROLE OF CALCINEURIN AND LVGCCs IN LTH

The data from the present study suggest that increased intracellular Ca^{2+} plays a key role in LTH. One likely source of elevated intracellular Ca^{2+} is influx through postsynaptic NMDA receptors (Ezzeddine and Glanzman, 2003). Activated LVGCCs, due to AMPA receptor-mediated postsynaptic depolarization, represent another potential source of the elevated intracellular Ca^{2+} necessary for LTH. Our finding that ascomycin blocks the induction of LTH indicates that calcineurin is one of the proteins whose activity is stimulated by increased intracellular Ca^{2+}. Interestingly, calcineurin activity has been implicated in both LTD (Mulkey et al., 1994) and depotentiation (Jouvenceau et al., 2003) in the mammalian brain, as well as in some forms of extinction in mammals (Lin et al., 2003; Baumgartel et al., 2008). We do not yet know what downstream pathways are stimulated by calcineurin during LTH. One action linked with calcineurin activity in the mammalian brain is endocytosis of AMPA receptors, which is believed to be a key event in LTD (Beattie et al., 2000). Endocytosis of AMPA-type receptors could well be one effect of calcineurin activity during habituation of the GWR (see Glanzman, 2009), because exocytosis of AMPA-type receptors has previously been shown to play a role in synaptic facilitation and enhancement of the withdrawal reflex in *Aplysia* (Chitwood et al., 2001; Li et al., 2005, 2009).

POTENTIAL ROLE OF LTD IN LTH IN *APLYSIA*

The synaptic mechanisms that underlie LTH of the withdrawal reflex remain to be identified. One potential mechanism that is consistent with the data in this and our previous study (Ezzeddine and Glanzman, 2003) is LTD induced by elevated postsynaptic Ca^{2+} (Malenka and Bear, 2004). A form of LTD of the sensorimotor synapse that depends partly on elevated postsynaptic Ca^{2+} has been demonstrated in dissociated cell culture (Lin and Glanzman, 1996); but this form of synaptic depression has not yet been experimentally linked to LTH. A problem for the idea that activity-dependent LTD is a mechanism of LTH is that induction of activity-dependent LTD requires 1 Hz electrical stimulation, which is quite different from the rate of behavioral stimulation that results in LTH. Further complicating the issue of the synaptic mechanism of LTH is the demonstration by Montarolo et al. (1988) that homosynaptic activation of the sensorimotor synapse with a rate of stimulation designed to mimic the synaptic stimulation that occurs during LTH training fails to induce long-term (24 h) depression of the synapse. The apparent failure of naturalistic homosynaptic activity to induce LTD of the sensorimotor synapse suggests that homosynaptic activation of the sensorimotor pathway, by itself, may be insufficient for LTH in *Aplysia*. The possibility that LTH may depend, at least in part, on one or more heterosynaptic pathways is supported by the finding that spaced applications of the endogenous inhibitory neuropeptide, FMRFamide, can produce depression of the

sensorimotor synapse that persists for ≥24 h (Montarolo et al., 1988). Both heterosynaptic, inhibitory (GABAergic), and homosynaptic pathways have been shown to play roles in habituation of the crayfish tailflip escape response (Krasne and Teshiba, 1995).

MECHANISTIC SIMILARITIES BETWEEN HABITUATION AND EXTINCTION

Habituation, like extinction, is the waning of a response due to repeated presentation of an unchanging stimulus. The major phenomenological distinction between habituation and extinction is that habituation is the waning of an innate response, whereas extinction is the waning of a conditioned response. Despite this distinction, the behavioral similarity between habituation and extinction has led to the speculation that these two forms of memory may share common mechanisms (McSweeney and Swindell, 2002). Although controversial, there is experimental support for this idea (Kamprath et al., 2006). We have found that LTH in *Aplysia* requires the activity of calcineurin and LVGCCs. Both of these processes have also been implicated in extinction (Lin et al., 2003; Barad et al., 2004; Suzuki et al., 2004, 2008; Cain et al., 2005; Baumgartel et al., 2008) (but see Schafe, 2008). Possibly, therefore, an understanding of the cellular mechanisms that mediate LTH of the GWR in *Aplysia* will contribute important mechanistic insights into the considerably more complex phenomenon of extinction.

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Memory formation in reversal learning of the honeybee

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In reversal learning animals are first trained with a differential learning protocol, where they learn to respond to a reinforced odor (CS+) and not to respond to a non-reinforced odor (CS–). Once they respond correctly to this rule, the contingencies of the conditioned stimuli are reversed, and animals learn to adjust their response to the new rule. This study investigated the effect of a protein synthesis inhibitor (emetine) on the memory formed after reversal learning in the honeybee *Apis mellifera*. Two groups of bees were studied: summer bees and winter bees, each yielded different results. Blocking protein synthesis in summer bees inhibits consolidation of the excitatory learning following reversal learning whereas it blocked the consolidation of the inhibitory learning in winter bees. These findings suggest that excitatory and inhibitory learning may involve different molecular processes in bees, which are seasonally dependent.

Keywords: olfactory learning, honey bee, season, protein synthesis inhibitor, reversal learning, memory

INTRODUCTION

In classical conditioning, animals learn to associate an originally neutral stimulus (CS) with a biologically significant stimulus (US) if the CS is followed by the US (forward pairing). Animals are also capable of acquiring an opposite contingency for a given CS, i.e., the absence of the US. Following Pavlov's (1927) terminology, differential conditioning consists of two such contingencies, where the stimulus which precedes the appearance of the US (CS+) retains an excitatory valence, and the one which predicts the absence of the US (CS–) retains an inhibitory one. In reversal learning the animal is first introduced to differential conditioning and once such discrimination has been learned, the stimuli's contingencies are reversed and the animal learns to adapt its response to the new rule. Following Pavlov (1927), forward pairing of CS with reinforcement generates excitatory learning whereas extinction leads to inhibitory learning. Thus reversal learning is a paradigm entailing rather more complex learning than a simple acquisition and extinction, as the animal has to form such new associations on the background of inverted contingencies. The molecular underpinnings of acquisition and extinction learning are believed to differ, particularly in regard to the requirement of protein synthesis. In a wide range of experimental preparations, protein synthesis inhibition was found to block memory formation of acquisition learning (e.g., Davis and Squire, 1984; Abel et al., 1997; Lattal and Abel, 2001). In extinction on the other hand, the administration of protein synthesis inhibitors yielded conflicting results which probably depend on the experimental protocol used (e.g., Flood et al., 1977; Berman and Dudai, 2001; Stollhoff et al., 2005; Duvarci et al., 2006). Altogether, reversal learning provides an adequate paradigm to study both acquisition and extinction learning and memory.

The honeybee (*Apis mellifera*) serves as a valid model for the study of the underlying mechanisms of learning and memory (Menzel et al., 2006) for which many paradigms of conditioning were tested. It was found that the results follow the rules of classical conditioning as known from laboratory mammals (Bitterman et al., 1983; Menzel and Bitterman, 1983; Menzel, 1990). Odors are used as CSs, and sucrose solution as US for hungry bees. Several forms of memory developing in series and in parallel have been described leading to lifelong memory under appropriate conditions (Menzel, 1990).

Memory formation has been shown to consist of distinctive phases; each depends on different molecular pathways: short-term and mid-term memories depend on existing proteins, and two forms of long-term memory (LTM) are controlled by different signaling cascades (Menzel and Müller, 1996; Menzel, 1999; Müssig et al., 2010). Notably, early LTM (eLTM) depends on translation and late LTM (lLTM) depends on transcription processes (Wüstenberg et al., 1998; Friedrich et al., 2004). When applied shortly prior to acquisition, emetine, a protein synthesis blocker which inhibits translation processes, is known to inhibit eLTM consolidation in the honeybee. The effects of transcription and translation inhibitors have been studied so far only in simple forward pairing paradigms (Wüstenberg et al., 1998; Menzel et al., 2001; Friedrich et al., 2004) and in extinction paradigms (Stollhoff et al., 2005; Stollhoff and Eisenhardt, 2009).

In the honeybee, reversal learning was found to have a heritable component which is manifested in the rapidity to reverse from the former CS– to the new CS+ association (Ferguson et al., 2001). However, Ben-Shahar et al. (2000) found differences in the extinction rate of the former CS+ during the reversal phase, which were derived from the bees' behavioral state: nurses showed faster rates of extinction than foragers. Taken together, these finding suggest that two dissociable processes constitute the reversal learning, i.e., excitatory learning and inhibitory learning.

Using local anesthetics to block the main output region of the mushroom body (MB), Devaud et al. (2007) were able to demonstrate that the acquisition of reversal learning requires an intact MB activity, whereas simple differential learning (the first phase in reversal paradigm) was spared. It was also shown that experiencing olfactory reversal learning improves the bee's future performance

in solving further discrimination reversals (Komischke et al., 2002), a feature that might serve to optimize bee's foraging efficiency when food-source profitability changes. However, those studies were designed so, that the temporal spacing of each phase from the next allowed only the formation of short-term and mid-term memories in this paradigm.

Here the effect of emetine on the eLTM formed after reversal learning was investigated in order to elucidate the consolidation of excitatory and inhibitory associations formed after reversal learning, into eLTM. To this end, each learning phase took place on a different day, when translation-dependent memories are formed. Two groups of honeybees were used: summer bees and winter bees, because it was observed in earlier experiments (Menzel et al., 2001) that inhibiting transcription factors yields different results in summer and winter bees, specifically, winter bees did not develop long-lasting memory following spaced conditioning. We found that blocking protein synthesis during consolidation of reversal learning inhibits the consolidation of the excitatory learning in summer bees whereas consolidation of inhibitory learning was blocked in winter bees.

MATERIALS AND METHODS

GENERAL PROCEDURES RELATED TO BEHAVIOR

The experiments were conducted in Berlin, Germany using honeybees (*A. mellifera carnica*) from the colonies of the laboratory. Experiments were carried out in summer time (July/August 2009), using bees raised in outdoor hives, and in winter time (November/December 2009), using bees kept in small flight cages (1 m^3) in a glasshouse. One day prior to the experimental procedure, foraging bees were caught at the hive entrance when leaving the hive; they were then immobilized by cooling and harnessed in small metal tubes. In the evening bees were fed to satiation with a 1-M sucrose solution. On each experimental day, bees were fed in the afternoon to satiation and then kept in a dark and humid box at room temperature (~22°C, ~70% humidity).

CONDITIONING OF THE PER

All acquisition and retrieval trials shared a standardized protocol; each acquisition trial began by positioning a test bee in front of an exhaust fan. Odor stimuli (CS) were applied after 10 s (duration 4 s) and were delivered through 5 ml syringe, each containing a filter paper soaked with 4 μL of pure odorant, 2-octanone, and 1-hexanol (Sigma-Aldrich Chemie GmbH). Computer-controlled magnetic valves were used for the delivery of the odorants, allowing constant air flow. The presentation of the US started 3 s after odor onset by touching the antennae with a toothpick soaked in sucrose solution to induce proboscis extension. US delivery lasted for 4 s during which animals were allowed to lick sucrose solution with the proboscis (hence 1 s overlap between CS and US).

On unrewarded trials (CS–) all conditions remained the same, except there was no presentation of the US (sucrose). A positive response was scored if the proboscis was extended during the CS and before the US.

REVERSAL LEARNING PROTOCOL

On the first day animals were subjected to a differential conditioning protocol with two odorants A and B (2-octanone and 1-hexanol), one forward paired with the US (sucrose solution), the other unrewarded (day 1: A+ vs. B–). Each odorant was presented six times in a pseudo-randomized order and the sequence of odor presentation was identical for all subjects (ABBABAABABBA). Odor identities were counter balanced across subjects.

The intertrial interval was 10 min. On the following day the reinforcement pattern was reversed (day 2: A– vs. B+) whereas all other conditions remained constant. Retention tests were carried out on the third day, where both odorants were presented in the absence of reward.

Acquisition curves are presented as percentages of bees showing conditioned PER for each pair of CS+ and CS– presentations, which constitute one block trial.

EMETINE TREATMENT

Emetine (catalog #45160; Fluka, Buchs, Switzerland) was dissolved in PBS (in mM: 137 NaCl, 2.7 KCl, 10.1 Na_2HPO_4, 1.8 KH_2PO_4, pH 7.2). One microliter of emetine (10 mM) was injected manually into the flight muscle using a calibrated glass capillary. Animals were injected 30 min before the reversal conditioning. Control bees were injected with 1 μl of PBS.

DATA ANALYSIS

Only animals that survived until the retention test and then showed an unconditioned response to sucrose were included. The ordinates give the probability of PER responses. The McNemar χ^2 test (Zar, 1997) was used (SigmaStat) for within-group comparison of the CR to the different odors. The *G*-test for contingency tables (log likelihood ratio) was used when testing the differences in CR for each odor for between group comparisons.

CONTROL EXPERIMENTS

Control experiments were designed in order to rule out a general effect of emetine on performance. On the first day bees were subjected to a differential conditioning protocol as described above. On the following day, bees were assigned randomly to two groups and were injected with either emetine or PBS, and after 30 min a retention test for both odors (in the absence of a reward) was carried out. On the third day bees underwent another retention test for both odors.

RESULTS

SUMMER EXPERIMENTS

Emetine inhibits the new excitatory learning when applied in summer

On the first experimental day, summer bees were trained to differentiate between two odorants, one being rewarded (A+) whereas the other was presented alone (B–). Each odor was presented six times; by the last differential learning trials the proportions of CRs to the A+ and B– were 76 and 2%, respectively (McNemar's Test: $\chi^2 = 112.00$, $p < 0.001$, df = 1; **Figure 1A**).

On the following day, 30 min prior to reversal learning training, bees were randomly assigned to two groups; one being injected with emetine and the other with PBS (phosphate buffer used as saline for emetine). All bees were then trained to the reversed rule (A– vs. B+). By the last differential learning trials the proportions of CRs to A– and B+ were 4 and 49% in the PBS group (McNemar's Test: $\chi^2 = 29.03$, $p = <0.001$, df = 1) and 8 and 39%

in the emetine group, respectively (McNemar's Test: $\chi^2 = 21.04$, $p = <0.001$, df = 1; **Figure 1B**). Emetine injections had no effect on acquisition during this phase.

On the third day, 24 h after the reversal learning, all bees were subjected to a retention test for both odorants (**Figure 1C**). The group injected with PBS scored significantly higher for odor B than for odor A (McNemar's Test: $\chi^2 = 4.267$, $p < 0.05$, df = 1) indicating that the reversal rule had been learned and was remembered. In contrast, the emetine injected group scored the same for both odors (McNemar's Test: $\chi^2 = 0.050$, NS, df = 1), indicating that this group did not remember the reversed rule. Moreover, the emetine injected group scored significantly lower for odor B in comparison with the PBS injected group (G test: $G = 4.254$, $p < 0.05$, df = 1), which indicates an emetine-treatment induced interference with consolidation of excitatory learning.

Control experiments: when applied 24 h after differential learning, emetine has no effect on memory retrieval

On the first experimental day, summer bees underwent a differential conditioning protocol, as described above. By the last differential learning trials the proportions of bees exhibiting the CR to the A+ and B– were 74 and 5%, respectively (McNemar's Test: $\chi^2 = 53.01$, $p < 0.001$, df = 1; **Figure 2A**). On the following day, 30 min prior to a retention test, bees were randomly assigned to two groups; one

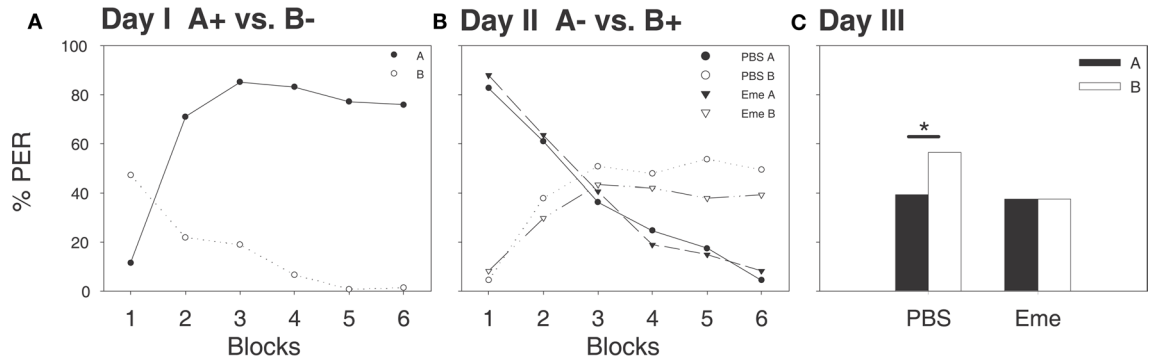

FIGURE 1 | In summer the systemic application of emetine 30 min before reversal learning inhibits consolidation of the new excitatory learning. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by either of the two odorants A (filled shapes and bars) and B (open shapes and bars). **(A)** On day 1, bees were untreated and trained to the differential conditioning rule (A+ vs. B–), each stimulus was presented six times, shown here in six blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B–). At the last trials a significant difference in CRs between odors was observed (McNemar's Test, $p < 0.005$). **(B)** On day 2, 30 min after emetine (triangles) or PBS (circles) injections, a reversal protocol was applied (A– vs. B+), each stimulus was presented six times, shown here in six blocks, (solid lines for A+ vs. dashed lines for B–) at the last trials both PBS and emetine groups exhibited a reversed preference (McNemar's Test, $p < 0.005$ for both groups). **(C)** On day 3, both groups were subjected to a retention test for both odorants in the absence of a reward. The PBS treated control group showed a significant preference for odor B (McNemar's Test, N = 56, $p < 0.05$), the emetine treated experimental group showed no preference (McNemar's Test, N = 64, NS) and bees responded significantly less often to odor B than in the PBS group (G test, $p < 0.05$).

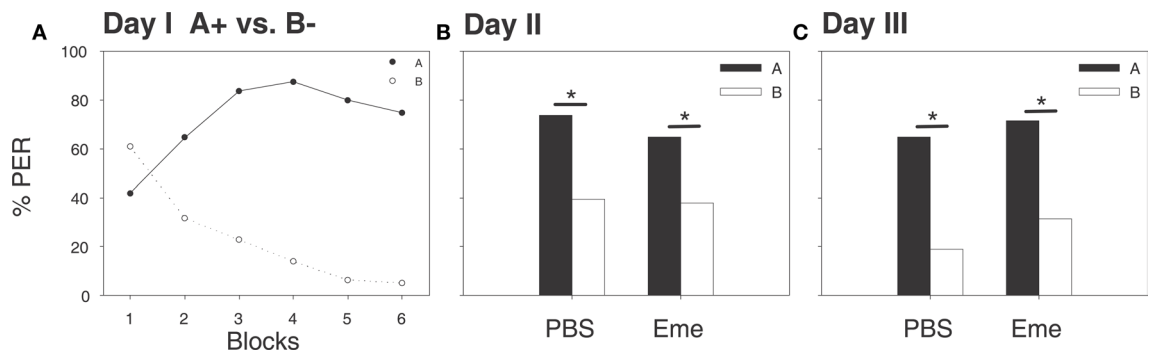

FIGURE 2 | The systemic application of emetine 30 min before a retention test has no effect on memory retrieval after 24 h. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by the two odorants A (filled shapes and bars) and B (open shapes and bars). **(A)** On day 1, bees were untreated and trained to differential conditioning (A+ vs. B–) each stimulus was presented six times, shown here in six blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B–). For the last trial a significant difference in the percentage of bees exhibiting the CR between odors was observed (McNemar's Test, $p < 0.001$). **(B)** On day 2, 30 min after emetine or PBS injections, a retention test was carried out in the absence of reward. Both groups exhibited a significant preference for odor A (McNemar's Test – PBS group: $p < 0.05$; Eme group: $p < 0.01$). **(C)** On day 3, all groups were subjected to another retention test for both odorants. Both groups scored significantly higher for odor A than for odor B (McNemar's Test – PBS group: $N = 37$, $p < 0.05$; Eme group: $N = 35$, $p < 0.01$).

being injected with emetine and the other with PBS. At the retention test both groups scored significantly higher to odor A than to odor B, as shown by their CRs; PBS group 73 and 39%, respectively (McNemar's Test: $\chi^2 = 8.47$, $p < 0.005$, df = 1), emetine group 75 and 27%, respectively (McNemar's Test: $\chi^2 = 13.13$, $p < 0.001$, df = 1; **Figure 2B**). There was no significant difference in the proportions of bees exhibiting a CR between the two experimental groups (*G* test odor A: G PBS vs. G Eme = 0.039, NS, df = 1; *G* test odor B: G PBS vs. G Eme = 1.31, NS, df = 1). Another retention test was carried out on the third day. Again both groups scored significantly higher for odor A than for odor B, PBS group 64 and 37%, respectively (McNemar's Test: $\chi^2 = 5.78$, $p < 0.05$, df = 1) emetine group 71 and 31%, respectively (McNemar's Test: $\chi^2 = 7.68$, $p < 0.01$, df = 1; **Figure 2C**). Again both groups did not differ in their proportion of bees exhibiting a CR to both odorants (*G* test odor A: G PBS vs. G Eme = 0.035, NS, df = 1; *G* test odor B: G PBS vs. G Eme = 0.032, NS, df = 1). A general non-specific effect of emetine on learning and memory can thus be ruled out.

WINTER EXPERIMENTS

Emetine inhibits the new inhibitory learning when applied in winter

The same protocol was applied to winter bees (**Figure 3**). By the last differential learning trials on day 1 the proportions of bees exhibiting the CR to the A+ and B− were 63 and 7%, respectively (McNemar's Test: $\chi^2 = 68.01$, $p = <0.001$, df = 1; **Figure 3A**). On the following day, 30 min prior to reversal learning training, bees were randomly assigned to two groups; one being injected with emetine and the other with PBS (phosphate buffer used as saline for emetine). All bees were then trained the reversed rule (A− vs. B+). By the last differential learning trials the proportions of bees exhibiting the CR to the A− and B+ were 22 and 57% in the PBS group (McNemar's Test: $\chi^2 = 14.06$, $p = <0.001$, df = 1), respectively, and 25 and 61% in the emetine group (McNemar's Test: $\chi^2 = 17.05$, $p = <0.001$, df = 1), respectively (**Figure 3B**). As in the summer experiments, emetine injections had no effect on the acquisition curves during this phase. On the third day a retention test for both odorants was carried out (**Figure 3C**). As in summer bees, the group injected with PBS scored significantly higher for odor B than for odor A (McNemar's Test: $\chi^2 = 9.091$, $p < 0.05$, df = 1) indicating that bees learned to associate odor B with reward. As in summer, no significant difference between the two odorants was observed in the emetine injected group (McNemar's Test: $\chi^2 = 0.571$, NS, df = 1). However, as opposed to the results achieved in the summer where emetine inhibited the excitatory association, here the emetine injected group scored significantly higher for odor A, when compared with the PBS group (*G* test: $G = 4.0422$, $p < 0.05$, df = 1).

Control experiments: when applied 24 h after differential conditioning, emetine has no effect on memory retrieval

On the first experimental day, winter bees underwent a differential conditioning protocol, as described above. By the last differential learning trials the proportions of bees exhibiting CRs to the A+ and B− were 65 and 15%, respectively (McNemar's Test: $\chi^2 = 27.57$, $p < 0.001$, df = 1; **Figure 4A**). On the following day, 30 min prior to a retention test, bees were randomly assigned to two groups; one being injected with emetine and the other with PBS. At the retention test both groups scored significantly higher to odor A than to odor B; PBS group 64 and 38%, respectively (McNemar's Test: $\chi^2 = 4.26$, $p < 0.05$, df = 1), emetine group 74 and 42%, respectively (McNemar's Test: $\chi^2 = 4.76$, $p < 0.05$, df = 1; **Figure 4B**). There was no significant difference in the proportions of bee exhibiting the CR between the two experimental groups (*G* test odor A: PBS vs. Eme = 0.74, NS, df = 1; *G* test odor B: PBS vs. Eme = 0.15, NS, df = 1). Another retention test was carried out on the third day, again, both groups scored significantly higher for odor A than for odor B, PBS group 70 and 41%, respectively (McNemar's Test: $\chi^2 = 5.06$; $p < 0.05$; df = 1) emetine group 73 and 38%, respectively

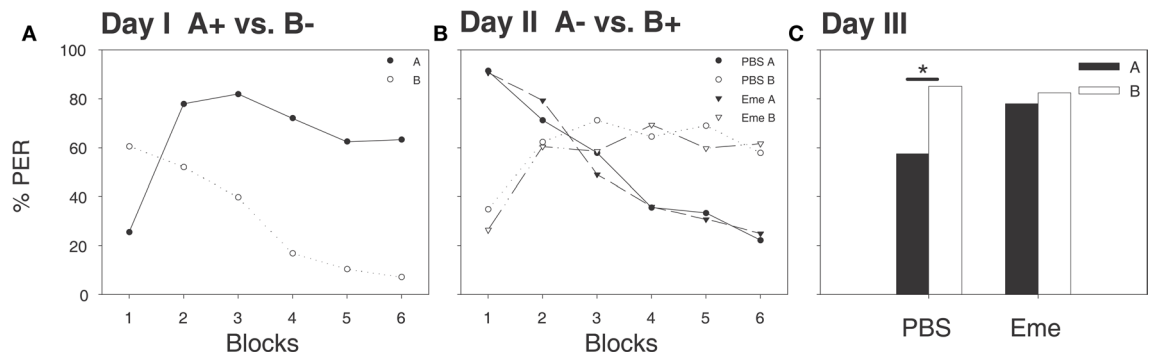

FIGURE 3 | In winter the systemic application of emetine 30 min before reversal learning inhibits consolidation of the new inhibitory learning. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by the two odorants A (filled shapes and bars) and B (open shapes and bars). **(A)** On day 1, bees were untreated and differentially conditioned (A+ vs. B−) each stimulus was presented six times, shown here in six blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B−). At the last trials a significant difference in CRs between odors was observed (McNemar's Test, $p < 0.005$). **(B)** On day 2, 30 min after emetine (triangles) or PBS (circles) injections, a reversal protocol was applied (A− vs. B+), each stimulus was presented six times, shown here in six blocks, (solid lines for A+ vs. dashed lines for B−), at the last trials both PBS and emetine groups exhibited a reversed preference (McNemar's Test, $p < 0.05$ for both groups). **(C)** On day 3, all groups were subjected to retention tests for both odorants in the absence of a reward. The PBS group showed a significant preference for odor B (McNemar's Test, $N = 40$, $p < 0.05$), the emetine group showed no preference (McNemar's Test, NS, $N = 45$) and scored significantly higher for odor A than PBS group (*G* test, $p < 0.05$).

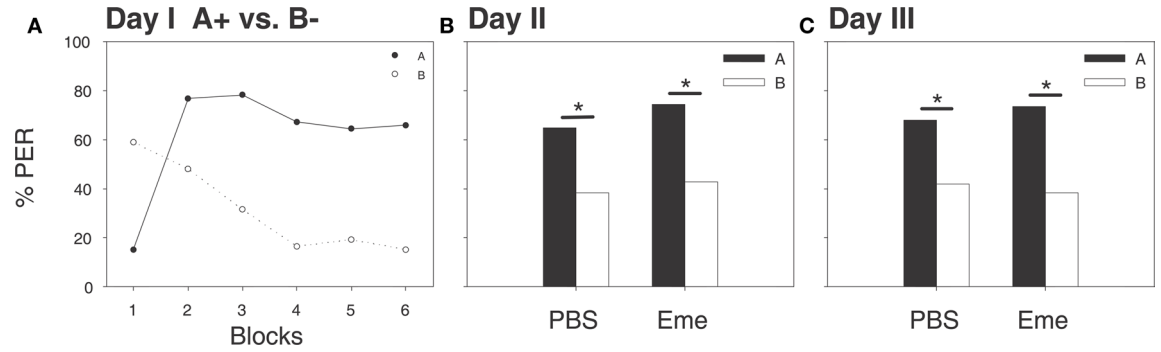

FIGURE 4 | The systemic application of emetine 30 min before a retention test has no effect on memory retrieval after 24 h. Shown are percentages of bees which exhibited proboscis extension responses (PER) evoked by the two odorants A (filled shapes and bars) and B (open shapes and bars). **(A)** On day 1, bees were untreated and differentially conditioned (A+ vs. B–) each stimulus was presented six times, shown here in six blocks, (solid line and filled circles for A+ vs. dashed line and open circles for B–). At the last trials a significant difference in CRs between odors was observed (McNemar's Test, $p < 0.001$). **(B)** On day 2, 30 min after emetine or PBS injections, a retention test was carried out in the absence of reward; both groups exhibited a significant preference for odor A (McNemar's Test: PBS group: $N = 31$, $p < 0.05$; Eme group $N = 34$, $p < 0.01$). **(C)** On day 3, all groups were subjected to another retention test for both odorants. Both groups scored significantly higher for odor A than for odor B (McNemar's Test: PBS group: $p < 0.05$; Eme group: $p < 0.01$).

(McNemar's Test: $\chi^2 = 6.05$, $p < 0.05$, df = 1; **Figure 4C**). Again both groups did not differ in the proportion of bees exhibiting the CR to both odorants (*G* test odor A: G PBS vs. G Eme = 0.05, NS, df = 1; *G* test odor B: G PBS vs. G Eme = 0.09, NS, df = 1). As in summer, a general non-specific effect of emetine on learning and memory can thus be ruled out.

DISCUSSION

Two learning processes take place while an animal experiences a reversed CS–US contingency: a new excitatory learning and a new extinction learning of the original memory. Unlike a regular extinction, reversal learning involves the continued delivery of a reinforcer and a manifestation of a new preference is hence formed. It has been long known that new memories must be stabilized if they are to persist; this process is called consolidation and requires a cascade of intracellular events (McGaugh, 2000; Dudai, 2004).

The administration of amnestic agents during a discrete time window following learning can disturb the formation of long-term memories. In the honeybee, the systemic administration of emetine, a translation inhibitor, shortly before an absolute appetitive conditioning yields no effect on the learning process but blocks consolidation of long-term memory when tested at 24 h after acquisition (Stollhoff et al., 2005).

The effect of protein synthesis inhibitors on memory formation has been investigated so far in honeybees in either simple forward conditioning, or in regular extinction paradigms (Wüstenberg et al., 1998; Friedrich et al., 2004; Stollhoff et al., 2005). In addition, eLTM is affected by actinomycin D, a transcription-inhibitor, under spaced conditioning but not under massed conditioning (Menzel et al., 2001). Under a regular extinction paradigm, the emetine effect depends on the number of retrieval trials presented. When applied systematically 30 min before the presentation of two retrieval trials (non-rewarded CS presentations), it blocks the extinction learning at a 24-h retention test, whereas for five retrieval trials the spontaneous recovery at 24 h retention test is blocked (Stollhoff et al., 2005).

The present study tested the different effects of protein synthesis inhibitor on reversal learning in two groups of honeybees, summer and winter bees.

The main findings from these experiments are that the requirements for protein synthesis in winter bees and summer bees appear to differ with respect to the kind of memory consolidation. In general, emetine did not fully block reversal learning in either summer bees, or in winter bees. In summer bees emetine injected shortly before reversal learning impaired the manifestation of the new CS–US relation but did not affect the extinction of the original preference, when tested 24 h later. In the winter bees however, emetine yielded an inverse effect: the manifestation of the new CS–US relation remained intact, whereas the extinction of the original preference was blocked, when tested 24 h later. These results suggest a double dissociation with respect to the protein synthesis requirements in reversal learning: emetine targets different memories (excitatory memory vs. inhibitory memory), and this effect is different with respect to the line of bees used (summer vs. winter).

It has already been suggested that seasonal variations in honeybees might result in a range of changes from behavior over neurotransmitter and pheromones levels to protein metabolism (Crailsheim, 1986; Currie and Jay, 1988; Harris and Woodring, 1992; Balderrama et al., 1996). Winter bees used in this study were kept under rather artificial conditions. They were housed in small flight cages under circadian illumination, humidity, and temperature conditions that mimicked summer. The bees were foraging for sucrose and pollen, and the colony did not form a winter cluster. The queen continued or started to lay eggs at a low rate. It was observed that in contrast to summer bees, these bees did not form transcription-dependent lLTM after multiple spaced conditioning trials (Menzel et al., 2001). Thus the hormonal status of winter bees that are exposed to simulated summer conditions must be different from real summer bees. So far it has been believed that these differences affect consolidation of lLTM but our study shows that they also affect consolidation of translation-dependent eLTM

in a learning-dependent way. In the future it would be interesting to investigate the effects of transcription inhibitors on the lLTM of reversal learning.

Translation-dependent memory consolidation requires existing mRNA and a mechanism that targets the synthesized proteins to the respective synaptic sites. Our findings suggest that the excitatory and inhibitory memory traces after olfactory reversal conditioning are differently dependent on cellular mechanisms that express the seasonal hormonal changes. In *Drosophila* the short-term memory trace of excitatory aversive conditioning and that of extinction learning of such excitatory learning (thus a form of inhibitory learning) appear to depend on different molecular mechanisms of the same neurons, the gamma lobe Kenyon cells of the MB (Schwärzel et al., 2002). It is also known in *Drosophila* that the transition from short-term to long-term olfactory aversive memory is accompanied by a shift from gamma lobe related Kenyon cells of the MB to vertical lobe related cells (Pascual and Preat, 2001). It is not known, however, whether the consolidation of such excitatory and inhibitory memory traces in *Drosophila* involves only translation or both transcription and translation. We also do not know yet for the bee whether the effects we see may also require transcription. Recently, using a series of single-gene *Drosophila* mutants, Qin and Dubnau (2010) found that extinction of olfactory aversive 1 day memory depends on different molecular mechanisms than those involved in associative learning. Other supporting evidence for the dissociation between classical learning and extinction learning arise from vertebrate studies in which pharmacological and genetics disruptions were shown to affect extinction but not classical conditioning (e.g., Cain et al., 2002; Marsicano et al., 2002). Taken together, we interpret our data as supporting the concept developed for *Drosophila* with respect to the mechanistic and molecular separations between excitatory and inhibitory memory traces. If the transition from short to long-term memory would lead to a separation at the network level, a specific control by hormonal factors expressing the differences between summer and winter bees may be more easily understood. In such a scenario the transfer of excitatory and inhibitory memory traces to the specific networks for long-term memory store would be differently controlled by these hormonal factors.

In this study, differences between summer and winter bees are also evident in the acquisition curves and the retention tests of the reversal learning, irrespective of the experimental groups. Summer bees display general lower levels of proboscis extension response during the acquisition of the reversal learning, compared to winter bees. This also holds true for the retention test on the third day. Such a disparity might result from different brain levels of the biogenic amine octopamine, which is known to influence response threshold to sucrose (Page and Erber, 2002), and its brain levels are correlated both with age and behavioral specialization of bees (Schulz and Robinson, 1999; Wagener-Hulme et al., 1999). In addition, injections of octopamine to specific brain regions served as a substitute for sucrose in an associative learning (Hammer and Menzel, 1998), again pointing to its involvement in the processing of sucrose reward.

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A neurogenetic dissociation between punishment-, reward-, and relief-learning in *Drosophila*

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What is particularly worth remembering about a traumatic experience is what brought it about, and what made it cease. For example, fruit flies avoid an odor which during training had *preceded* electric shock *punishment*; on the other hand, if the odor had *followed* shock during training, it is later on approached as a signal for the *relieving* end of shock. We provide a neurogenetic analysis of such relief learning. Blocking, using UAS-*shibire*^{ts1}, the output from a particular set of dopaminergic neurons defined by the TH-Gal4 driver partially impaired punishment learning, but left relief learning intact. Thus, with respect to these particular neurons, relief learning differs from punishment learning. Targeting another set of dopaminergic/serotonergic neurons defined by the DDC-Gal4 driver on the other hand affected neither punishment nor relief learning. As for the octopaminergic system, the *tbh*^{M18} mutation, compromising octopamine biosynthesis, partially impaired sugar-reward learning, but not relief learning. Thus, with respect to this particular mutation, relief learning, and reward learning are dissociated. Finally, blocking output from the set of octopaminergic/tyraminergic neurons defined by the TDC2-Gal4 driver affected neither reward, nor relief learning. We conclude that regarding the used genetic tools, relief learning is neurogenetically dissociated from both punishment and reward learning. This may be a message relevant also for analyses of relief learning in other experimental systems including man.

Keywords: dopamine, fruit fly, octopamine, olfaction, reinforcement signaling, relief learning

INTRODUCTION

Having no idea as to what will happen next is not only bewildering, but can also be dangerous. This is why animals learn about the predictors for upcoming events. For example, a stimulus that had preceded a traumatic event can be learned as a predictor for this event and is later on avoided. Such predictive learning qualitatively depends on the relative timing of events: a stimulus that occurred once a traumatic event had subsided later on supports opposite behavioral tendencies, such as approach, as it signals what may be called *relief* (Solomon and Corbit, 1974; Wagner, 1981) or *safety* (Sutton and Barto, 1990; Chang et al., 2003). Such opposing memories about the beginning and end of traumatic experiences are common to distant phyla (e.g., dog: Moskovitch and LoLordo, 1968, rabbit: Plotkin and Oakley, 1975, rat: Maier et al., 1976, snail: Britton and Farley, 1999, adult fruit fly: Tanimoto et al., 2004; Yarali et al., 2008, 2009; Murakami et al., 2010, larval fruit fly: Khurana et al., 2009), including man (Andreatta et al., 2010). This timing-dependency may reflect a universal adaptation to what one may call the "causal texture" of the world, such that whatever precedes X is likely to be the cause of X, and whatever follows X may be responsible for X's disappearance (Dickinson, 2001). Correspondingly, pleasant experiences, too, support opposing kinds of memory for stimuli that respectively precede and follow them (e.g., pigeon: Hearst, 1988; honeybee: Hellstern et al., 1998). Thus, to fully appreciate the behavioral consequences of affective experiences, it is necessary to study the mnemonic effects of their beginning and their end.

To do so, the fruit fly offers a fortunate possibility for fine grained behavioral analyses, combined with a small, experimentally accessible brain. Once trained with odor-electric shock pairings, fruit flies avoid this odor as a signal for *punishment* (Tully and Quinn, 1985); training with a reversed timing of events, that is first shock and then the odor, on the other hand, results in approach toward this odor as a predictor for *relief* (in adults: Tanimoto et al., 2004; Yarali et al., 2008, 2009; Murakami et al., 2010; in larvae: Khurana et al., 2009). Presenting an odor together with a sugar *reward* establishes conditioned approach, too (Tempel et al., 1983).

Punishment and reward learning are well-studied, including how the respective kinds of reinforcement are signaled. Shock activates a set of fruit fly dopaminergic neurons (Riemensperger et al., 2005), defined by the TH-Gal4 driver; blocking the output from these neurons impairs punishment learning, but not reward learning (in adults: Schwaerzel et al., 2003; Aso et al., 2010; in larvae: Honjo and Furukubo-Tokunaga, 2009; Selcho et al., 2009; regarding the former larval study, Gerber and Stocker (2007) filed caveats which may challenge the associative nature of the used paradigm). Also, loss of function of the dopamine receptor DAMB selectively impairs punishment rather than reward learning in fruit fly larvae (Selcho et al., 2009). Accordingly, in the cricket and the honey bee as well, punishment rather than reward learning is impaired by dopamine receptor antagonists (Unoki et al., 2005, 2006; Vergoz et al., 2007). Finally, activating a set of dopaminergic neurons, defined by the TH-Gal4 driver in adult (Claridge-Chang et al., 2009; Aso et al., 2010) and reportedly also in larval (Schroll et al., 2006) fruit flies

substitutes for punishment during training. Altogether, these results point to dopamine as covered by the applied genetic tools, to be necessary and sufficient to signal punishment.

As for reward signaling, this reinforcing role seems to be fulfilled by octopamine. In the honeybee, activity of a sugar responsive octopaminergic neuron "VUMmx1," innervating the olfactory pathway, is sufficient to substitute for the rewarding, but not the reflex-releasing, effects of sugar during training (Hammer, 1993), as does injecting octopamine at various sites along the olfactory pathway (Hammer and Menzel, 1998). In turn, interfering with the honey bee or cricket octopamine receptors impairs reward learning, but leaves punishment learning intact (Farooqui et al., 2003; Unoki et al., 2005, 2006; Vergoz et al., 2007). Accordingly, in the fruit fly, compromising octopamine biosynthesis via the *tbh*^{M18} mutation impairs reward learning, but not punishment learning (Schwaerzel et al., 2003; Sitaraman et al., 2010). Finally, in larval fruit flies, the output from a particular set of octopaminergic/tyraminergic neurons, defined by the TDC2-Gal4 driver seems to be required selectively for reward learning (see Honjo and Furukubo-Tokunaga, 2009, but see above); in turn, activating these neurons reportedly substitutes for the reward during training (Schroll et al., 2006).

These findings together suggest a double dissociation between the roles of dopamine and octopamine in signaling punishment and reward, respectively. This double dissociation however may need qualification, as the function of the fruit fly dopamine receptor dDA1 turns out to be required for both kinds of learning (in adults: Kim et al., 2007; in larvae: Selcho et al., 2009). The picture becomes more complicated with the additional role of dopaminergic neurons in signaling the state of hunger, which is a determinant for the behavioral expression of the sugar-reward memory in adult fruit flies (Krashes et al., 2009; in other insects, too, octopamine and dopamine affect the behavioral expression of memory, Farooqui et al., 2003; Mizunami et al., 2009; also in crabs: Kaczer and Maldonado, 2009). Finally, in a fruit fly operant place learning paradigm, where high temperature acts as punishment and preferred temperature as potential reward, neither dopamine nor octopamine signaling seems to be critical (Sitaraman et al., 2008, 2010). Thus, the scope of what octopamine and dopamine do for punishment and reward learning, memory, and retrieval remains open, including (except for the seminal case of the VUMmx1 neuron in the bee, Hammer, 1993, and a recent study on dopaminergic signaling in the fly, Aso et al., 2010) the assignment of these putative roles to specific amine-releasing and receiving neurons and the receptors involved, as well as the utility of the genetic tools available. Here, we ask for the neurogenetic bases of relief learning, comparing the underpinnings of relief learning to punishment and reward learning.

MATERIALS AND METHODS

FLIES

Drosophila melanogaster were reared as mass culture at 25°C, 60–70% relative humidity, under a 14:10 h light:dark cycle.

We used *shibire*^{ts1} for temperature-controlled, reversible blockage of synaptic output (Kitamoto, 2001). *shibire*^{ts1} expression was directed to different sets of neuron by crossing the males of the respective Gal4 strains (**Table 1**) to females of a UAS-*shibire*^{ts1} strain (Kitamoto, 2001; first and third chromosomes); thus the offspring were heterozygous for both the Gal4-driver and UAS-*shibire*^{ts1}. We refer to these flies with the name of the Gal4-driver together with "*shi*^{ts1}" (e.g., "TH/*shi*^{ts1}"). To obtain proper genetic controls, we crossed each of the UAS-*shibire*^{ts1} or the Gal4-driver strains to *white*^{1118} flies, thus obtaining flies heterozygous either for the Gal4-driver or for UAS-*shibire*^{ts1}. We refer to these as, e.g., "TH/+" and "*shi*^{ts1}/+," respectively.

To approximate the patterns of Gal4 expression, we used the respective drivers (**Table 1**) to express the UAS-controlled transgene *mCD8GFP*, which encodes for a green fluorescent protein (GFP) to insert into cellular membranes. To do this, we crossed males from each driver strain to females of a UAS-*mCD8GFP* strain (Lee and Luo, 1999; second chromosome) and stained the brains of the progeny against the Synapsin protein to visualize the neuropils and against GFP to approximate the pattern of Gal4 expression. Note however that the pattern of GFP-immunoreactivity does not necessarily reflect which neurons would be targeted had another effector, e.g., *shibire*^{ts1} been expressed using the same Gal4 driver (Ito et al., 2003): first, UAS-*mCD8GFP* and UAS-*shibire*^{ts1} may support different levels and patterns of background expression without any Gal4; this background expression then adds up with the driven expression when the Gal4 is present. Second, the level of *mCD8GFP* expression sufficient for immunohistochemical detection may well be different

Table 1 | The Gal4 driver strains that were used.

	Gal4 driver	Gal4 expression in	Chromosome	References
TH	Regulatory sequences of *t*yrosine *h*ydroxylase gene	Dopaminergic neurons	Third	**Friggi-Grelin et al. (2003),** Schwaerzel et al. (2003), Riemensperger et al. (2005), Schroll et al. (2006), Zhang et al. (2007), Sitaraman et al. (2008), Claridge-Chang et al. (2009), Honjo and Furukubo-Tokunaga (2009), Krashes et al. (2009), Mao and Davis (2009), Selcho et al. (2009), Aso et al. (2010)
DDC	Regulatory sequences of *d*opa *dec*arboxylase gene	Dopaminergic/ serotonergic neurons	Third	**Li et al. (2000)**, Sitaraman et al. (2008)
TDC2	Regulatory sequences of the neuronal *t*yrosine *dec*arboxylase gene	Octopaminergic/ tyraminergic neurons	Second	**Cole et al. (2005)**, Schroll et al. (2006), Busch et al. (2009), Honjo and Furukubo-Tokunaga (2009), Sitaraman et al. (2010)

Bold font indicates the original report of the respective Gal4 strain.

from the level of *shibire^{ts1}* expression sufficient to block neuronal output; thus potentially, not all neurons that are visualized by immunohistochemistry may be affected by *shibire^{ts1}* or *vice versa*.

To test for an effect of an octopamine biosynthesis deficiency, we used the mutant strain *tbh^{M18}* (Monastirioti et al., 1996; also see Schwaerzel et al., 2003; Saraswati et al., 2004; Scholz, 2005; Brembs et al., 2007; Certel et al., 2007; Hardie et al., 2007; Sitaraman et al., 2010). These flies have reduced or no octopamine (Monastirioti et al., 1996), due to the deficiency of the tyramine β-hydroxylase enzyme, which catalyzes the last step of octopamine biosynthesis (**Figure 2**). Since the original *tbh^{M18}* strain (Monastirioti et al., 1996) contains an additional mutation in the *white* gene, we instead used a recombinant strain with a wild-type *white^{+}* allele, which was generated by Schwaerzel et al. (2003). As genetic control, we used a non-recombinant strain with wild-type *tbh^{+}* and *white^{+}* alleles, which was generated in parallel; we refer to this strain simply as "Control."

IMMUNOHISTOCHEMISTRY

Brains were dissected in saline and fixed for 2 h in 4% formaldehyde with PBST as solvent (phosphate-buffered saline containing 0.3% Triton X-100). After a 1.5 h incubation in blocking solution (3% normal goat serum [Jackson Immuno Research Laboratories Inc., West Grove, PA, USA] in PBST), brains were incubated overnight with the monoclonal anti-Synapsin mouse antibody SYNORF1, diluted 1:20 in PBST (Klagges et al., 1996) and polyclonal anti-GFP rabbit antibody, diluted 1:2000 in PBST (Invitrogen Molecular Probes, Eugene, OR, USA). These primary antibodies were detected after an overnight incubation with Cy3 goat anti-mouse Ig, diluted 1:250 in PBST (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) and Alexa488 goat anti-rabbit Ig, diluted 1:1000 in PBST (Invitrogen Molecular Probes, Eugene, OR, USA). All incubation steps were followed by multiple PBST washes. Incubations with antibodies were done at 4°C; all other steps were performed at room temperature. Finally, brains were mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and examined under a confocal microscope (Leica SP1, Leica, Wetzlar, Germany).

BEHAVIORAL ASSAYS

Flies were collected from fresh food vials and kept for 1–4 days at 18°C and 60–70% relative humidity before experiments. For reward learning as well as for the punishment learning experiments shown in **Figures 6B,B′**, flies were instead starved overnight for 18–20 h at 25°C and 60–70% relative humidity in vials equipped with a moist tissue paper and a moist filter paper. Those experiments that did not use *shibire^{ts1}* were performed at 22–25°C and 75–85% relative humidity. For inducing the effect of *shibire^{ts1}*, flies were first exposed to 34–36°C and 60–70% relative humidity for 30 min; then the experiment took place under these same conditions, which are referred to as "@ high temperature." The condition referred to as "@ low temperature" in turn involved exposing the flies to 20–23°C and 75–85% relative humidity for 30 min; then the experiment followed also under these conditions.

The experimental setup was in principle as described by Tully and Quinn (1985) and Schwaerzel et al. (2003). Flies were trained and tested as groups of 100–150. Trainings took place under dim red light which does not allow flies to see, tests were in complete darkness.

As odorants, 90 µl benzaldehyde (BA), 340 µl 3-octanol (OCT), 340 µl 4-methylcyclohexanol (MCH), 340 µl *n*-amyl acetate (AM) and 340 µl isoamyl acetate (IAA) (CAS 100-52-7, 589-98-0, 589-91-3, 628-63-7, 123-92-2; all from Fluka, Steinheim, Germany) were applied in 1 cm-deep Teflon containers of 5, 14, 14, 14, and 14 mm diameters, respectively. For the experiments in **Figures 6A,B,C** MCH and OCT were diluted 100-fold in paraffin oil (Merck, Darmstadt, Germany, CAS 8012-95-1), whereas for **Figures 6A′,B′**, AM and IAA were diluted 36-fold. All other experiments used undiluted BA and OCT.

For punishment learning (**Figure 1A**), flies received six training trials. Each trial started by loading the flies into the experimental setup (0:00 min). From 4:00 min on, the control odor was presented for 15 s. Then, from 7:15 min on, the to-be-learned odor was presented also for 15 s. From 7:30 min on, electric shock was applied as four pulses of 100 V; each pulse was 1.2 s-long and was followed by the next with an onset-to-onset interval of 5 s. Thus the to-be-learned odor preceded shock with an onset-to-onset interval of 15 s. The control odor on the other hand preceded the shock by an onset-to-onset interval of 210 s, which does not result in a measurable association between the two (Tanimoto et al., 2004; Yarali et al., 2008, loc. cit. Figures 1D and 2F, Yarali et al., 2009, loc. cit. Figure 1B). For relief learning (**Figure 1B**), keeping all other parameters unchanged, we reversed the relative timing of events: that is, the to-be-learned odor was presented from 8:10 min on, thus following shock with an onset-to-onset interval of 40 s. At 12:00 min, flies were transferred out of the setup into food vials, where they stayed for 16 min until the next trial. At the end of the sixth training trial, after the usual 16 min break, flies were loaded back into the setup. After a 5 min accommodation period, they were transferred to the choice point of a T-maze, where they could escape toward either the control odor or the learned odor. After 2 min, the arms of the maze were closed and flies on each side were counted. A preference index (PREF) was calculated as:

$$\mathrm{PREF} = (\#_{\mathrm{Learned\ odor}} - \#_{\mathrm{Control\ odor}}) \times 100/\#_{\mathrm{Total}} \quad (1)$$

# indicates the number of flies found in the respective maze-arm. Two groups of flies were trained and tested in parallel (**Figure 1D**). For one of these, e.g., 3-octanol (OCT) was the control odor and BA was to be learned; the second group was trained reciprocally. PREFs from the two reciprocal measurements were then averaged to obtain a final learning index (LI):

$$\mathrm{LI} = (\mathrm{PREF}_{\mathrm{BA}} + \mathrm{PREF}_{\mathrm{OCT}})/2 \quad (2)$$

Subscripts of PREF indicate the learned odor in the respective training. Positive LIs indicate conditioned approach to the learned odor; negative values reflect conditioned avoidance.

Reward learning (**Figure 1C**) used two training trials. Each trial started by loading the flies into the setup (0:00 min). One minute later, flies were transferred to a tube lined with a filter paper which was soaked the previous day with 2 ml of 2 M sucrose solution, and then was left to dry over night. This tube was scented with the to-be-learned odor. After 45 s, the to-be-learned odor was removed, and after 15 additional seconds flies were taken out of the tube. At the end of a 1 min waiting period, they were transferred into another tube lined with a filter paper which was soaked with pure water and then dried. This second tube was scented with the control

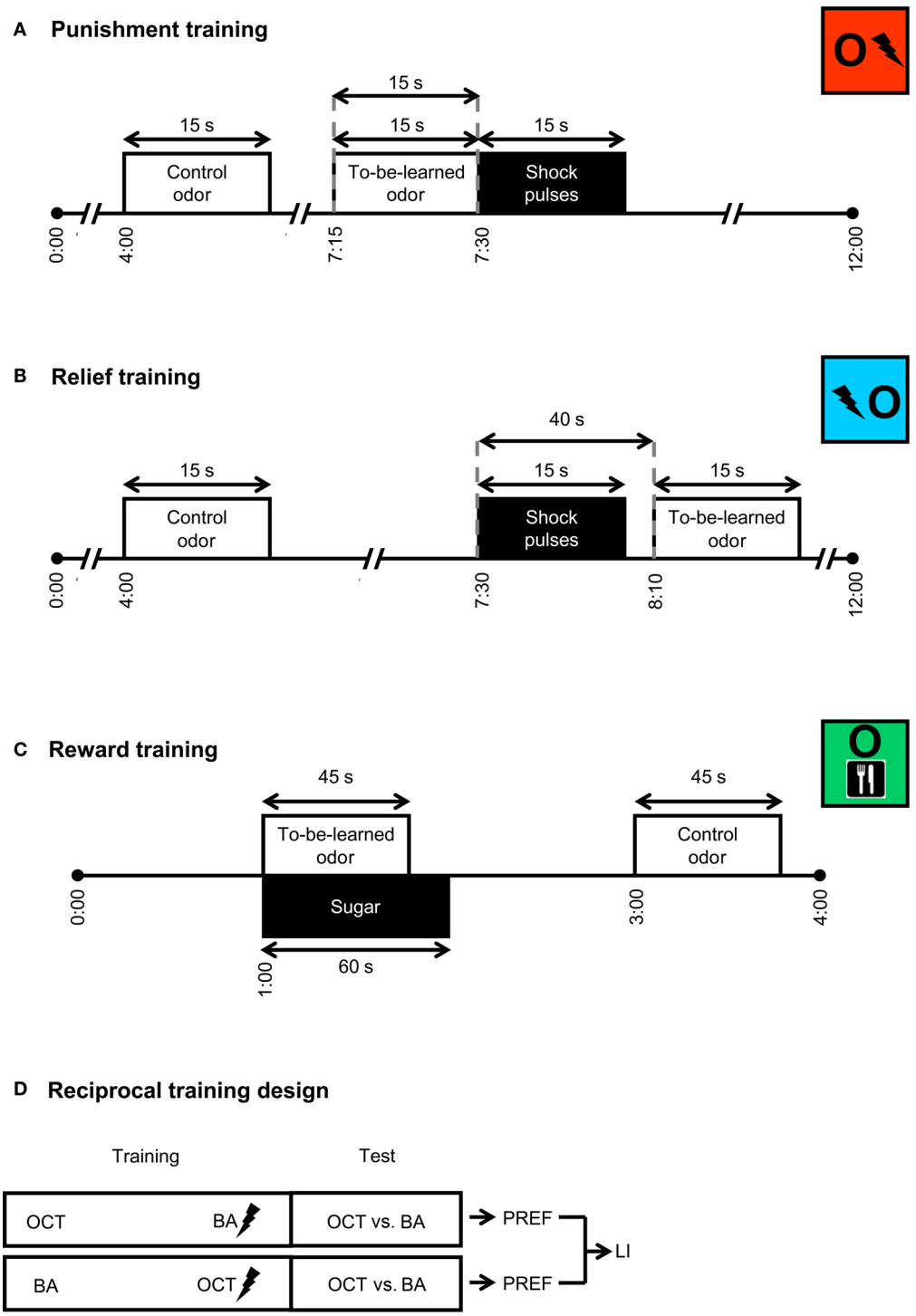

FIGURE 1 | Training. For punishment training **(A)**, flies received two odors and pulses of electric shock. A control odor was presented long before shock; a to-be-learned odor *preceded* shock with an onset-to-onset interval of 15 s. For relief training **(B)**, while all other parameters were unchanged, the to-be-learned odor *followed* shock with an onset-to-onset interval of 40 s. For reward training **(C)**, flies were successively exposed to a to-be-learned odor in the presence of sugar and then to a control odor without any sugar. Although not shown here, in half of the cases, reward training started with the control odor instead of the to-be-learned odor and sugar. For each kind of training, we used a reciprocal design **(D)**: two groups were trained in parallel; for one of these, e.g., 3-octanol (OCT) was the control odor and benzaldehyde (BA) was to be learned; the other group was trained reciprocally. Each group was then given the choice between the two odors. Based on the flies' distribution, preference indices (PREF) were calculated. Based on the two reciprocal PREF values, we calculated a learning index (LI). The situation is sketched for punishment learning, but also applies to relief and reward learning.

odor. After 45 s, control odor was removed and 15 s later, flies were taken out of this second tube. The next trial started immediately. This transfer between the two kinds of tube during training should prevent the learning of an association between the control odor and the sugar. For half of the cases, training trials started with the to-be-learned odor and sugar; in the other half, control odor was given precedence. Once the training was completed, after a 3 min waiting period, flies were transferred to the choice point of a T-maze between the control odor and the learned odor. After 2 min, the arms of the maze were closed, flies on each side were counted and a preference index (PREF) was calculated according to Eq. 1. As detailed above (also see **Figure 1D**), two groups were trained reciprocally and the LI was calculated based on their PREF values according to Eq. 2.

Finally, a modified punishment training procedure (not shown in **Figure 1**) imitated the reward learning as in **Figure 1C**, but sugar presentation was replaced by 12 pulses of 100 V electric shock, each lasting 1.2 s and separated by an onset-to-onset interval of 5 s.

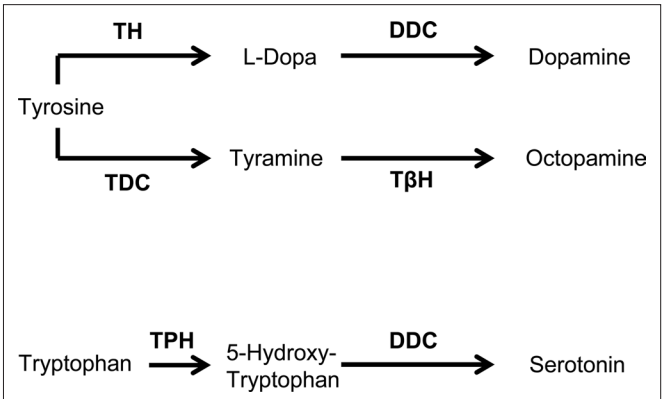

FIGURE 2 | Biosynthesis of dopamine, tyramine, octopamine, and serotonin. DDC, dopa decarboxylase; TβH, tyramine β-hydroxylase; TDC, tyrosine decarboxylase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase. Modified from Monastirioti (1999).

STATISTICS

All data were analyzed using non-parametric statistics and are reported as box plots, showing the median as the midline and 10, 90, and 25, 75% as whiskers and box boundaries, respectively. For comparing scores of individual groups to 0, we used one-sample sign tests. Mann–Whitney *U*-tests and Kruskal–Wallis tests were used for pair-wise and global between-group comparisons, respectively. When multiple tests of one kind were performed within a single experiment, we adjusted the experiment-wide error-rate to 5% by Bonferroni correction: we divided the critical $P < 0.05$ by the number of tests. One-sample sign tests were done using a web-based tool (http://www.fon.hum.uva.nl/Service/Statistics/Sign_Test.html). All other statistical analyses were performed with the software Statistica (Statsoft, Tulsa, OK, USA). Sample sizes are reported in the figure legends.

RESULTS

BLOCKING OUTPUT FROM TWO DIFFERENT SETS OF DOPAMINERGIC NEURONS

First, we compared relief learning to punishment learning in terms of the roles of dopaminergic neurons. We confirmed that blocking the output from a particular set of dopaminergic neurons, using the temperature-sensitive UAS-*shibire^{ts1}* in combination with the TH-Gal4 driver (Friggi-Grelin et al., 2003, **Table 1**; **Figures 2 and 3A**), impairs punishment learning: when trained and tested at high temperature, TH/*shi^{ts1}* flies showed less negative learning scores than the genetic controls (**Figure 4A** @ high temperature: Kruskal–Wallis test: $H = 11.44$, d.f. = 2, $P < 0.05$). This impairment in punishment learning, however, was obviously partial in the TH/*shi^{ts1}* flies (**Figure 4A** @ high temperature: one-sample sign tests: $P < 0.05/3$ for each genotype), as was the case in previous studies (Schwaerzel et al., 2003; Aso et al., 2010). This residual learning ability may be due to incomplete coverage of dopaminergic neurons by the TH-Gal4 driver (Friggi-Grelin et al., 2003; Sitaraman et al., 2008; Claridge-Chang et al., 2009; Mao and Davis, 2009; see the Discussion for details) and/or to an incomplete block of neuronal output by *shibire^{ts1}*. At low temperature, as *shibire^{ts1}* was benign, TH/*shi^{ts1}* flies performed comparably to the genetic controls in punishment learning (**Figure 4A** @ low temperature: Kruskal–Wallis test: $H = 2.06$, d.f. = 2, $P = 0.36$).

Importantly, blocking output from TH-Gal4 neurons, a treatment which did impair punishment learning, left relief learning intact: with training and test at high temperature, we found relief learning scores of TH/*shi^{ts1}* flies to be indistinguishable from the genetic controls (**Figure 4B** @ high temperature: Kruskal–Wallis test: $H = 0.10$, d.f. = 2, $P = 0.96$). Accordingly pooling the data, we found conditioned approach (**Figure 4B** @ high temperature: one-sample sign test for the pooled data set: $P < 0.05$). One might argue that the generally low relief learning scores may not allow detecting a possible partial impairment due to neurogenetic intervention. This however does not apply to **Figure 4B**, as relief learning in the TH/*shi^{ts1}* flies does not even tend to be inferior to the genetic controls (similarly, see **Figures 5B, 6C, and 7B**). We note that punishment and relief learning procedures differ only with respect to the timing of the to-be-learned odor during training; otherwise they entail the same handling and stimulus–exposure. Therefore, intact relief learning in the TH/*shi^{ts1}* flies (**Figure 4B**) excludes sensory and/or motor problems as potential cause for the impairment in punishment learning (**Figure 4A**, left).

Next, we used an independent driver, DDC-Gal4 (Li et al., 2000; **Table 1**; **Figures 2 and 3B**), to express UAS-*shibire^{ts1}* in a set of dopaminergic/serotonergic neurons. Blocking the output from these neurons left punishment learning unaffected: when trained and tested at high temperature, DDC/*shi^{ts1}* flies showed learning scores comparable to the genetic controls (**Figure 5A** @ high temperature: Kruskal–Wallis test: $H = 2.14$, d.f. = 2, $P = 0.34$). Thus pooling the scores across genotypes, we observed conditioned avoidance (**Figure 5A** @ high temperature: one-sample sign test for the pooled data set: $P < 0.05$). This lack of effect on punishment learning may be caused by (i) the DDC-Gal4 driver not covering all dopaminergic neurons; (ii) incomplete overlap to those dopaminergic neurons targeted by the TH-Gal4 (Sitaraman et al., 2008; Claridge-Chang et al., 2009; Mao and Davis, 2009; see the

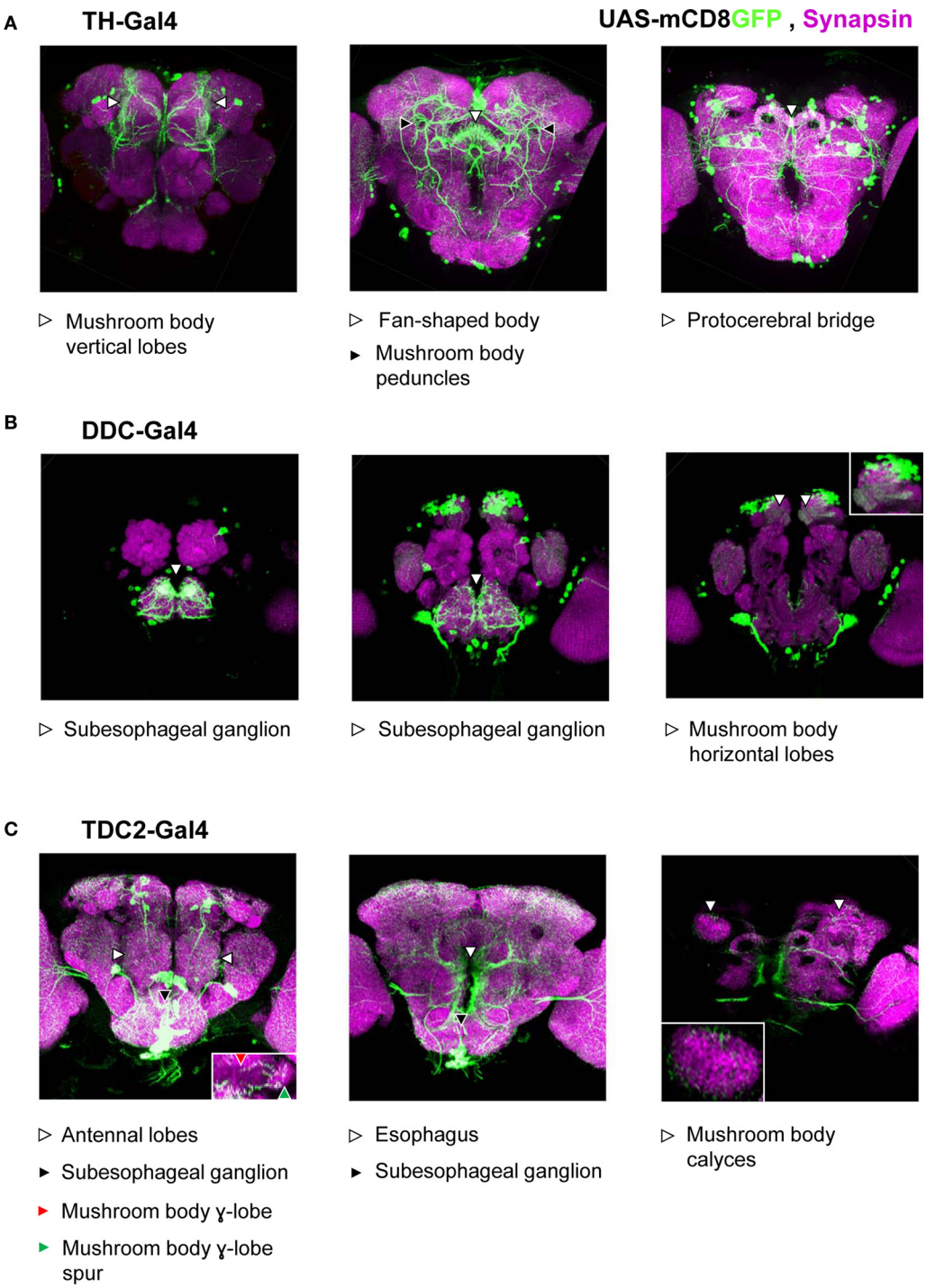

FIGURE 3 | Approximated patterns of Gal4 expression by the used drivers. We drove the expression of a membrane bound green fluorescent protein (mCD8GFP) using three different Gal4 drivers. Patterns of GFP-immunoreactivity (green) should approximate the respective patterns of Gal4-expression; Synapsin-immunoreactivity (magenta) shows the organization of the neuropils. We display projections of frontal optical sections of 0.9 µm, each. In each row, the leftmost panel shows the anterior-most projection; in each panel, dorsal is to the top. When driven by TH-Gal4 **(A)**, GFP was expressed in neurons that innervate the mushroom body vertical lobes and peduncles (left and middle panels) as well as the fan-shaped body (middle panel) and the protocerebral bridge (right panel). We found no innervation of the antennal lobes or the mushroom body calyces (but see Mao and Davis, 2009). Under the control of the DDC-Gal4 driver **(B)**, GFP was expressed in neurons that innervate the subesophageal ganglion (left and middle panels) as well as the horizontal lobes of the mushroom body (right; see also the inset). Neurons that express GFP, driven by TDC2-Gal4 **(C)** innervated the antennal lobes (left panel), mushroom body γ-lobes and their spurs (left panel, inset), the subesophageal ganglion (left and middle panels), the areas surrounding the esophagus (middle panel), and the mushroom body calyces (right panel; see also the inset).

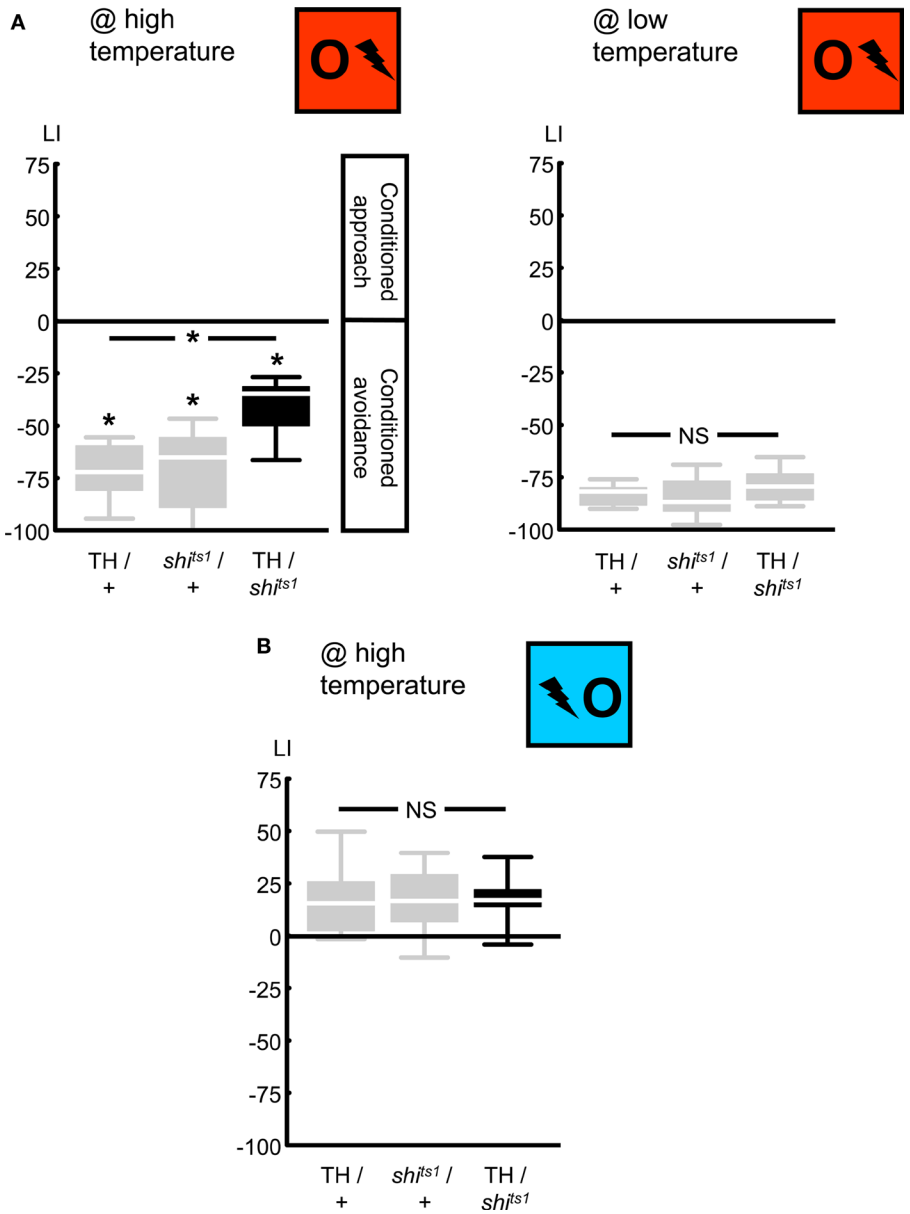

FIGURE 4 | Targeting a set of dopaminergic neurons, using the TH-Gal4 driver. We expressed *shibire*^{ts1} in the set of dopaminergic neurons defined by the TH-Gal4 driver. Punishment learning was partially impaired at high temperature **(A**, left**)**, but not at low temperature **(A**, right**)**. Contrarily, relief learning remained unaffected even at high temperature **(B)**. *$P < 0.05$ and NS: $P > 0.05$ while comparing between genotypes. While comparing scores of each genotype to 0 *$P < 0.05/3$, to keep the experiment-wide error-rate at 5% (i.e., Bonferroni correction). Sample sizes were $N = 8$, each in **(A)** and 13, each in **(B)**. Box plots show the median as the midline; 25 and 75% as the box boundaries and 10 and 90% as whiskers.

Discussion for details), (iii) incomplete block of synaptic output by *shibire*^{ts1}; (iv) a dominant-negative effect of DDC-Gal4, which is non-additive with the effect of *shibire*^{ts1} expression in these neurons (see below).

In any case, we probed for an effect of blocking output from the DDC-Gal4 neurons on relief learning and found none: after training and test at high temperature, learning scores were not different between genotypes (**Figure 5B** @ high temperature: Kruskal–Wallis test: $H = 1.24$, d.f. $= 2$, $P = 0.54$). We thus pooled the data and found weak yet significant conditioned approach (**Figure 5B** @ high temperature: one-sample sign test for the pooled data set: $P < 0.05$). We note that the DDC/+ flies tended to show less pronounced punishment and relief learning when compared to the TH/+ flies (compare **Figure 4** *versus* **Figure 5**) as well as when compared to the *shi*^{ts1}/+ flies (**Figure 5**). In the case of punishment learning, as we used a Kruskal–Wallis test across all three experimental groups, this effect of the DDC-Gal4 driver construct may have obscured an actual effect of blocking the output from DDC-Gal4-targeted neurons (compare *shi*^{ts1}/+ to DDC/*shi*^{ts1} in **Figure 5A**). For relief learning, however, no corresponding trend is noted (compare

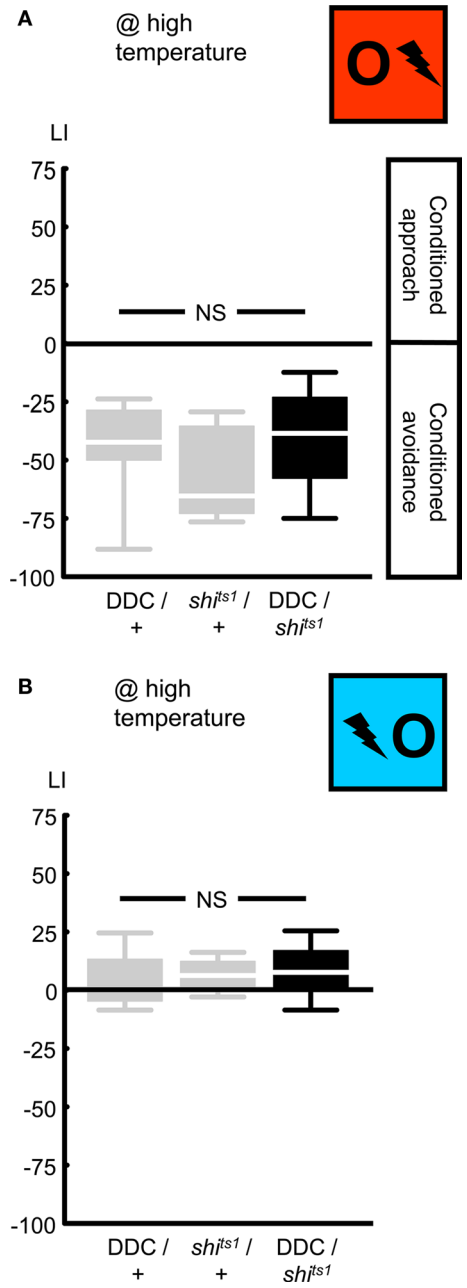

FIGURE 5 | Targeting a set of dopaminergic/serotonergic neurons, using the DDC-Gal4 driver. We expressed *shibire*^{ts1} in the set of dopaminergic/serotonergic neurons defined by the DDC-Gal4 driver. At high temperature, neither punishment learning **(A)**, nor relief learning **(B)** was affected. NS: $P > 0.05$, while comparing between genotypes. Sample sizes were from left to right $N = 13, 11, 12$ in **(A)** and 12, 11, 12 in **(B)**. Box plots are as detailed in **Figure 4.**

shi^{ts1}/+ to DDC/*shi*^{ts1} in **Figure 5B**). In any case, with respect to the role of the neurons defined by DDC-Gal4, our results do not offer an argument to dissociate punishment from relief learning.

To summarize, concerning the neurons defined by TH-Gal4, we found a clear dissociation between punishment and relief learning (**Figure 4**), while for the DDC-Gal4 neurons the situation remains inconclusive (**Figure 5**). We would like to stress that this does not at all exclude a role for the dopaminergic system in relief learning, given that first, in neither experiment did we cover all dopaminergic neurons at once, and second, as a general concern, blockage of neuronal output by *shibire*^{ts1} may well be incomplete (see the Discussion for details).

COMPROMISING OCTOPAMINE BIOSYNTHESIS

Next, we compared relief learning to reward learning in terms of the role of octopamine. We first confirmed that compromising octopamine biosynthesis via the *tbh*^{M18} mutation in the key enzyme tyramine β-hydroxylase (Monastirioti et al., 1996; **Figure 2**) impairs reward learning: after odor-sugar training, using the odors 3-octanol (OCT) and 4-methylcyclohexanol (MCH), the *tbh*^{M18} mutant showed significantly less conditioned approach than the genetic Control (**Figure 6A**: *U*-test: $U = 544.00$, $P < 0.05$). Residual reward learning ability was however detectable in the *tbh*^{M18} mutant (**Figure 6A**: one-sample sign tests: $P < 0.05/2$ for each genotype). This contrasts to the report of Sitaraman et al. (2010), who had shown a complete loss of reward learning using the same odors; the discrepancy may be due to the different genetic backgrounds used in the two studies (i.e., the present study uses the strains from Schwaerzel et al., 2003, whereas Sitaraman et al., 2010 uses those from Certel et al., 2007). Schwaerzel et al. (2003) found no reward learning ability in the *tbh*^{M18} mutant, using the odors ethyl acetate and isoamyl acetate (IAA); indeed, using *n*-amyl acetate (AM) and IAA as odors, we also found a complete loss of reward learning in the *tbh*^{M18} mutant (**Figure 6A′**: *U*-test: $U = 33.00$, $P < 0.05$; one-sample sign tests: $P < 0.05/2$ for Control, and $P = 0.58$ for the *tbh*^{M18} mutant). Surprisingly however, when the odors OCT and benzaldehyde (BA) were used, *tbh*^{M18} mutant flies showed fully intact reward learning (**Figure 6A″**: *U*-test: $U = 204.50$, $P = 0.27$; one-sample sign test for the pooled data set: $P < 0.05$). This lack of effect in **Figure 6A″** should not be due to the relatively low learning indices of the Control flies, since in **Figure 6A**, we could detect even a partial effect of the *tbh*^{M18} mutation despite such low Control scores. Note that using the present two-odor reciprocal training design (**Figure 1D**), the contribution of each odor to the LI, and hence the question whether the *tbh*^{M18} mutation affects learning about any one given odor but not the other, remains unresolved. We can however conclude that the reward learning impairment of the *tbh*^{M18} mutant can be partial, complete, or absent, depending on the combination of odors used and likely also on the genetic background; this suggests residual octopaminergic function and/or an octopamine-independent compensatory mechanism (see the Discussion for details).

To test for an effect of the *tbh*^{M18} mutation on punishment learning, we used a modified training, which entailed the same pre-starvation, handling, and stimulus–exposure as reward learning, except the sugar presentation was replaced by shock pulses. In such modified punishment learning, the *tbh*^{M18} mutant performed comparably to the genetic Control, using either the odors OCT and MCH (**Figure 6B**: *U*-test: $U = 47.00$, $P = 0.15$; one-sample sign test for the pooled data set: $P < 0.05$) or AM and IAA (**Figure 6B′**: *U*-test: $U = 38.00$, $P = 0.82$; one-sample sign test for the pooled data set: $P < 0.05$). Thus, confirming Schwaerzel et al. (2003), we can conclude that reward and punishment learning are dissociated in terms

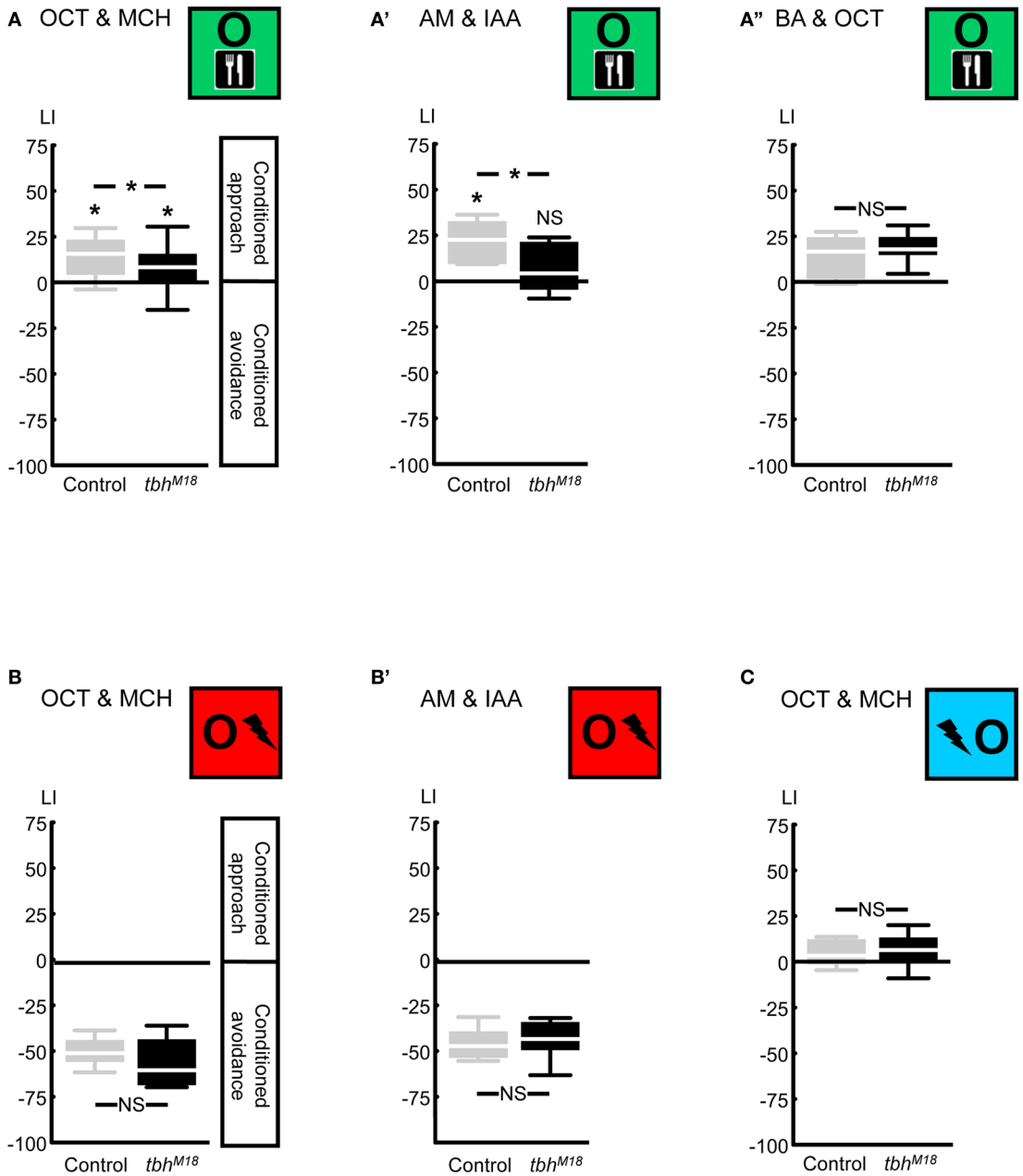

FIGURE 6 | Compromising octopamine biosynthesis using the *TβH* mutant. We used the *tbh*^{M18} mutant, which has reduced or no octopamine. When the odors 3-octanol (OCT) and 4-methylcyclohexanol (MCH) were used, reward learning was partially impaired **(A)**. Using the odors *n*-amyl acetate (AM) and isoamyl acetate (IAA) revealed complete lack of reward learning in the *tbh*^{M18} mutant **(A′)**. When the odors OCT and benzaldehyde (BA) were used, *tbh*^{M18} mutant was intact in reward learning **(A″)**. A modified punishment learning procedure, which was identical to reward learning, except that the shock pulses were replaced by sugar presentation, revealed no impairment in the *tbh*^{M18} mutant, when either the odors OCT and MCH **(B)** or AM and IAA **(B′)** were used. Finally, under those conditions for which reward learning of the *tbh*^{M18} mutant was partially impaired, i.e., using the odors OCT and MCH, relief learning remained unaffected **(C).** For this experiment, the odors AM and IAA were not used, as these do not support relief learning (Yarali et al., 2008, loc. cit. Figure 5D). $*P < 0.05$, NS: $P > 0.05$, while comparing between genotypes. While comparing scores of each genotype to 0 $*P < 0.05/2$, NS: $P > 0.05/2$ (i.e., Bonferroni correction). Sample sizes were from left to right $N = 40$, 39 in **(A)**, 11, 13 in **(A′)**, 23, 22 in **(A″)**, 12, 12 in **(B)**, 9, 9 in **(B′),** and 20, 20 in **(C)**. Box plots are as detailed in **Figure 4.**

of the effect of the *tbh*^{M18} mutation. In addition, normal performance of the *tbh*^{M18} mutant in this modified punishment learning makes deficiencies in odor perception or motor control unlikely as causes for the reward learning impairment (**Figures 6A,A′**).

In order to test for an effect of the *tbh*^{M18} mutation on relief learning, we used the odors OCT and MCH, because the odors AM and IAA do not support relief learning (Yarali et al., 2008, loc. cit. Figure 5D). Under conditions for which the *tbh*^{M18} mutant did

show a reward learning impairment, however partial (i.e., using the odors OCT and MCH), relief learning ability remained unaffected: learning scores were statistically indistinguishable between genotypes (**Figure 6C**: *U*-test: $U = 168.00$, $P = 0.40$), with no apparent trend for lower scores in the tbh^{M18} mutant. We thus pooled the data and found weak yet significant conditioned approach (**Figure 6C**: one-sample sign test for the pooled data set: $P < 0.05$).

BLOCKING THE OUTPUT FROM A SET OF OCTOPAMINERGIC/TYRAMINERGIC NEURONS

As an additional, independent assault toward the octopaminergic system, we blocked the output from a set of octopaminergic/tyraminergic neurons, using UAS-$shibire^{ts1}$, in combination with the TDC2-Gal4 driver (Cole et al., 2005; **Table 1**; **Figures 2 and 3C**). We first tested for an effect on reward learning: when trained and tested at high temperature, TDC2/shi^{ts1} flies performed comparably to the genetic controls (**Figure 7A** @ high temperature: Kruskal–Wallis test: $H = 3.03$, d.f. = 2, $P = 0.22$). Accordingly pooling the learning scores across genotypes, we found conditioned approach (**Figure 7A** @ high temperature: one-sample sign test for the pooled data set: $P < 0.05$). This lack of effect on reward learning may be because the TDC2-Gal4 driver does not target all octopaminergic neurons (Busch et al., 2009; see the Discussion for details) and/or the output from the targeted neurons is not completely blocked by the $shibire^{ts1}$.

Nevertheless, we probed for an effect on relief learning and found none: after training and test at high temperature, learning scores were statistically indistinguishable between genotypes (**Figure 7B** @ high temperature: Kruskal–Wallis test: $H = 2.43$, d.f. = 2, $P = 0.30$). Accordingly pooling the data, we found conditioned approach (**Figure 7B** @ high temperature: one-sample sign test for the pooled data set: $P < 0.05$). To summarize, while reward and relief learning are apparently dissociated when considering the tbh^{M18} mutant, we can put no distinction between these two kinds of learning in terms of the role of the neurons covered by the TDC2-Gal4 driver. Again, this does not rule out a role for the octopaminergic system in relief learning, as these conclusions refer only to the specific genetic manipulations used.

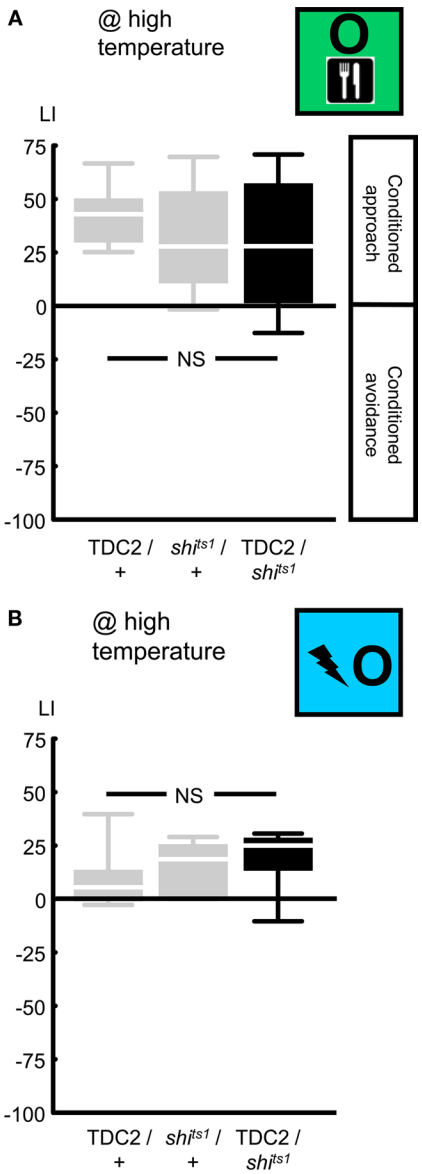

FIGURE 7 | Targeting a set of octopaminergic/tyraminergic neurons, using the TDC2-Gal4 driver. We expressed $shibire^{ts1}$ in the set of octopaminergic/tyraminergic neurons defined by the TDC2-Gal4 driver. At high temperature, neither reward learning **(A)** nor relief learning **(B)** was impaired. NS: $P > 0.05$, while comparing between genotypes. Sample sizes were from left to right $N = 24, 27, 27$ in **(A)** and 11, each in **(B)**. Box plots are as detailed in **Figure 4.**

DISCUSSION

We compared relief learning to both punishment learning and reward learning, focusing on the involvement of aminergic modulation by dopamine and octopamine.

As previously reported (Schwaerzel et al., 2003; Aso et al., 2010), directing the expression of UAS-$shibire^{ts1}$ to a particular set of dopaminergic neurons defined by the TH-Gal4 driver partially impaired punishment learning (**Figure 4A**). Relief learning however was left intact (**Figure 4B**). Expressing UAS-$shibire^{ts1}$ with another driver, DDC-Gal4, on the other hand affected neither punishment nor relief learning (**Figure 5**).

All dopaminergic neuron clusters in the fly brain are targeted by the TH-Gal4 driver; some clusters however, are covered only partially, e.g., 80–90% of the anterior medial "PAM cluster" neurons are left out (Friggi-Grelin et al., 2003; Sitaraman et al., 2008; Claridge-Chang et al., 2009; Mao and Davis, 2009). Contrarily, the DDC-Gal4 driver, along with serotonergic neurons, likely targets most of the PAM cluster dopaminergic neurons, while possibly leaving out dopaminergic neurons in other clusters (Sitaraman et al., 2008; **Figure 3B**). In a mixed classical-operant olfactory punishment learning task, Claridge-Chang et al. (2009) found no impairment upon blocking the activity of most PAM cluster neurons with an inwardly rectifying K^+ channel (UAS-*kir2.1*), driven by HL9-Gal4. Although relying on both a different Gal4 driver and a different effector, this result is in agreement with the intact punishment learning we found when expressing UAS-$shibire^{ts1}$ with the DDC-Gal4 driver (**Figure 5A**). Thus, as

far as short-term punishment learning is concerned, there is so far no evidence for a role for the PAM cluster neurons (for middle-term punishment learning, see Aso et al., 2010). Nevertheless, targeting the remaining dopaminergic neuron clusters by the TH-Gal4 driver only partially impairs punishment learning (Schwaerzel et al., 2003; Aso et al., 2010; **Figure 4A**). Conceivably, the TH-Gal4 driver may leave out few dopaminergic neurons in clusters other than PAM; these may then carry a punishment signal, redundant to that carried by the TH-Gal4-targeted neurons. This scenario would readily accommodate Schroll et al.'s (2006) report that activity of the TH-Gal4-targeted neurons in larval fruit flies substitutes for punishment. The intact relief learning upon expressing UAS-*shibire^{ts1}* with TH-Gal4 can also be explained by this scenario. Alternatively, the level of *shibire^{ts1}* expression driven by TH-Gal4 may fall short of effectively blocking the neuronal output required for relief learning, and/or an additional, *shibire^{ts1}*-resistant neurotransmission mechanism may be employed in relief learning. Further, if punishment were to be signaled by a shock-induced *in*crease in the activity of the TH-Gal4 neurons and relief was to be signaled by a *de*crease in their activity below the baseline at the shock offset, incomplete blockage of output from these neurons could partially impair punishment learning, while leaving relief learning intact. In face of these caveats, we find it too early to exclude any role of dopamine or of the TH-Gal4 neurons. What then is a safe minimal conclusion? Given that while punishment learning is partially impaired (**Figure 4A**) relief learning does not even tend to be impaired (**Figure 4B**), these two kinds of learning do differ in terms of whether and which role the TH-Gal4-covered neurons play. This does dissociate punishment and relief learning in terms of their underlying mechanisms.

Turning to the octopaminergic system, we confirmed Schwaerzel et al. (2003) in that the *tbh^{M18}* mutant with compromised octopamine biosynthesis is impaired in reward learning (**Figures 6A,A′**), but not in punishment learning (**Figures 6B,B′**). The effect on reward learning was however conditional on the kinds of odor used (**Figures 6A,A′,A″**). Under the conditions that significantly impaired reward learning, we found relief learning intact (**Figure 6C**). Although the *tbh^{M18}* mutant we used revealed no octopamine content in immunohistochemical and high pressure liquid chromatography (HPLC) analyses (Monastirioti et al., 1996), it may retain an amount of octopamine below the detection thresholds of these methods but sufficient to signal reward and/or relief. Furthermore, HPLC analysis reveals a ~10-fold increase in the amount of octopamine-precursor tyramine in this mutant (Monastirioti et al., 1996); this excessive tyramine may compensate for the lack of octopamine (Uzzan and Dudai, 1982).

As an additional approach, we blocked the output from a set of octopaminergic/tyraminergic neurons, expressing UAS-*shibire^{ts1}* with the TDC2-Gal4 driver; this impaired neither reward, nor relief learning (**Figure 7**). The TDC2-Gal4 driver targets, along with tyraminergic neurons, octopaminergic neurons in three paired and one unpaired neuron clusters (Busch et al., 2009). Among these, the unpaired "VM cluster" harbors octopaminergic neurons innervating on the one hand the subesophageal ganglion (SOG), and on the other hand the antennal lobes, mushroom bodies, and the lateral horn (Busch et al., 2009); such connectivity would enable signaling gustatory reward onto the olfactory pathway. Indeed, in the honey bee, activation of a single octopaminergic neuron, VUMmx1, with such innervation pattern, is sufficient to carry the reward signal for olfactory learning (Hammer, 1993). Surprisingly however, although all octopaminergic neurons in the VM cluster are targeted by the TDC2-Gal4 (Busch et al., 2009), using this driver with UAS-*shibire^{ts1}*, we found reward learning intact (**Figure 7A**). This may be because the level UAS-*shibire^{ts1}* expression falls short of completely blocking the neuronal output. Alternatively, given that activation of the TDC2-Gal4-targeted neurons in fruit fly larvae reportedly substitutes for reward (Schroll et al., 2006), the VM cluster neurons may indeed carry a reward signal, but other octopaminergic neurons outside this cluster, left out by the TDC2-Gal4 driver (Busch et al., 2009) may redundantly do so. Either kind of argument could also explain the lack of effect on relief learning (**Figure 7B**). Thus, although we find no evidence for a role for the octopaminergic system in relief learning, we refrain from excluding such a role. Still, given that the *tbh^{M18}* mutation affects reward learning, but not relief learning, these two forms of learning are to some extent dissociated in their genetic requirements.

Obviously, the question whether dopaminergic and octopaminergic systems are involved in relief learning remains open. Follow up studies should extend our neurogenetic approach to further tools. For example, dopamine biosynthesis can be specifically compromised in the fly nervous system using a *tyrosine hydroxylase* mutant in combination with a hypoderm-specific rescue construct (Hirsh et al., 2010). Also, for two different dopamine receptors, DAMB and dDA-1, loss of function mutations are available (Kim et al., 2007; Selcho et al., 2009). Notably, by means of the dDA-1 receptor loss of function mutant, the role of the dopaminergic system in reward learning was revealed (Kim et al., 2007; Selcho et al., 2009), which had been overlooked with the tools used in the present study. In addition, a pharmacological approach would be useful. Antagonists for the vertebrate D1 and D2 receptors have been successfully used in the fruit fly (Yellman et al., 1997; Seugnet et al., 2008) and other insects (Unoki et al., 2005, 2006; Vergoz et al., 2007) (regarding the octopamine receptors: Unoki et al., 2005, 2006; Vergoz et al., 2007). Such pharmacological approach could be extended to other aminergic, as well as peptidergic systems and could also test for the effects of human psychotherapeuticals. The results of such studies may then guide subsequent analyses at the cellular level.

To summarize, while this study has shed no light on how relief learning works, it did show that relief learning works in a way neurogenetically *different* from both punishment learning and reward learning, likely at the level of the roles of aminergic neurons. Interestingly, at this level also punishment and reward learning are dissociated. However, all three kinds of learning also share genetic commons, for example with respect to the role of the *synapsin* gene, likely critical for neuronal plasticity (Godenschwege et al., 2004; Michels et al., 2005; Knapek et al., 2010; T. Niewalda, Universität Würzburg, personal communication). Thus, punishment-, relief-, and reward-learning may conceivably rely on common molecular mechanisms of memory trace formation, which however are triggered by experimentally dissociable reinforcement signals, and/or operate in distinct neuronal circuits. This may be a message relevant also for analyses of relief learning in other experimental

systems, including rodent (Rogan et al., 2005), monkey (Tobler et al., 2003; Belova et al., 2007; Matsumoto and Hikosaka, 2009), and man (Seymour et al., 2005; Andreatta et al., 2010).

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Behavioral characterization of individual olfactory memory retrieval in *Drosophila melanogaster*

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Memory performance depends not only on effective learning and storage of information, but also on its efficient retrieval. In *Drosophila*, aversive olfactory conditioning generates qualitatively different forms of memory depending on the number and spacing of conditioning trials. However, it is not known how these differences are reflected at the retrieval level, in the behavior of individual flies during testing. We analyzed conditioned behaviors after one conditioning trial and after massed and spaced repeated trials. The single conditioning produces an early memory that was tested at 1.5 h. Tested at 24 h after training, the spaced and the massed protocols generate two different forms of consolidated memory, dependent, or independent of *de novo* protein-synthesis. We found clearly distinct patterns of locomotor activity in flies trained with either spaced or massed conditioning protocols. Spaced-trained flies exhibited immediate and dynamic choices between punished and unpunished odors during the test, whereas massed-trained flies made a delayed choice and showed earlier disappearance of the conditioned response. Flies trained with single and spaced trials responded to the punished odor by decreasing their resting time, but not massed-trained flies. These findings demonstrate that genetically and pharmacologically distinct forms of memory drive characteristically different forms of locomotor behavior during retrieval, and they may shed light on our previous observation that memory retrieval in massed-trained flies is socially facilitated. Social interactions would enhance exploratory activity, and then reduce the latency of their conditioned choice and delay its extinction.

Keywords: insect, aversive conditioning, memory retrieval, behavior, odor choice, locomotion

INTRODUCTION

Investigations of memory aim to understand how individuals adapt decision-making and other forms of behavior to environmental circumstances, as a function of their previous experience. Memory processing involves three steps: learning and acquisition of memory, memory storage, and memory retrieval. The respective contributions of these steps to memory performance are hard to separate, even in controlled laboratory studies, because measurement of memory performance is accessible mainly through expression of the conditioned behavior during retrieval. Consequently, an indispensable approach for studying memory is to precisely describe the conditioned response.

In *Drosophila*, olfactory aversive conditioning has been thoroughly studied for more than 30 years (Berry et al., 2008), with several operant or Pavlovian versions of the paradigm (Quinn et al., 1974; Tully and Quinn, 1985; Pascual and Preat, 2001; Mery and Kawecki, 2005; Claridge-Chang et al., 2009). The most widely used Pavlovian discriminatory set up involves the presentation of two odors: one associated with electric shocks and the other with no shocks (Tully and Quinn, 1985; Pascual and Preat, 2001). Memory performance is measured in groups of flies using a T-maze, in which flies have a set time to choose between both odors. At the end of the test, a mean performance index is calculated by counting the number of flies that made the correct and the incorrect choice. This paradigm provides a relatively easy and rapid assessment of learning and memory capacities, allowing screening, and characterization of mutants (for a review, see Waddell and Quinn, 2001). It can induce different forms of memory: labile short-term and middle-term memory (STM and MTM) after single or repeated learning trials, and two forms of consolidated memory that depend on the spacing of repeated learning trials (Tully et al., 1994; Isabel et al., 2004). Long-term memory (LTM), formed after spaced trials, requires *de novo* protein-synthesis, in contrast to anesthesia-resistant memory (ARM), which is formed after single or massed trials (Tully et al., 1994). It is still not clear if these two forms of memory coexist after spaced conditioning (Tully et al., 1994) or if only LTM is expressed after spaced conditioning (Isabel et al., 2004). Importantly, the olfactory conditioned response has been estimated mainly by the presence of flies in the punished or unpunished odor at the end of the test. However, the response has never been precisely characterized, either for individuals or groups of flies, by the different behavioral events that can influence the position of flies during the test.

The vast majority of studies using this protocol measure the responses of groups of flies during memory retrieval. Consequently, our understanding of memory phase dynamics in this system

does not take into account potential differences between collective group behavior and decision-making by individual flies. However, a new version of the operant olfactory aversive conditioning paradigm has recently been set up to test shock-trained odorant avoidance of single flies (Claridge-Chang et al., 2009). Furthermore, using the classical paradigm we recently demonstrated that for the two long-lasting memories, group performances are influenced by interactions between flies during the test, with specific effects on memory performance (Chabaud et al., 2009). In contrast to LTM, ARM retrieval is impaired when tested individually but facilitated when tested in a group of conditioned flies. We show that the social interactions involved in the facilitation of retrieval are specific to conditioned flies, suggesting that they may use stress-like signals that enhance their attention or motivation to respond to the punished odor. The ARM conditioned flies tested individually might also be in a state of perceived social isolation, which is known to contribute to poorer overall cognitive performance, as reviewed elsewhere (Cacioppo and Hawkley, 2009). The social context of the test can be seen as one of a number of physiological states known to modulate memory performance, such as the effect of sleep deprivation upon aversive learning (Seugnet et al., 2008) or the effect of food motivation state upon appetitive learning and memory retrieval in *Drosophila* (Colomb et al., 2009; Krashes et al., 2009), and the gustatory context of the test of olfactory memory for *Drosophila* larvae (Gerber and Hendel, 2006).

To understand which component of the decision-making process is deficient in single flies tested for ARM, we decided to characterize, in detail, the conditioned responses of individual flies during the test. We compared the conditioned responses of individuals trained for ARM (24 h after massed conditioning) with those of individuals trained for early memory (EM 1.5 h after a single conditioning) and for LTM (24 h after spaced conditioning). For each memory type, we measured several behavioral parameters potentially related to memory retrieval, such as the time spent in each odor, walking, or resting states, and levels of exploratory activity between and within odors. In doing so, we analyzed the conditioned response over time during the test.

Our data reveal the behavioral features that sustain the difference in memory performance between massed- and spaced-trained flies. In particular we show that, when tested individually, spaced-trained flies make dynamic choices, with repeated avoidance of the punished odor from the beginning of the test. In contrast, conditioned avoidance in massed-trained flies is both delayed and less persistent than in spaced-trained flies, suggesting memory extinction occurs during the test. The behavior of individual massed-trained flies suggest that the social facilitation of ARM retrieval acts by decreasing the latency and/or retarding the extinction of the conditioned response during testing within a group.

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

The subjects used were adult *Drosophila melanogaster* from the wild-type strain *Canton-Special*. Flies were raised at 18°C on standard *Drosophila* medium, then trained and tested under red light at 25°C and at 80% relative humidity.

OLFACTORY CLASSICAL CONDITIONING

Discriminatory olfactory aversive conditioning was performed on samples of 30–40 flies between 2 and 3 days old. One conditioning trial consists of pairing a first odor with electric shocks (twelve 1.2 s pulses of 60 V over 1 min), followed by presentation of the second odor without electric shocks. Odors are referred to as punished and unpunished. Each odor presentation is always followed by 45 s of airflow without odor. 4-methylcyclohexanol and 3-octanol were used alternately as punished odor and unpunished odor, for every other set of flies (Tully and Quinn, 1985; Pascual and Preat, 2001). Flies trained with this paired procedure were compared with control flies trained with an unpaired protocol, in which electric shocks are delivered 2 min before the first odor. With this schedule, flies are not expected to learn any backward association between electric shocks and odor (Tanimoto et al., 2004).

Early memory, which we measured 1.5 h after a single conditioning trial, corresponds both to MTM and early ARM (Tully et al., 1990). Flies trained with the single conditioning protocol were compared to control flies that were trained with one trial of the unpaired procedure.

The massed conditioning protocol consists of five consecutive trials, without inter-trial intervals. ARM formed after massed conditioning was measured 24 h after training and was compared with control performance obtained with five repetitions of the unpaired procedure.

The spaced conditioning consists of five consecutive trials, with 15 min inter-trial intervals. It is still not clear if memory measured 24 h after training is composite, with the induction of both ARM and LTM (Tully et al., 1994), or if only LTM is present (Isabel et al., 2004). We showed, however, that if memory is composite, LTM expression appears to be dominant during retrieval (Chabaud et al., 2009). To facilitate the reading of this paper, we thus used the term LTM for the memory measured 24 h after spaced conditioning. Flies trained with the spaced conditioning protocol were compared to control flies that were trained with similar repetitions of the unpaired procedure.

MEMORY TESTS

Tests were performed in a T-Maze set on a homogenous-lighting table (Waldmann, 50 Hz) covered by three transparent red sheets, which allowed diffusion of a red light that illuminated the T-Maze sufficiently to observe flies with the naked eye. For individual memory assays, single flies were collected without anesthesia from trained groups, and introduced alone in a T-maze apparatus (Tully and Quinn, 1985) to choose between octanol or methylcyclohexanol odor over a period of 3 min. Six flies per group (three males and three females) were tested individually. The remaining flies of a group (about 30) were then tested together in the T-maze for 3 min to provide a control, and to verify the positive effect of the group on ARM scores (Chabaud et al., 2009).

DATA RECORDING

Data were recorded with The Observer® software (Noldus). We recorded the position of flies in the different parts of the T-maze, during the 3-min memory test, i.e., the arm carrying the unpunished odor (correct choice), the central part where flies were introduced, and the arm carrying the previously punished odor

(incorrect choice). We also noted their position within each arm, dividing the arms into three zones (**Figure 1**). Time spent walking or resting was also recorded.

Individual memory performance was estimated in two ways. First we calculated a "time score" for each fly, equal to the time spent by the fly in the unpunished odor minus the time spent in the punished odor, divided by the time spent in both odors during the 3-min test. The mean time score and its SE were then calculated for each conditioning protocol. Secondly, the evolution of odor choice during the test was analyzed using the position of the fly at different time points (at the first choice, 30 s, 1, 2, and 3 min). At these different time points, flies were either in the punished odor, in the unpunished odor, or in the central part of the T-maze. We calculated a global "position score" at each time point of the test, on all individual data of the different treatments, equal to the sum of flies in the unpunished odor minus the sum of flies in the punished odor, divided by the sum of both numbers.

To verify memory performance in groups and the positive effect of the group on ARM scores, we had to compare the memory score of individuals with that of the group from which they came. When flies are tested in groups, their memory is estimated using the position score, defined above. To estimate individual memory with a memory score similar in essence to that of the group score, we pooled individual positions (12 flies per pool, 6 conditioned to 3-octanol and 6 to 4-methylcyclohexanol). A position score was calculated as for the group score, for each pool of 12 flies. Mean individual position score of a given sample was the mean of the scores of the pools. In all cases, memory scores were multiplied by 100 and thus evaluated on a scale from −100 to 100. For the unpaired control procedure, "memory" scores were calculated as for the paired procedure, with the first odor, delivered 2 min after electric shocks, being considered as the "punished" odor.

STATISTICAL ANALYSES

The chi-square test (χ^2) was used to analyze (i) the distributions of duration scores (**Figure 3B**), (ii) the position scores (**Figures 4 and 5**), and (iii) the distribution of flies as a function of the number of odor changes (**Figure 6**). The Haber correction (χ^2_{Haber}) was applied for 1 df chi-square tests (Zar, 1999).

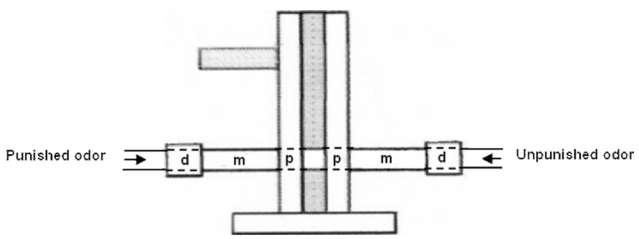

FIGURE 1 | Scheme of the virtual division in three zones on each of the two arms of the T-maze. The proximal zone (p), measuring 1.8 cm long, is located at the entrance of the arm and near the central part of the T-maze, where flies are introduced at the beginning of the test and where both odors are extracted. The median part of the arm (m) measures 5.5 cm long. The distal part of the arm (d), measuring 2.2 cm, is located at the end of the arm, fitted inside a small plexiglas connector that insures the junction with the airflow tubing. The internal diameter is constant and equal to 1.5 cm.

After normalization (arcsine transformation for proportions), and after checking assumptions of normality (Shapiro–Wilk test) and homogeneity of variances (Levene test), mean scores of conditioned group vs control group, and conditioned group vs conditioned individuals (**Figure 2**) were compared with a two-tailed Student's *t*-test at the Dunn–Sidak corrected significance level $\alpha = 0.025$ for the use of data in two comparisons (Zar, 1999). For all the other data, the assumption of normality of data was not satisfied even after normalization (arcsine transformation for proportions, logarithmic and square root transformations for latencies, and number of odor changes). Therefore, we used non-parametric tests. The median test (z) was used to characterize the symmetry of the distribution of duration scores around the median for control flies (**Figure 3B**). The normal approximation to the Mann–Whitney test corrected for continuity (Z) was used to compare two independent samples (conditioned vs control groups) when minimum and maximum sample sizes exceeded 20 and 40, respectively (Zar, 1999; **Tables 1 and 3**; **Figure 7**). The Wilcoxon sign and rank test (V) was used to compare dependent data (**Table 2**). Correlations between the time memory score and the proportion of time spent resting or spent in the proximal zone of the punished odor, were analyzed using the Spearman rank correlation. Results of all statistical analyses refer to the significance level $\alpha = 0.05$, and were calculated with Statistica 9 or Xlstat softwares.

RESULTS

CONFIRMING THE SOCIAL EFFECT ON MEMORY PERFORMANCE

We first verified that, for the three conditioning procedures, conditioned groups show higher scores than their specific unpaired control groups following 3 min tests (**Figure 2**; *t*-test : $t \geq 6.38$, $P < 0.0001$. We also confirmed the existence of social facilitation of retrieval when ARM-trained flies were tested in groups (Chabaud et al., 2009), with a higher ARM score when tested in groups than when tested as individuals ($t = 2.91$, $P < 0.008$). No group effect was observed in EM and LTM performance ($t \leq 1.35$, $P \geq 0.19$).

DISTRIBUTION OF INDIVIDUAL MEMORY SCORES

Individual memory was estimated using the time score. For this parameter, we first checked that each associative training procedure induced a significant individual avoidance of the punished odor compared to corresponding control procedures (**Figure 3A**; Mann–Whitney test, $Z \geq 2.51$, $P \leq 0.012$), with similar memory performances for males and females (data not shown; $EM_{\delta} = 65 \pm 9$ and $EM_{\varphi} = 62 \pm 9$, $LTM_{\delta} = 39 \pm 8$ and $LTM_{\varphi} = 48 \pm 8$, $ARM_{\delta} = 20 \pm 10$ and $ARM_{\varphi} = 25 \pm 10$; Mann–Whitney test, $Z \leq 1.17$, $P \geq 0.24$).

For all unpaired control procedures, time scores were close to 0, indicating that unpaired training induces neither avoidance nor preference for any odors (**Figure 3A**). Control individual scores were, however, not normally distributed and showed bipolar and symmetrical distributions around the means (**Figure 3B**; median test: $z \leq 1.96$, $P \geq 0.23$, 1 df). Thus, control individuals chose one of the two odors randomly during the test and tended to stay in this odor for most of the test period.

Following single or spaced conditioning protocols, time score distributions were significantly different from those of the corresponding controls (**Figure 3B**; $\chi^2 \geq 24.73$, $P \leq 3.10^{-5}$, 4 df).

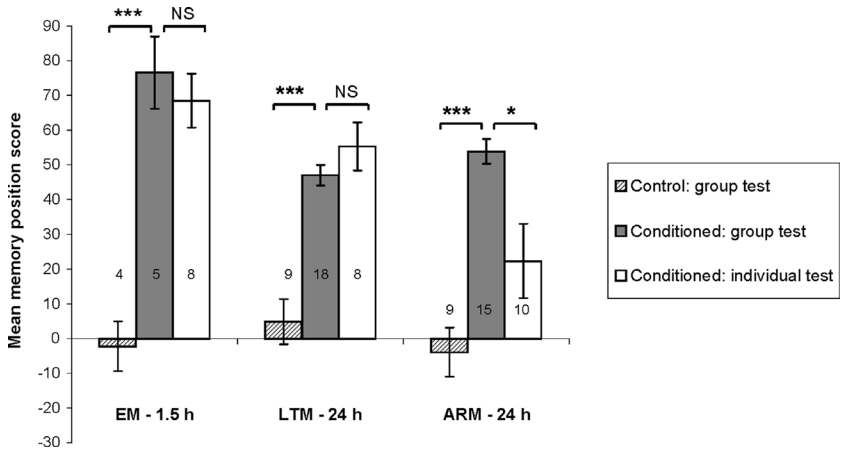

FIGURE 2 | Confirming the social effect on memory performance. Bars represent mean values of memory position score ± SE. For each conditioning procedure (EM, early memory; LTM, long-term memory; ARM, anesthesia-resistant memory), position scores are compared between control and conditioned flies tested in group, and between conditioned flies tested in group or individually, using Student's *t*-test. *P*-values are compared to $\alpha' = 0.025$. ***$P < 0.001$; *$P < 0.05$; NS: $P \geq 0.05$. Numbers of groups tested and pools of flies tested individually are reported on the graph.

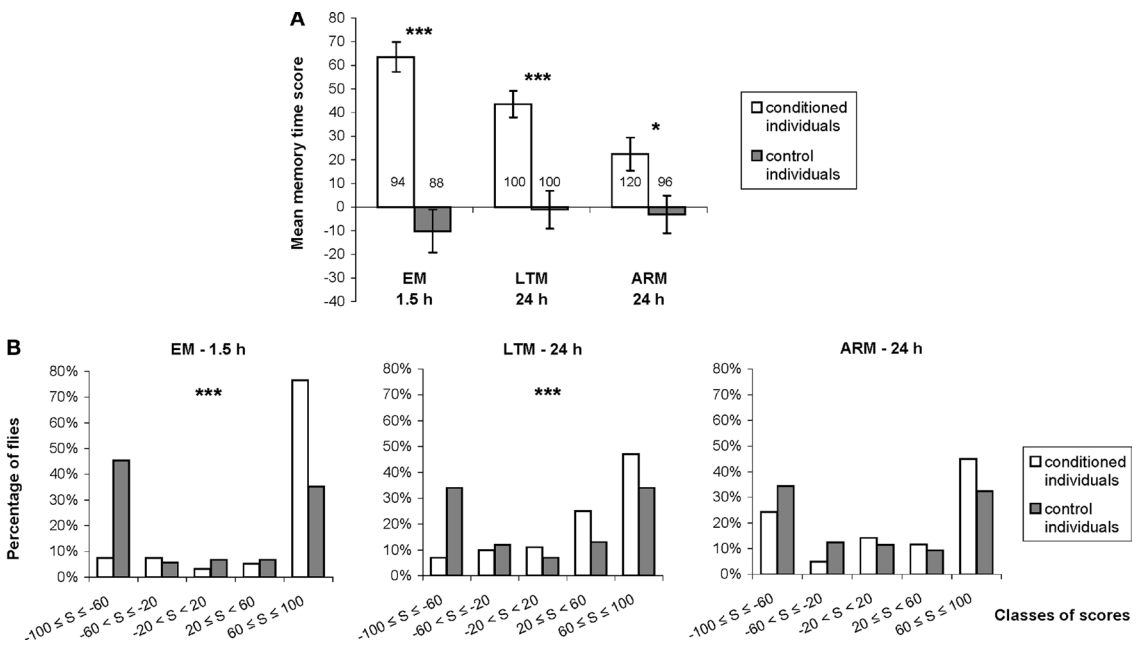

FIGURE 3 | Distribution of individual memory scores. (A) We compared mean individual time scores between flies trained with associative (conditioned individuals) and unpaired (control individuals) protocols, using single cycle conditioning (EM), spaced conditioning (LTM), and massed conditioning (ARM). See Section "Materials and Methods" for the calculation of time score. Data represent the mean ± SE of the mean. Numbers of flies tested are reported on the graph. Mann–Whitney test: stars indicate significant differences between conditioned and control individuals, at *P*-values inferior to 0.05 (*), 0.01 (**), or 0.001 (***). **(B)** Graphs showing the distribution of individual time scores in five classes ranging from –100 to 100, represented by the percentage of flies in each class of scores for conditioned and control flies, trained and tested for EM, LTM, and ARM. Chi-square: stars indicate significant differences between scores distribution of conditioned and control individuals, at *P*-values inferior to 0.05 (*), 0.01 (**), or 0.001 (***).

The majority of EM-trained flies showed high scores, higher, or equal to 60. As expected, the LTM-trained flies performed more poorly, as shown by a significantly higher proportion of flies in the immediately inferior class (20–60) compared to EM-trained flies ($\chi^2 = 12.89$, $P < 10^{-4}$, 1 df). In ARM-trained flies, low performance did not correspond to an increased in intermediate scores, but resulted from a subpopulation of flies with very low scores (inferior to –60); these flies were significantly more numerous than in EM-and LTM-trained flies ($\chi^2 = 18.03$, $P < 10^{-3}$, 2 df). The distribution of ARM individual scores was therefore more bipolar, and not significantly different from the distribution of ARM-control individuals (**Figure 3B**; $\chi^2 = 6.57$, $P = 0.16$, 4 df). This suggests that a fraction of individually tested, ARM-trained flies effectively make a random odor choice.

EVOLUTION OF POSITION MEMORY SCORES ALONG THE TEST

To circumvent the cumulative nature of the time score, the evolution of the conditioned response was analyzed using position data. Individuals conditioned with the single-trial (EM) or the spaced-trial (LTM) protocols showed a significantly higher score from the beginning of the test compared to that of control individuals (**Figures 4A,B**; $\chi^2_{Haber} \geq 8.91$, $P < 0.005$, 1 df). In contrast, ARM-trained individuals made their first choice at random, like control individuals ($\chi^2_{Haber} = 0.019$, $P > 0.80$, 1 df), and from 30 s they gained a significantly higher score than controls ($\chi^2_{Haber} \geq 3.85$, $P < 0.05$, 1 df). This demonstrates a delayed conditioned response compared to EM- or LTM-trained flies. Thus, the bipolar distribution of duration scores for control and ARM-trained flies (**Figure 3B**) might be linked to their random first choice. We therefore analyzed the effect of the first choice on the evolution of position scores during the test.

A SIGNIFICANT EFFECT OF THE FIRST CHOICE ON THE EVOLUTION OF INDIVIDUAL ARM SCORES

For all control procedures, the influence of the first choice disappeared progressively during the test; scores were not significantly different by the 1-min point of test (control for ARM and EM; **Figures 5B,F**; $\chi^2_{Haber} \leq 3.48$, $P > 0.05$, 1 df) or 2 min (control for LTM; **Figure 5D**; $\chi^2_{Haber} = 2.37$, $P = 0.10$, 1 df). Control flies chose the first odor at random but did not necessarily stay in this odor during the entire test.

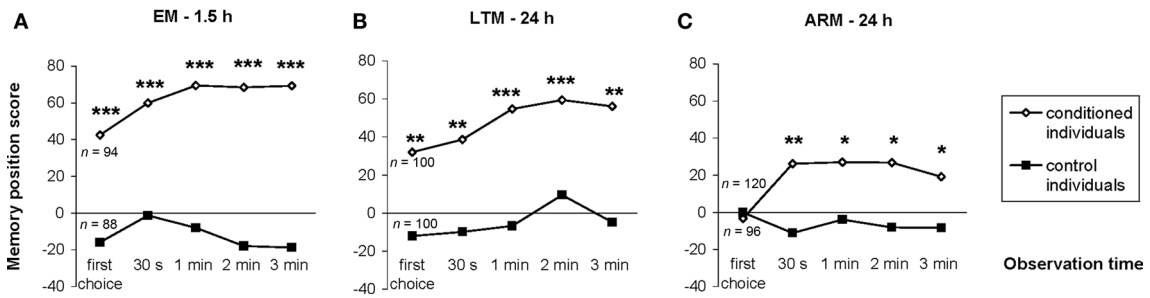

FIGURE 4 | Evolution of individual memory scores during the test period. The memory position score of a given sample of individually tested flies was the difference between the numbers of flies in the punished and unpunished odors divided by the total number of flies. It was calculated at each observation time until the end of the 3-min test, for conditioned and control flies trained and tested for EM **(A)**, LTM **(B)** and ARM **(C)**. *n* corresponds to the number of flies tested. Chi-square test: stars indicate significant differences between conditioned and control individuals, at *P*-values inferior to 0.05 (*), 0.01 (**), or 0.001 (***). For the latency of the first choice, see Section "Latency of the First Choice does not Depend on the Odor Status."

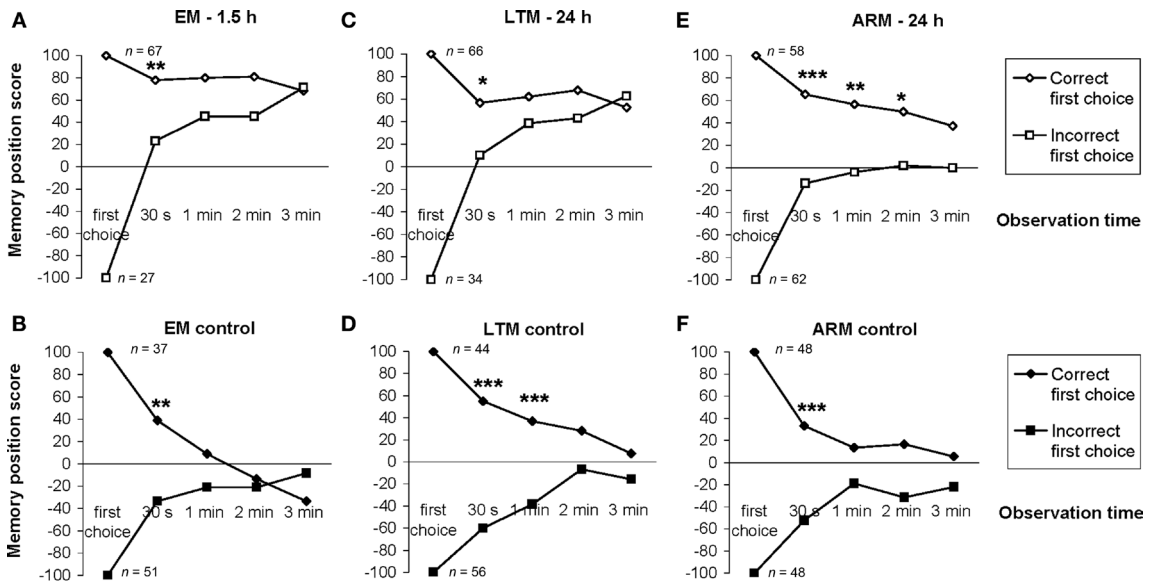

FIGURE 5 | Effect of the first choice on the evolution of the individual memory score. Position scores were calculated for two categories of flies as a function of their first odor choice. As the test starts at the first choice, 100% of the flies making the correct choice are in the unpunished odor and this category has, therefore, a score equal to 100. Then part of the flies move from one odor to the other, so at the 30-s point the score of the category is less than 100. **(A,C,E)** EM-, LTM-, and ARM-trained flies respectively. **(B,D,F)** Control flies for EM, LTM, and ARM, respectively. *n* corresponds to the number of flies. Chi-square test: stars indicate significant differences between scores of flies making a correct or incorrect first choice, at *P*-values inferior to 0.05 (*), 0.01 (**), or 0.001 (***).

For the associative training procedures, EM- and LTM-trained flies that made the incorrect first choice had higher scores than control flies at 30 s (**Figure 5A vs 5B, Figure 5C vs 5D**; $\chi^2_{Haber} \geq 5$, $P < 0.05$, 1 df), but this difference was only marginal in ARM-trained flies (**Figure 5E vs 5F**, $\chi^2_{Haber} = 3.1$, $P = 0.051$, 1 df). This suggests that the memories generated after the EM and LTM conditioning protocols allow flies to recognize and leave the punished odor significantly before 30 s of test, but to a lesser extent after the ARM conditioning protocol.

In EM- and LTM-trained flies, the effect of the incorrect first choice disappeared during the test; scores of "initially mistaken" flies approached those of "initially correct" flies, with no significant differences observed after 1 min of testing (**Figures 5A,C**; $\chi^2_{Haber} \leq 3.21$, $P > 0.05$, 1 df). In contrast, for ARM-trained flies, an incorrect first choice had an irreversible effect on the score, which remained close to 0 from 30 s to the end of the test (**Figure 5E**; comparison with zero value: $\chi^2_{Haber} \leq 0.35$, $P > 0.50$, 1 df). Initially correct flies retained significantly higher scores than initially mistaken flies until 2 min of testing (**Figure 5E**; $\chi^2_{Haber} \geq 6.15$, $P < 0.02$, 1 df), after which their scores decreased; the difference was no longer significant at 3 min (**Figure 5E**; $\chi^2_{Haber} = 3.40$, $P > 0.05$, 1 df).

To better understand the mechanisms that sustain the evolution of memory scores, we then analyzed the exploratory behavior of flies during the test. In particular, we scrutinized the latency of the first choice, the frequency of odor changes as a function of the first choice, and locomotor activity in both odors.

LATENCY OF THE FIRST CHOICE DOES NOT DEPEND ON THE ODOR STATUS

Flies left the central part of the T-maze and made their first odor choice in a median time of 3.1–3.5 s, without significant differences between paired and control protocols (data not shown; Mann–Whitney test, $Z \leq 0.90$, $P \geq 0.36$). Moreover, the latency of the first choice did not depend on the odor (whether punished or not; data not shown; $Z \leq 1.79$, $P \geq 0.07$), except for EM-trained flies that took a little more time to enter the punished odor (i.e., median time of 4.1 vs 3.1 s to enter the unpunished odor; $Z = 2.65$, $P = 0.008$).

DIFFERENT PATTERNS OF TRANSITION BETWEEN THE ODORS ARE A FUNCTION OF MEMORY TYPE

During the 3-min test, the mean number of changes between odors was significantly higher in LTM-trained flies than in LTM-control flies (**Table 1**. Mann–Whitney test: $P = 3.10^{-5}$). The transition rates of EM- and ARM-trained flies did not differ significantly from their control flies.

The distribution of flies as a function of the number of changes between the odors is represented on **Figure 6**, comparing initially correct and initially mistaken flies. For all control procedures, the distributions did not depend on the first odor choice, suggesting that flies continued to randomly stop in one of the two odors until the end of the test (**Figures 6B,D,F**; $\chi^2 \leq 8.003$, $P \geq 0.15$, 5 df). One-third of flies stayed in the first odor (i.e., class 0 odor change) and nearly one-third more stayed in the second, opposite, odor (i.e., class 1 odor change). This explains why the position score of control flies tends toward 0 after the first choice (**Figures 5B,D,F**).

In the case of trained flies, for all training protocols the distributions depended on the first odor choice (**Figures 6A,C,E**; $\chi^2 \geq 13.2$, $P \leq 0.033$, 6 df). A higher percentage of flies stayed in the first and the second odor when it was the unpunished odor (**Figures 6A,C,E**, class 0 odor change; $\chi^2_{Haber} \geq 10.20$, $P < 0.01$, 1 df; class 1 odor change; $\chi^2_{Haber} \geq 4.55$, $P < 0.05$, 1 df).

The fraction of mistaken flies that never left the punished odor during the test (**Figures 6A,E**, class 0 odor change) likely underlies the effect of incorrect first choice on the evolution of scores during the test, especially for ARM-trained flies (**Figure 5E**). Initially mistaken flies were more numerous in ARM-trained flies than in EM-trained and LTM-trained flies (**Figures 6A,C,E**, class 0 odor change; respectively 24, 15, and 0% initially mistaken flies).

Flies leaving the punished odor (**Figures 6A,C,E**, class 1 odor change) after an incorrect first choice, were responsible for the fast increase in scores observed for all three types of conditioning, though this was to a lesser extent for ARM flies (**Figures 5A,C,E**). In ARM-trained flies, no avoidance of the punished odor was observed following the second odor change (**Figure 6E**, class 2 odor changes; $\chi^2_{Haber} \leq 0.84$, $P \geq 0.30$, 1 df). This could explain the stagnation of the score close to 0 after an incorrect first choice, and the decrease of the score after a correct first choice (**Figure 5E**). Similarly, EM-trained flies did not avoid the punished odor from the second odor change (**Figure 6A**, class 2 odor changes; $\chi^2_{Haber} \leq 1.34$, $P \geq 0.20$, 1 df). The conditioned response was present for a longer time in LTM-trained flies; it was only after the third odor change that flies no longer showed significant avoidance (**Figure 6C**, class 3 odor changes; $\chi^2_{Haber} \leq 2.18$, $P \geq 0.10$, 1 df). In LTM-trained flies, avoidance of the punished odor is therefore a more dynamic choice than in EM- and ARM-trained flies.

LOCOMOTOR ACTIVITY DURING THE TEST

Locomotor activity decreased through the duration of the test, as indicated by the significant increase of resting time after 1 min of test, in both trained flies and their respective controls (**Table 2**; 1st minute vs 2nd + 3rd minute; Wilcoxon test, $P < 10^{-4}$). This decrease in locomotor activity explains the limited mean number of odor changes (**Table 1**), and the fact that individual memory scores reach a plateau from 1 min of test (**Figure 4**).

When in the punished odor, EM- and LTM-trained flies spent less time resting than the corresponding control flies (**Table 3**). This difference was not observed in ARM-trained flies. This result shows that decreased resting in the punished odor is a component of the conditioned response of EM- and LTM-trained flies.

Table 1 | Number of changes between odors as a function of conditioning.

	EM – 1.5 h	LTM – 24 h	ARM – 24 h
Conditioned individuals	1.3 ± 0.2	2.8 ± 0.3	1.7 ± 0.2
	(*n* = 94)	(*n* = 100)	(*n* = 120)
Control individuals	1.3 ± 0.1	1.4 ± 0.2	2.4 ± 0.3
	(*n* = 88)	(*n* = 100)	(*n* = 96)
Mann–Whitney test	*Z* = 1.07	*Z* = 4.20	*Z* = 1.55
	(*P* = 0.28)	**(*P* = 3.10^{-5})**	(*P* = 0.12)

Mean number of changes between punished and unpunished odors during the 3-min test ± SE of the mean. Numbers of flies are bracketed. Significant P-values are in bold.

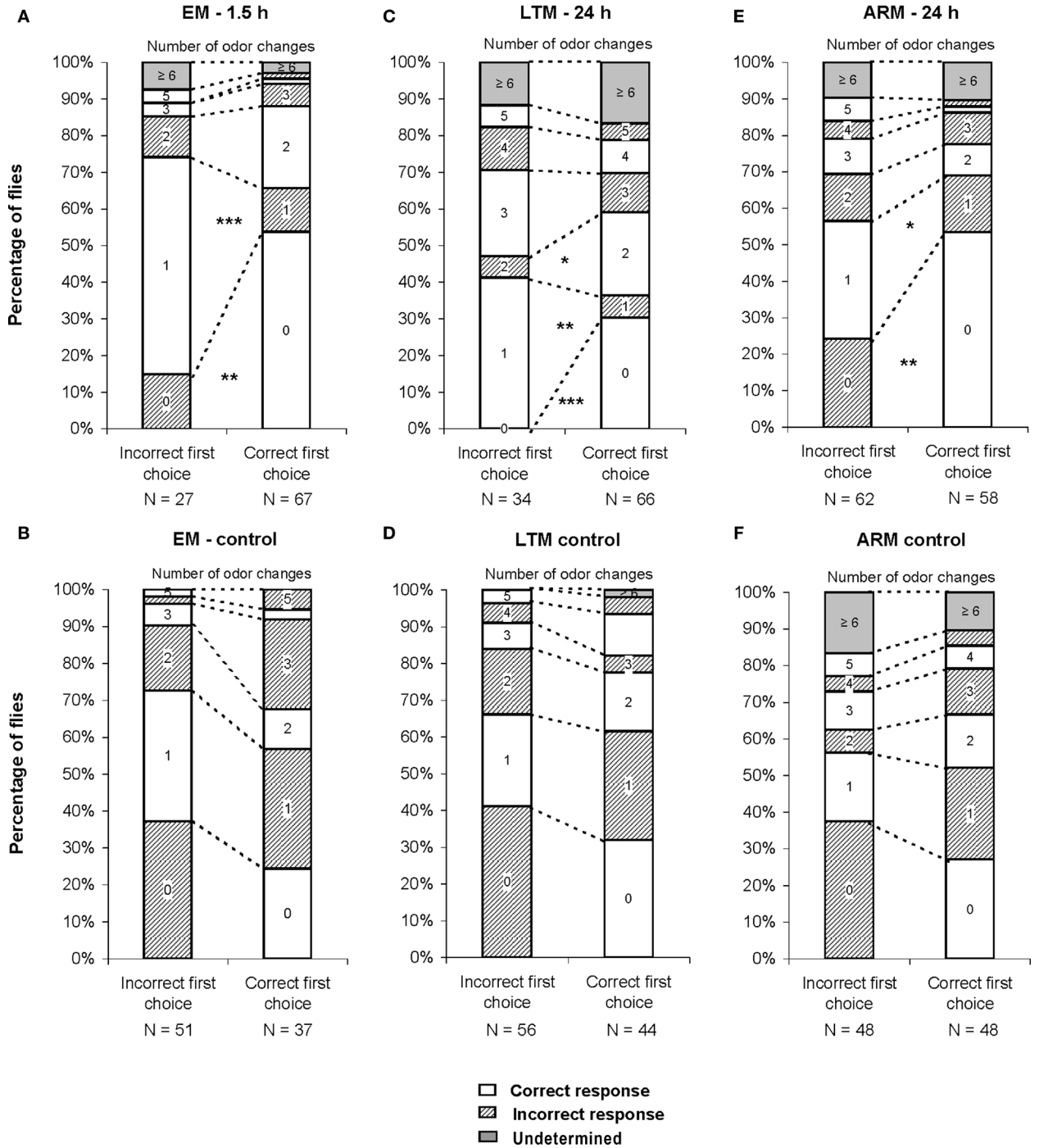

FIGURE 6 | Number of odor changes as a function of the first choice. The graphs represent the distribution of flies as a function of the number of odor changes performed during the test, for each category of first choice (correct vs incorrect). **(A,C,E)** Conditioned flies, trained for EM, LTM, and ARM. **(B,D,F)** control flies, exposed to unpaired protocols for EM, LTM, and ARM. Flies of class 0 are those staying in the first chosen odor until the end of the test, flies of class 1 are those staying in the opposite odor after one change, etc. Chi-square test: stars indicate, for each class of odor change, significant differences between the number of flies in the unpunished odor (correct response) or in the punished odor (incorrect response) at the end of the test, at *P*-values inferior to 0.05 (*), 0.01 (**), or 0.001 (***). *N* is the sample size.

Control flies spent equivalent time resting in both odors. In contrast, flies of the three conditioned groups appeared to spend more time resting when in the unpunished odor than when in the punished one (this could not be statistically tested because only a portion of the flies visited both odors, thus data were either dependent or independent according to the fly's

Table 2 | Evolution of the time spent at rest during the test.

	0–1 min (%)	1–3 min (%)	Wilcoxon test
EM – 1.5 h	15 ± 2	53 ± 3	$V_{94} = 119$, $P < 10^{-4}$
LTM – 24 h	10 ± 1	39 ± 2	$V_{100} = 110$, $P < 10^{-4}$
ARM – 24 h	11 ± 1	42 ± 3	$V_{120} = 170$, $P < 10^{-4}$
EM control	21 ± 2	55 ± 3	$V_{88} = 190$, $P < 10^{-4}$
LTM control	13 ± 2	37 ± 2	$V_{100} = 351$, $P < 10^{-4}$
ARM control	13 ± 2	39 ± 3	$V_{96} = 257$, $P < 10^{-4}$

Mean percentage of time spent at rest during periods of 0–1 and 1–3 min of testing, for conditioned and control flies trained and tested for EM, LTM, and ARM. Data represent the mean ± SE of the mean. For all samples, the Wilcoxon sign and rank V test indicates that the time spent at rest is significantly higher during the second period of testing (1–3 min). Sample sizes are the index numbers of the V parameter.

Table 3 | Proportion of time spent at rest.

	EM – 1.5 h	LTM – 24 h	ARM – 24 h
PUNISHED ODOR			
Conditioned individuals	6 ± 2%	12 ± 2%	15 ± 2%
	($n = 58$)	($n = 81$)	($n = 89$)
Control individuals	29 ± 3%	22 ± 2%	17 ± 2%
	($n = 79$)	($n = 86$)	($n = 83$)
Mann–Whitney test	$Z = 5.45$	$Z = 2.40$	$Z = 0.11$
	($P = 5.10^{-8}$)	**($P = 0.012$)**	($P = 0.91$)
UNPUNISHED ODOR			
Conditioned individuals	32 ± 3%	27 ± 2%	27 ± 3%
	($n = 90$)	($n = 100$)	($n = 105$)
Control individuals	27 ± 4%	21 ± 2%	19 ± 3%
	($n = 69$)	($n = 76$)	($n = 78$)
Mann–Whitney test	$Z = 1.77$	$Z = 1.82$	$Z = 1.95$
	($P = 0.077$)	($P = 0.054$)	($P = 0.052$)

Data represent the mean percentage of time spent at rest relative to the total time spent in the odor during the 3-min test (±SE of the mean). The time spent at rest was compared between conditioned and control individuals with Mann–Whitney Z-test. Numbers of flies are bracketed. Significant P-values are in bold.

behavior). This time spent in the unpunished odor also tended to be higher in EM-, LTM-, and ARM-trained flies than in their corresponding control, but this was not significant despite large sample sizes. Altogether, these data provide no clear evidence that aversive conditioning increases the time spent resting in the unpunished odor.

EXPLORATION OF THE T-MAZE

When looking at the percentage of time allocated to the three defined zones of the arms of the T-maze by control flies (**Figure 7**), it appears that they spent most time (around 50%) in the proximal zone. They spent about 30–40% of time in the median zone, and only 10% of time in the distal zone of the arm.

This time allocation was modified in the punished odor in EM- and LTM-trained flies in comparison with the control flies. EM-trained flies showed a displacement from the median to the proximal zone (**Figure 7A**, Mann–Whitney test, proximal zone: $Z = 2.40$, $P = 0.016$; median zone: $Z = 2.14$, $P = 0.032$). LTM-trained flies spent also less time in the median zone, and more in the proximal zone, though this difference was not significant (**Figure 7B**. median zone: $Z = 2.32$, $P = 0.020$; proximal zone: $Z = 1.79$, $P = 0.074$).

This change in position may be a component of the conditioned response and it increases the probability that flies leave the punished odor. It was not observed in ARM-trained flies, which had the same exploratory behavior as the corresponding control flies (**Figure 7C**; Mann–Whitney test: $Z \leq 1.70$, $P \geq 0.088$).

In the unpunished odor, EM-, LTM-, and ARM- trained flies exhibited the same pattern of exploration as the respective control groups (**Figures 7A–C**; Mann–Whitney test: $Z \leq 1.64$, $P \geq 0.10$).

CORRELATION BETWEEN THE MEMORY SCORE AND BEHAVIORAL PARAMETERS

One question is whether the conditioned changes observed above are typical for EM, ARM, or LTM, or if they are dependent on the mean level of memory generated by the three training protocols, i.e., if individual flies' behavior is linked to its score value regardless of the training protocol. To investigate this hypothesis, we looked for correlations between the memory score and conditioned behavioral variables. For the three memory types, not surprisingly, the percentage of time spent resting in the punished odor was negatively correlated with the memory score (Spearman coefficient of correlation: $-56 \leq R \leq -61$, $P < 10^{-4}$), as well as the percentage of time spent in the median zone of the punished odor ($-50 \leq R \leq -77$; $P < 10^{-4}$). These two conditioned changes in locomotor and exploratory activity were therefore quantitative and not typical for any one memory form.

DISCUSSION

CHARACTERISTICS OF THE INDIVIDUAL CONDITIONED RESPONSE AS A FUNCTION OF MEMORY TYPE

In our assay, memory performance is measured as the time allocated to the punished vs the unpunished odor. This response involves first recognizing the odor, recalling its conditioned character and then translating the information into a behavioral response. Only the latter can be observed, but our work suggests that detailed characterization of conditioned behavior during retrieval can provide us with information on earlier stages of the memory retrieval process.

EM-trained and LTM-trained flies retrieve memory efficiently, and do so from the beginning of the test. All stages of the recognition-retrieval-response process are completed in approximately 3–4 s. In contrast, the random first choice of ARM-trained flies (**Figure 4C**) suggests that they do not immediately recognize the conditioned repulsive character of the odor. Apparently, ARM is not efficient enough to prevent flies from entering and exploring the arm of the punished odor (**Figures 6E and 7**), but is sufficient to encourage some of these flies to leave (**Figure 6E**) suggesting that they remember the repulsive character of the odor, but with a degree of latency.

The first choice has a major impact on memory performance in ARM-trained flies, as it is definitive for half of the flies entering the unpunished odor, and for one-quarter of the flies

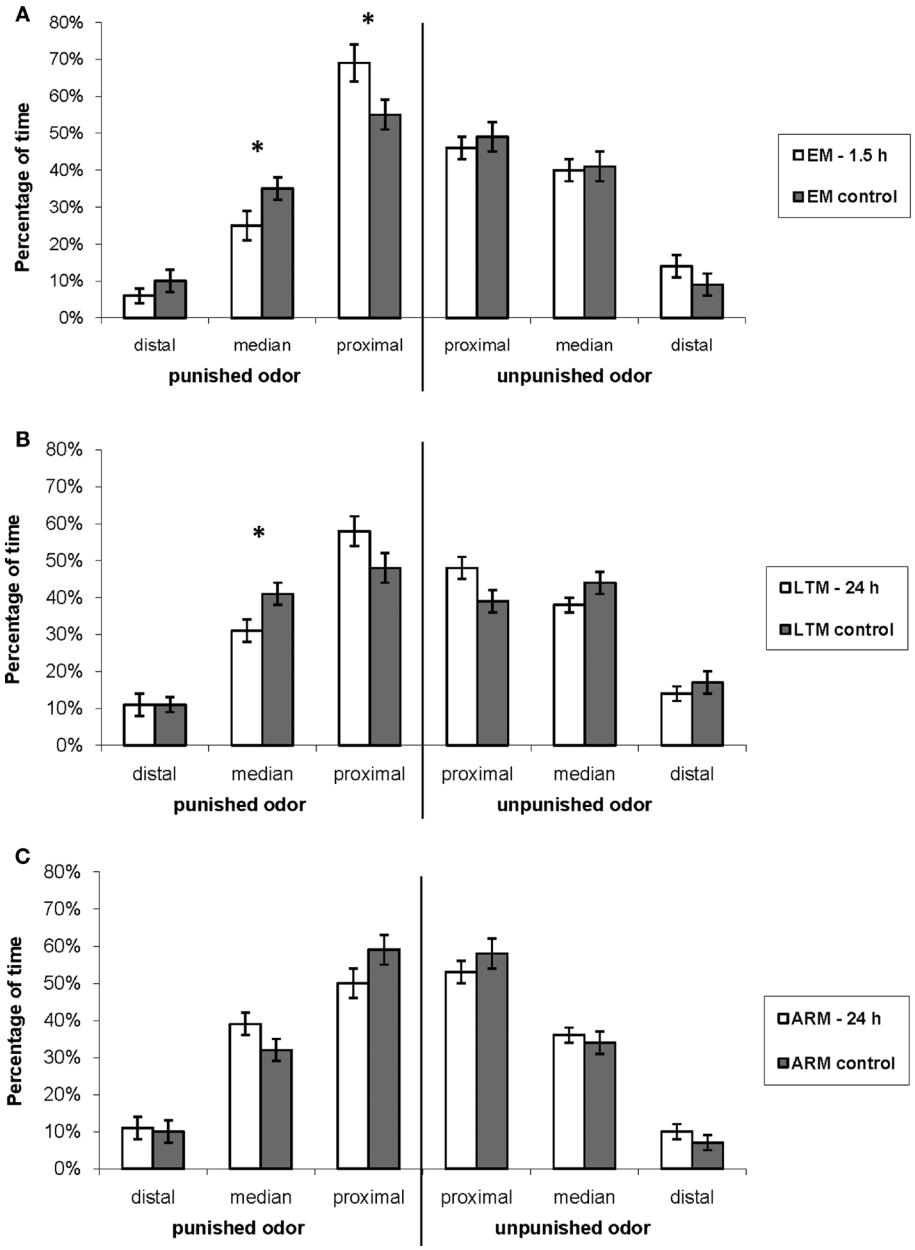

FIGURE 7 | Exploration of the T-maze arm when flies are in the punished or in the unpunished odor. Mean percentage of time allocated to the distal, median, and proximal zones of the arm relative to the total time spent in each of the odor types during the 3-min test, for flies trained and tested **(A)** for EM, **(B)** LTM, and **(C)** ARM (blank bars), and their respective controls (gray bars). Data represent the mean ± SE of the mean. Stars indicate a significant difference between conditioned and control flies, tested with the Mann–Whitney *Z*-test (* $P < 0.05$). Sample sizes are those of **Table 3**.

entering the punished odor (**Figure 6E**). In contrast, no LTM-trained fly stayed in the punished odor after a first incorrect choice (**Figure 6C**). After two stays in the punished odor, ARM-trained flies did not avoid it anymore (**Figure 6E**). This low level of leaving the punished odor, initially or later during the test, can be explained by weaker conditioned reactions. Resting behavior and progression into the arm carrying the punished odor are less inhibited in ARM-trained flies than in EM- and LTM-trained flies (**Table 3**; **Figure 7**). Such deficiency in ARM recall is initially present, as shown by the random choice of odors in this group, and may well be aggravated by memory extinction. Memory can be rapidly extinguished by repeatedly exposing *Drosophila* to the punished odor in the absence of punishment (Tully and Quinn, 1985; Schwaerzel et al., 2002; Lagasse et al., 2009). In the bee *Apis mellifera*, appetitive memory extinction begins from the first presentation of the conditional stimulus without reinforcement (Bitterman et al., 1983; Sandoz and Pham-Delègue, 2004). This suggests that ARM-trained flies might be susceptible to

memory extinction during the test. In contrast to the classical protocol of memory extinction in *Drosophila,* in which a single trial produces neither reconsolidation nor extinction (Lagasse et al., 2009), our set up may correspond to an operant form of memory extinction in individual flies, by allowing the flies to repeatedly experience the punished odor in the absence of negative reinforcement.

In the case of EM, which is thought to be a composite of early ARM and MTM (Tully et al., 1990), some characteristics of the conditioned response were similar to that of the ARM conditioned response. These characteristics included a similar low transition rate between the odors, the existence of a proportion of flies (albeit smaller than for ARM) that never correct their initially mistaken choice, and apparent memory extinction that occurs from the second odor change, earlier than for LTM (**Figures 6A,C,E**). The similarities between EM and ARM conditioned responses could be due to the fact that ARM is already formed 1.5 h after single conditioning, or could more simply be due to similar mechanisms of memory storage, without *de novo* protein-synthesis. However, EM-trained flies had a much higher score than ARM-trained flies, associated with a higher magnitude of behavioral changes including less time spent resting in the punished odor and less time spent in the median zone of the arm carrying the punished odor. These quantitative differences are likely due to less forgetting in EM-trained flies, because EM-trained flies are tested only 1.5 h after conditioning.

Long-term memory-trained flies shift more often between the odors than their control, which is not the case in EM-trained and ARM-trained flies (**Table 1**). However, such shifting does not hamper good individual LTM performance. LTM-trained flies leave, then re-enter the unpunished odor frequently and are able to avoid staying in the punished odor several times during the test (**Figure 6C**). In LTM-trained flies, avoidance of the punished odor therefore appears to be a dynamic process. After a certain number of odor changes, they may be sensitive to a mechanism of memory extinction, but this occurs less rapidly than in ARM-trained flies since random selection of odor in LTM flies occurs only after a higher number of odor changes than in ARM flies.

We observed that forgetting is more gradual for LTM-trained flies and more rapid for ARM-trained flies. This is shown by the distribution of LTM-trained flies in high and intermediate memory score classes. Some ARM-trained flies exhibit high memory scores but the remaining ones are distributed like the corresponding control flies (**Figure 3B**) due to the important deficit in memory recall (see Hypotheses for the Positive Effect of the Group on ARM Performance). In contrast with ARM, the clearly distinct and robust conditioned response 24 h after spaced conditioning would be due to the specific mechanism of protein-synthesis dependent LTM storage. Altogether, the differences found between ARM- and LTM-trained flies in the present study suggest that distinct memories are indeed expressed 24 h after massed and spaced procedures, favoring the model of exclusivity of ARM and LTM (Isabel et al., 2004), or at least that LTM expression is dominant over ARM expression if ARM and LTM coexist (Tully et al., 1994).

HYPOTHESES FOR THE POSITIVE EFFECT OF THE GROUP ON ARM PERFORMANCE

We showed recently that ARM-trained flies tested in groups have higher memory retrieval performance than when tested individually (Chabaud et al., 2009). A fly tested individually has not forgotten the learned odor but has a memory retrieval deficit. We know this because its memory score is good when it is tested in a group of trained flies, even when the group has been trained to the reverse odor combination (Chabaud et al., 2009). The data presented here clarify this hypothesis of retrieval deficit in individually tested flies, by pointing out an absence or a latency of memory retrieval, depending on the individuals. Individual ARM-trained flies tend to stay in the punished odor though they rest less in the punished odor than in the unpunished one, indicating that they may perceive the danger (**Table 3**). Their deficient exploratory behavior might be interpreted as a deficiency in decision-making associated with a physiological state of perceived social isolation (Cacioppo and Hawkley, 2009), a factor that has not yet been investigated in *Drosophila*.

The present analysis highlights the characteristics of the conditioned response that might be positively affected when flies are tested in groups. The presence of other trained flies could limit random first choice, or might reduce resting and exploration of the arm carrying the punished odor, and, thus, results in decreased time spent there. Interactions produced by ARM-trained flies in groups, possibly mediated by stress signals (Chabaud et al., 2009), would enhance their mates' attention while making the initial choice, and would create a less favorable context for memory extinction. It would be interesting to record the behavior of a single ARM-trained fly during testing within a group. Such an analysis is, however, beyond the capability of our current laboratory set up.

CONCLUSION

This work is the first behavioral analysis of individual punished odor avoidance in the T-maze, a set up used for over 30 years for neurogenetic studies of memory, based on group memory scores in *Drosophila*. It documents how individual flies produce quantitatively and/or qualitatively distinct avoidance response as a function of the conditioning procedure used and the type of memory formed, and sheds light on the precise behaviors that are negatively affected by social isolation in the case of aversive memory retrieval. Our study raises new questions about the processes underlying memory retrieval and decision-making in the fly brain.

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