



FROM STIMULUS TO BEHAVIORAL DECISION-MAKING

EDITED BY: Gérard Manière and Gérard Coureaud
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FROM STIMULUS TO BEHAVIORAL DECISION-MAKING

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The proper detection of stimuli coming from the environment is vital for any organism, in vertebrates as in invertebrates. Animals use different sensory modalities, independently or simultaneously, to detect them, such as audition, vision, olfaction, taste and touch. Indeed, in their ecosystems, animals constantly perceive various sensory signals. These stimuli come either from the environment (light, temperature, humidity, salinity...), or from other organisms (calls, colors, movements, pheromones, allomones, ...).

This eBook combines studies and comments (research articles, reviews, opinions ...), dealing with how animals detect a precise simple or complex sensory signal, confer a value to that signal, and respond to it by devoted and adaptive behaviors. This eBook provides a general overview for the detection of identified stimuli, and the mechanisms that lead to decision-making in animals. It also highlights the differences and similarities that may exist between vertebrates and invertebrates.

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Editorial: From Stimulus to Behavioral Decision-Making

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Editorial on the Research Topic

From Stimulus to Behavioral Decision-Making

In their natural environments, animals are continuously exposed to sensory complexity, due to the huge number and diversity of stimuli emitted and contained in their surroundings, within and between sensory modalities (somesthesis, olfaction and taste, audition, vision), and due to fluctuating and sometimes unpredictable conditions. Two main kinds of environmental factors have consequences on behavior in both solitary and gregarious species, abiotic factors (e.g., temperature, pressure, salinity, hygrometry, light/darkness, etc.) and biotic factors produced by organisms themselves (movements, displays, calls, colors, pheromones, etc.). These various stimuli regulate the life of animals and their interactions in the ecosystem. The present Research Topic aimed at providing illustrations, reflections, and elements of discussion about some of the stimuli that lead to decision making in vertebrates and invertebrates and about mechanisms that support their emission and/or action.

In response to environmental changes, animals adapt their physiology (homeostasis, etc.) and behavior (food seeking, avoidance of predators, social interactions, etc.) to survive. Responses to environmental stimuli may be either spontaneous, i.e., result from predispositions to perceive and process particular signals, or dependent on experience, i.e., be acquired through simple exposure, or associative/non-associative learning episodes. Learning may contribute to reduce or discard behavioral experiences that have negative consequences, as shown here in the original paper by Yang et al.. They found that the larvae of zebrafish could acquire by operant conditioning a new visual stimulus like pure-black pattern (conditioned stimulus) when it is associated with electric shock (unconditioned stimulus). The contrast of the visual stimulus (grayscale visual pattern) then modulates the learning responses and retention of the visual information in the larvae.

Animal behavior is also regulated by alternating day-night cycles over the nycthemeral period of 24 h, which can lead to the expression of circadian rhythmic behaviors. Some animals indeed have a daytime activity, whereas others have a nocturnal one. A fascinating question is how various sources of information are combined to sense a single time of the day in an animal. Actually, behavioral activities are mediated by internal clocks supported by neurophysiological mechanisms from the peripheral and/or central nervous system. Among these mechanisms, molecular processes may contribute to the internal clock and generation of the locomotor circadian rhythm. The mini-review by Somers et al. shows in particular that in *Drosophila*, locomotor activity depends on two brain oscillators, one that controls the morning locomotor activity and another that governs the evening activity. These clocks are autonomous oscillators, and the phase can be adjusted by light or thermal stimuli.

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Animals including humans may currently benefit from five main senses (more or less functional, depending on the species and on the developmental stages) allowing them to detect, discriminate, represent, and respond behaviorally to external stimuli.

Olfactory or gustatory organs in animals allow for detecting chemical cues merging from the ecosystem. These organs are fundamental, e.g., for food searching, identification of conspecifics, recognition of sexual partners, or detecting dangers such as predators or noxious compounds. For instance, some insect species are known to be major pest of crops. Many methods aim to use mating disruption to control insect populations using synthetic sex pheromones. In his review, Cattaneo shows the interest of the identification and functional characterization of the chemosensory receptors of *Cydia pomonella* (Lepidoptera: Tortricidae), a pest of apple, pear, and walnuts. A better knowledge and understanding of the olfactory mechanisms of pests would allow for a better control of their action by using the olfactory system as a target. About odor-guided searching for food, de Fouchier et al. show in invertebrates that caterpillars from a phytophagous insect, the moth-model pest *Spodoptera littoralis* (Noctuidae), respond behaviorally to plant volatiles. These chemical cues are ligands of olfactory receptors identified by the authors. By a complementary modeling approach, the authors pinpoint some receptors whose activation is related to caterpillar attraction. In vertebrates, Pettersson et al. show that attraction to mammalian blood odor is not exclusive to top predators (such as tigers and wolves), but that such an attraction may be also displayed by a small-bodied mesopredator, here the meerkat (*Suricata suricatta*). The study reveals that this attraction is triggered by a single molecule contained in mammalian blood, which seems to be the TED single molecule (trans-4,5-epoxy-(E)-2-decenal).

At the periphery of the olfactory system, olfactory receptor neurons sense odor cues. These sensory inputs are then processed by the brain to promote attraction or avoidance responses. Claßen and Scholtz show that in *Drosophila*, the behavior induced by an olfactory stimulus like ethanol can be shifted depending on specific activation of particular neurons. Indeed, the activation of a cluster of octopaminergic neurons leads to an attraction toward ethanol, while the activation of another group of octopaminergic neurons results in avoidance of the same stimulus. Thus, octopaminergic neurons are directly involved in decision making and contrasted behaviors in *Drosophila*.

Social insects live in colonies and communicate with conspecifics to transmit crucial information carrying adaptive values, e.g., enabling colony provisioning, defense, and reproduction. In hives, bees that have found and located a food source in the surroundings transfer multiple sensory information using the waggle dance to bee dance followers. In the present *Frontiers* Research Topic, Moauro et al. show that bee dance followers have better memory retention and higher gustatory responsiveness to sucrose than non-follower bees. The followers are more sensitive to environmental stimuli compared to non-followers and could decipher more easily the nature and localization of the food source encoded by the bee waggle dance.

Concerning taste and underlying neurophysiological processing, research in animal models may have fundamental impacts allowing for improving knowledge in human nutritional physiology, brain structures, and related behaviors. In their proof-of-concept study, Coquery et al. show that potentially appetitive (sucrose) or aversive (quinine) gustatory stimulations may be combined with functional MRI in minipigs to promote brain responses in part concordant with results already obtained in humans. This may constitute a promising way to run preclinical investigations that compare the integration of gustatory stimuli in healthy individuals or individuals suffering from dysgeusia or eating disorders.

In audition, certain signals carry crucial values in terms of adaptation and survival, as, for instance, in the case of signals that indicate the immediate presence of a predator. Animals must therefore remain attentive in order to select relevant auditory information that helps in deciding for an escape response. In their original study, Chapuis and Chadderton reveal that mice can detect a target sound from another in a sound mixture and that the activity of the auditory cortex is modulated during the different attentional states.

Surviving from threats such as predators depends also in some species on vision, and on acquired or innate defensive responses to visual stimuli. However, the regulation of innate fear responses by the additional perception of pleasantness/unpleasantness still remains debatable. In their original research, Liu et al. test the consequences of gentle handling of the innate response of mice to a visual looming stimulus. They show that handling attenuates the defensive response of mice in correlation with cortical plasticity, as the de-excitation of specific layers of the superior colliculus, and change in the connection between the cortex and the subcortex.

Finally, visual attention is also important in humans, for instance, as a contributor to consumer decision making. While individuals' determinants of this attention remain weakly understood, Audrin et al. propose here an original study assessing how visual attention can be modulated by psychological values in the context of forced-choice experiments proposing luxury/non-luxury ready-to-wear products. They show that participants with high vs. low levels of materialism display with visual attention to distinct information carried by the products (symbolic dimension vs. actual characteristics) that may end in the choice of a same product but for different reasons.

Thus, innate or learned responsiveness to stimuli and consecutive behaviors allow animals to adapt to their ecosystem and to provide for their vital needs such as food searching, sexual interacting with partners, and escaping from predators. To that goal, animals may modify their physiology and behavior by detecting changes in the environment. By being exposed at the same time or sequentially to stimuli from different sensory modalities like olfactory, visual, and auditory events, animals must combine multisensory information to adjust their behavior. The peripheral and central nervous systems play a major role in the integration of this information allowing for making a decision and *in fine* to display a coherent and efficient behavioral response.

AUTHOR CONTRIBUTIONS

GM and GC conceived and supervised this Frontiers Research Topic and wrote and edited the editorial.

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Assessment of Appetitive Behavior in Honey Bee Dance Followers

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Honey bees transfer different informational components of the discovered feeding source to their nestmates during the waggle dance. To decode the multicomponent information of this complex behavior, dance followers have to attend to the most relevant signal elements while filtering out less relevant ones. To achieve that, dance followers should present improved abilities to acquire information compared with those bees not engaged in this behavior. Through proboscis extension response assays, sensory and cognitive abilities were tested in follower and non-follower bees. Individuals were captured within the hive, immediately after following waggle runs or a bit further from the dancer. Both behavioral categories present low and similar spontaneous odor responses (SORs). However, followers exhibit differences in responsiveness to sucrose and odor discrimination: followers showed increased gustatory responsiveness and, after olfactory differential conditioning, better memory retention than non-followers. Thus, the abilities of the dance followers related to appetitive behavior would allow them to improve the acquisition of the dance surrounding information.

Keywords: *Apis mellifera*, waggle dance, gustatory responsiveness, olfactory conditioning, proboscis extension response

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INTRODUCTION

The waggle dance is a stereotyped behavior performed by *Apis mellifera* foragers which consists in an eight-shape figure on the vertical comb inside the hive (von Frisch, 1967). This complex behavioral display is considered a multicomponent signal (Grüter and Farina, 2009) which not only attracts nestmates to the dance surrounding but also informs the presence of a profitable food source (von Frisch, 1967; Seeley, 1989). Honey bees can acquire information about the location of the feeding site by following these maneuvers from behind or laterally (Michelsen, 2003; Díaz et al., 2007). The dance followers can also perceive and learn the odors of the collected food during interactions with the dancer (von Frisch, 1967; Farina et al., 2005; Díaz et al., 2007). In this way, both naïve and experienced foragers can acquire information from the waggle dance (Biesmeijer and de Vries, 2001; Biesmeijer and Seeley, 2005).

The honey bee dance takes place in particular comb areas named “dance floor” (Tautz and Lindauer, 1997), located at approximately 4–20 cm from the hive entrance, where dancers and dance followers come into contact (von Frisch, 1967; Seeley, 1995). Dance maneuvers increase the activity of bees in the dancer’s vicinity (von Frisch, 1923; Božič and Valentinčič, 1991; Thom et al., 2007). Thus, in this informational context, the levels of motivation and attention of the bees located in the dance surrounding might be enhanced by the presence of the excited dancers. As a result, follower bees are motivated to start foraging.

The shift from in-hive tasks to foraging involves changes in the responsiveness to external stimuli (Robinson, 1987; Robinson and Page, 1989; Seeley, 1989). Furthermore, bees performing different tasks within the colony also present different response thresholds (Ramírez et al., 2010). In this sense, Katz and Naug (2016) have shown that dancer and follower bees present different sensitiveness according to the individual and colony nutritional states. Creating a mismatch between these nutritional states and using a conditioning assay, they evaluated the proboscis extension response of fed and starved bees. Followers showed to be less sensitive to changes in the colony nutritional condition than dancers. However, incoming foragers can adjust their response to the nutritional status of the colony (Lindauer, 1954; Seeley, 1989; Farina, 2000; De Marco, 2006). Therefore, the presence of individuals responding differentially according to nutritional states would allow a better adjustment of the foraging activity of the entire colony. However, until now, it is unknown how different are the chemosensory and olfactory learning abilities of those bees located in the hive areas where the information related to the incoming resources is transmitted. Bearing this in mind, our aim is to study the sensory and cognitive capacities of bees involved in dance following and unemployed bees located next to the dance floor that did not follow dances at the moment they were captured. For this, we carried out behavioral assays testing sucrose responsiveness, spontaneous response to odors and ability to discriminate odors.

MATERIALS AND METHODS

Study Site and Animals

The experiments were performed during the summer-autumn seasons of 2015 and 2016 at the Experimental Field of the University of Buenos Aires, Argentina (34° 32'S, 58° 26'W). We used four observation hives (two colonies during 2015, henceforth: H1 and H2; and the other two during 2016, henceforth: H3 and H4) that consisted of two frames with brood, a mated queen and about 4000 workers of the European honey bees (*Apis mellifera*) each. The hives were contained between acrylic walls that had a 40 × 25 cm² window covered by a hinged door that allowed access to the colony during the assays. The observation hives were located individually inside a flight chamber (6 m length × 3 m wide × 2 m height), which was closed only during the experiments to prevent interference with other bee colonies. During the rest of the day, the flight chamber was opened for bees to have access to natural food sources.

General Procedure

A group of foraging workers of each experimental colony was trained to visit an artificial feeder located 6 m from the hive that offered 50% weight/weight (w/w) unscented sucrose solution. Foragers that visited the feeder were marked with acrylic paint (ALBA-Argentina) on their thoraxes to distinguish them from the rest of the nestmates.

Dance maneuvers of bees that returned from the artificial feeder were observed carefully by eye. A successful follower attends 3–7 consecutive waggle-runs before leaving the hive (Judd, 1994). Despite of the short distance to the artificial

source, it could be observed waggle dances. Thus, we defined as dance followers those hive bees that were captured directly from the unloading area (dance floor) after following at least three waggle-runs. In addition, we avoided capturing followers that performed oral contact with the dancers in order to avert a gustatory experience which could affect the sensory and cognitive capacities evaluated here (see sections below; Farina et al., 2007; Martinez and Farina, 2008). Unemployed hive bees that were not observed to be involved in dance following (henceforth: non-follower bees) were caught at a distance of 10–20 cm from the dancers, depending on the size of the dancing area (considering the approximate followed waggle dance radius is about 1.5 cm), and were used to compare their spontaneous odor response (SOR), gustatory responsiveness and odor discrimination with the dance follower's responses. Bees that performed cell cleaning, food and wax processing or absolute repose were not captured. It is worth to note that bees of both categories were caught from combs without brood or food reserves.

The captured bees were anesthetized in the freezer (−18°C) for no more than 2 min and harnessed in small metal tubes that restrained body movement but allowed free movement of antennae and mouthparts (Frings, 1944; Bitterman et al., 1983; Matsumoto et al., 2012). After waking, bees were offered a drop of water to drink if they would be tested for their gustatory responsiveness or 30% w/w sucrose solution if they would be submitted to the differential conditioning protocol and then, housed in an incubator (30°C, 55% RH and darkness) for 90 min before assessing their response.

Spontaneous Odor Response

The proboscis extension reflex (PER) is a reliable indicator for studying response to odors or sugared rewards, such as those found in nectar naturally (Frings, 1944; Page et al., 1998). Even though an odor is considered a neutral stimulus and usually the proboscis is not released by itself, a novice bee can still show a spontaneous response towards certain odors in the laboratory context (Guerrieri et al., 2005). For this procedure, two pure odors commonly present in floral fragrances (Knudsen et al., 1993; Raguso and Pichersky, 1999; Nouvian et al., 2015), Linalool (LIO) and Phenylacetaldehyde (PHE; Sigma-Aldrich, Steinheim, Germany), were used to test the SOR of bees from H1 and H2. A device that delivered a continuous airflow was used for the odorant application. Individually harnessed bees were exposed to a constant clean airstream (2.5 ml s^{−1}) delivered 2 cm away from their heads. A filter paper (30 × 3 mm) was impregnated with 4 μl of the pure odorant and placed inside a syringe. Each odor was delivered during 6 s when the airflow was redirected to pass through the syringe by means of an electric valve. A SOR was measured when the bee fully extended its proboscis towards any of the two odors, during odor delivery (Farina et al., 2005). Bees that responded to the mechanical air stimulus (6 s of clean airflow before and 3 s after odor presentation) were discarded, as well as bees that did not respond to 50% w/w sucrose solution after the gustatory response assay. The elapsed time between the first and the second odor presentation was 15 min, and the order was alternated from bee to bee.

Gustatory Responsiveness

PER was also used to evaluate gustatory responsiveness, by determining a gustatory response score (GRS) per experimental bee (Page et al., 1998). It was tested on bees from H1 and H2 after going through SOR assay. At the beginning of the assay, water was offered in order to avoid confounding thirst effects. Bees were assayed by presenting sucrose solutions of increasing concentration (0.1, 0.3, 1, 3, 10, 30 and 50% w/w; Page et al., 1998). Usually, this protocol only tests the response to sucrose concentrations from 0.1% to 30% w/w; however, we added the 50% w/w solution in order to include those bees with high sucrose response thresholds. Between each concentration of sucrose solution, all bees were tested for their response to water. This was done to avoid potential effects of repeated sucrose stimulation that could lead to increased sensitization or habituation. The inter-stimulus interval between water and sucrose solution was an average of 3 min. At the end of the procedure, a GRS was obtained for each bee. This score was based on the times each bee responded to the different sucrose concentrations (Scheiner et al., 2001; Pankiw et al., 2004). The response was arbitrarily quantified with scores from *one* to *seven*, where one (1) represented a bee that only responded to the highest sucrose concentration (50% w/w), while a score of seven (7) represented an individual that responded to all concentrations tested. If a bee failed to respond to a concentration of sucrose in the middle of a response series (e.g., responded to 0.1, 0.3, 3 and 10% w/w, but did not respond to 1%), this response was considered to be an error and the bee was deemed to have responded to that concentration as well. A bee that did not respond to more than one of the sucrose concentration in the middle of a response series was excluded from the analyses (Mc Cabe et al., 2007; Martinez and Farina, 2008). The same happened for bees that did not respond to the 50% w/w concentration (positive control, score = 1) offered because we cannot assure if they were able to sense and respond. In addition, those bees that responded to all sucrose concentrations but to all water presentations, too, were excluded from analyses as they appeared not to be able to discriminate between sucrose solution and water, were too starved or just thirsty.

Differential PER Conditioning

Two additional hives, H3 and H4, were exposed to 1.5 ml of a pure odor for at least 3 days before performing PER conditioning (H3: PHE, H4: LIO). The scent was provided by two small Petri dishes placed at the bottom of the hive containing a filter paper (3 cm diameter) soaked with 0.75 ml of the pure scent. The Petri's top lid was perforated to prevent bees touching the compound while volatiles could be dispelled. The volatile exposed in the hive had the purpose that bees associate it with a non-appetitive hive context (Farina et al., 2005; Díaz et al., 2007).

The bees captured from both colonies were subjected to a differential PER conditioning to analyze olfactory discrimination to two pure odors during training procedure (Bitterman et al., 1983): the rewarded odor (rewarded conditioning stimulus, CS+) was paired with 50% w/w sucrose solution (unconditioned stimulus, US), and the non-rewarded odor was presented alone

(non-rewarded conditioned stimulus, CS-) and was the same used in the hive as the pre-exposed odor. The same device described in SOR section was used for this assay. The volatile compound was delivered through a secondary air stream (2.5 ml s⁻¹) injected into the main airflow during the delivery of the odor. The bees were exposed to these stimuli five times each in a pseudo-randomized order (CS-, CS+, CS+, CS-, CS-, CS+, CS-, CS+, CS+, CS-). For each colony, differential PER conditioning was carried out using a pre-exposed odor as CS- and a non-exposed odor as CS+ (H3: CS- = PHE, CS+ = LIO; H4: CS- = LIO, CS+ = PHE). The inter-trial interval lasted 10–15 min between the CS presentations, depending on the number of individuals tested at one time (usually, $n = 20\text{--}40$). Only those bees that showed an unconditioned response (the reflexive extension of proboscis after applying a 50% w/w sucrose solution to the antenna, UR) and that did not respond to the mechanical airflow stimulus were used for the test. During the experiment in the per setup, a fan extracted the released odors to avoid contamination. Each learning trial lasted 40 s and we presented the CS for 6 s. Reinforcement (50% w/w sucrose) was delivered for 3 s after the onset of the CS+. Those bees that showed a spontaneous response to the odor in the first trial were discarded from the experiment. To evaluate whether the bees had formed a medium-term memory after they had all gone through the learning assay, bees stayed harnessed for 15 min and were then subjected to a non-rewarded presentation of both training odors (testing phase).

Statistics

The effects of factors on all variables were assessed by means of Generalized Linear Models (GLM) or Generalized Linear Mixed Models (GLMM), depending on the type of factors included in the models. Models were fitted in R program (R Development Core Team, 2011) using the *glm* function for the former case and the *glmer* function of the *lme4* package (Bates et al., 2015) for the latter, in which fixed and random effects are specified via the model formula. We used the MuMIn package which contains functions to streamline information-theoretic model selection and carry out model averaging based on the information criteria (Burnham and Anderson, 2002). The *dredge* function was used to perform the automated model selection with subsets of the global model; the set of models was generated with all possible combinations of the fixed factors. *AICc*, which is a second-order information criterion, was used to rank the models and to obtain model weights (Akaike, 1973). Unless the Akaike weight for the best model was high enough ($w_k \geq 0.8$, personal criterion), we could not consider that the predictors not selected were unimportant; so, in the cases in which the $w_k < 0.8$, we performed multimodel inference using the *model.avg* function which calculates model averaged parameters with standard errors and confidence intervals to evaluate the significances.

The effect of the dance following behavior on SOR was assessed by means of a GLM with binomial error structure. Gustatory responsiveness was estimated through the GRS, which is a sum of the unconditioned responses to the sugar solutions presented in the procedure. Values include 1 through 7. The effect of the following or non-following behavior on GRS was

assessed by means of a GLM with Poisson error structure. In both cases, the initial model included *behavior* and *hive* as fixed factors, considering only additive effects. *Hive* was considered as a fixed factor because it had only two levels (Zuur et al., 2007). In the particular case of GRS, in which significant difference between the hives was found, we analyzed *behavior* factor in each hive separately by constructing data subsets.

The effect of behavior on olfactory discrimination was assessed by means of a GLMM with binomial error structure. The initial model to analyze the acquisition phase included *behavior*, *trial* and *hive* as fixed factors, contemplating only additive effects, and *bee* as a random factor. In the testing phase, none of the bees extended its proboscis towards the CS-. Therefore, the effect of behavior was only studied on conditioned response towards the CS+ and assessed by means of a GLM which included *behavior* and *hive* as fixed factors, considering additive effects.

RESULTS

Spontaneous Odor Response

We tested the SOR of follower and non-follower bees from H1 and H2. Each experimental bee was exposed to a single presentation of two odor stimuli in the per setup: LIO and PHE. Because we were interested in the response to the odors independently of their identity, we analyzed the SOR towards any of them. Then, we grouped the odors since there was no interaction with the behavior fixed factor (in all cases, there was a bit higher response to PHE; data not shown). For both hives, we found that, independently of the behavioral category analyzed (followers vs. non-followers), bees showed low SOR levels (ca. 25%) and there was no difference between them (H1: $N_F = 103$, $N_{NF} = 88$; H2: $N_F = 79$, $N_{NF} = 81$). Indeed, the minimal model did not include the behavioral factor (Figure 1, Supplementary Table S1; $SOR_{H1+H2} \sim 1$). Thus, both followers and non-followers did not show odor preference. Although there was no significant difference between colonies, Figure 1 shows their result separately because, analyzing GRS of the same bees (see below), *hive* factor was included in the minimal model.

Gustatory Response Scores

After SOR, our aim was to study if gustatory responsiveness was affected by the dance context. We evaluated the sucrose response thresholds of bees from H1 and H2 under the PER paradigm. The GRS was defined as the sum of positive responses throughout the presentation of increasing concentration of the sucrose solutions (Page et al., 1998). Our results show that the dance followers had a higher GRS than non-follower bees in both experimental colonies [median values: 4 for followers ($N_{H1} = 103$, $N_{H2} = 88$), and 3 for non-followers ($N_{H1} = 79$, $N_{H2} = 81$)]. It means that follower bees present higher sucrose responsiveness than non-followers: followers respond to lower sucrose concentrations (Figure 2, Supplementary Table S2; $GRS_{H1} \sim \text{Behavior}$, $Z = -2.462$, $p = 0.0138$; $GRS_{H2} \sim \text{Behavior}$, $Z = -3.899$, $p = 9.65e^{-05}$). Hence, the gustatory responsiveness seems to be dependent on the behavioral category which bees belonged to. Even though we found significant difference between colonies, it is important to highlight that the tendency

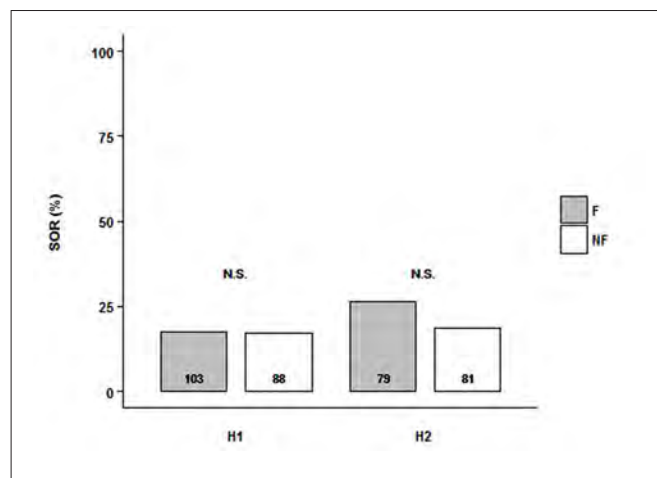


FIGURE 1 | Follower and non-follower dancing honey bees present similar probability of spontaneous odor response (SOR). Percentage of bees, followers (F, gray bars) and non-followers (NF, white bars) that extended their proboscises towards any of the two odors, Linalool (LIO) and Phenylacetaldehyde (PHE). Bees from Hive 1 (H1) and Hive 2 (H2) were tested. "N.S." indicates no statistical differences (*behavior*, as a factor, is not included in the final model; see "Results" section for details). The number of bees tested is shown inside the bars.

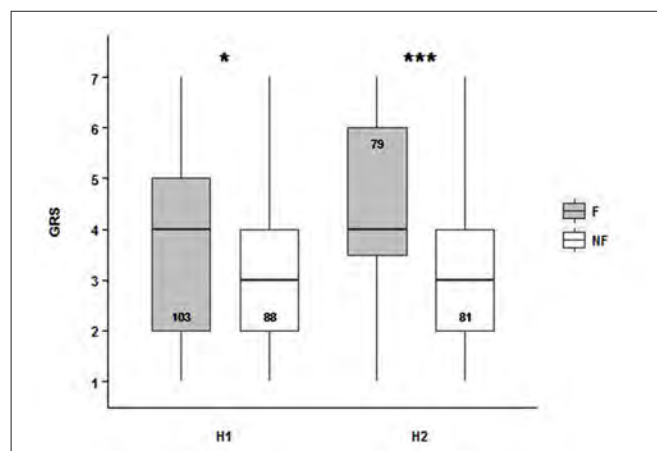
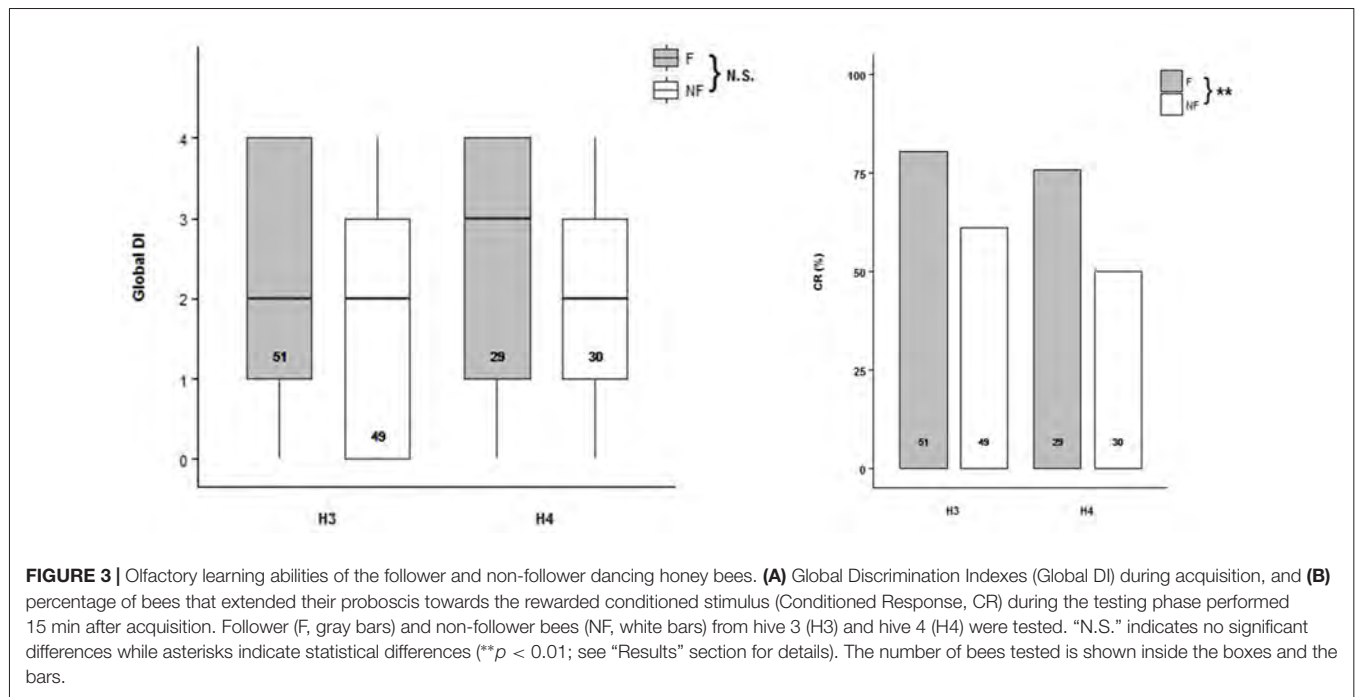


FIGURE 2 | Follower and non-follower dancing honey bees present different gustatory responsiveness. Gustatory response score (GRS) for followers (F, gray bars) and non-followers (NF, white bars) from Hive 1 (H1) and Hive 2 (H2). Medians (black line), quartiles as vertical boxes, and ranges with bars are shown. Median values: for followers, 4, and for non-followers, 3. Asterisks indicate statistical differences (* $p < 0.05$; *** $p < 0.001$; see "Results" section for details). The number of bees tested is shown inside the boxes.

for both of them, related to *behavior* factor, was the same, which means there was no interaction between the analyzed factors.

Odor Discrimination in Classical PER Conditioning

Odor discrimination were tested in bees from scent pre-exposed hives H3 and H4 (H3: $N_F = 51$, $N_{NF} = 49$; H4: $N_F = 29$, $N_{NF} = 30$) under PER conditioning procedure. Global Discrimination Index (DI) was defined for each bee as the number of trial pairs the bee succeeded in discriminating between the two odors, in other



words, if it extended its proboscis towards the CS+ but not to the CS− (Mengoni Goñalons et al., 2016). No difference was found during the acquisition between the two behavioral groups of bees (Figure 3A, Supplementary Table S3; $ACQ_{H3+H4} \sim \text{Trial}+1 | \text{Bee}$). However, the acquisition level reached by the follower bees at the last trial was between 10% and 15% higher than by the non-followers, in both hives (H3: $F = 73\%$, $NF = 63\%$; H4: $F = 72\%$, $NF = 57\%$). During the testing phase, the follower bees exhibited a higher Conditioning Response (CR) to CS+ than the non-follower ones (Figure 3B, Supplementary Table S4; $TEST_{H3+H4} \sim \text{Behavior}$, $Z = -2.724$, $p = 0.00646$). Therefore, followers showed better memory retention than the non-follower when they were evaluated 15 min after the olfactory conditioning.

DISCUSSION

Through PER assays, we evaluated olfactory and gustatory responsiveness besides the ability to discriminate odors through classical conditioning in honey bee workers with different probabilities to be recruited as foragers. A brief time after capturing both dance follower and non-follower bees showed similar SOR levels but significant differences in the sucrose responsiveness and odor memory retention. Specifically, dance followers presented higher gustatory responsiveness (higher GRS levels) and better memory retention after olfactory conditioning than non-followers.

We did not take into account the age of the experimental bees in this study. Previous reports suggest that age seems not to be relevant within this social context. For instance, individualized bees that followed dances showed a wide age range (i.e., from 9 to 32 days old; Balbuena et al., 2012). Likewise, a recent study evaluated the sucrose responsiveness of hive

bees captured from the dance floor or delivery area within an age interval of 2–15 days old (Mengoni Goñalons et al., 2016). No age-dependent relationship was found for the sucrose responsiveness measured at the per setup in that study. In this sense, the gustatory responsiveness of honey bees may be affected by the informational context where individuals were caught from (i.e., dance floor) more than by their age. Indeed, Martinez and Farina (2008) showed that hive bees captured after receiving food from a donor bee presented different sucrose responsiveness according to the food quality received during the oral contact.

In the present study, bees captured in the dance context (following dances but did not interact orally with a dancer) showed higher gustatory responsiveness compared with those bees captured at a distance of 10–20 cm from the dancer. High GRS values correlate positively with improved performances during olfactory conditioning as it was previously reported (Scheiner et al., 2004; Mengoni Goñalons and Farina, 2015). Consistent with this evidence, we found that dance followers showed improved levels of memory retention after an olfactory PER conditioning. However, when sucrose responsiveness correlates with memory retention, also correlates with learning performance (Scheiner et al., 2001, 2004; Mengoni Goñalons and Farina, 2015). Here, we only found a correlation between memory retention and gustatory responsiveness. Those previous studies used absolute conditioning procedures to test it. In this study, differential conditioning has been used. This protocol evaluates the ability to associate an odor to a reward, but also the capacity to distinguish it from another which is not linked to an unconditioned appetitive stimulus. Here, our unrewarded odor, CS−, is the same odor used that we used as the hive odor and it would represent a nonappetitive context for the experimental subjects. Thus, the differences in response to rewarded and

unrewarded odors would be bigger for those bees that are more motivated to acquire appetitive information. Although it was not significant, this tendency can be observed at the end of the learning performance for dance followers and clearly visualized while the trained odors were tested 15 min later. It is worth mentioning that we only evaluated memory retention at a medium-term scale. It is expected that highly motivated individuals not only learn faster but also recall longer, an issue which was not covered in the present study.

Changes in the motivation and attention levels of the dance followers would turn out to be more sensitive to any other environmental stimuli, a fact that might facilitate the decoding of spatial information transmitted as well as the acquisition of incidental cues such as floral odors carried by the waggle dancer. The role of the early odor-rewarded experiences acquired in the beehive as a stimulus that facilitates the decoding of waggle dance information at elder ages has been suggested (Balbuena et al., 2012). In that study, honey bees preferred to follow dancers scented with an early exposed and rewarded odorant, and even they were recruited to the feeding site scented with the early experienced odors more successful. As the presence of reward affects physiological states in honey bees within a short-term period (Hammer, 1997), the most vigorous dances, which indicate the presence of a highly profitable food source, might represent appetitive stimuli that facilitate a prompt acquisition of information within the dance context.

This study shows a correlation between sensory and cognitive performances and behavioral category based on the dance

context. Nevertheless, it does not show if there is a causal relationship. To do that, the life history of individual bees should be considered to determine whether bees with a low gustatory responsiveness tend to follow dances or even whether the sucrose responsiveness changes after dance following. Our results are a first approach to understand abilities of the dance surrounding bees, but this issue requires further analysis.

AUTHOR CONTRIBUTIONS

MAM, MSB and WMF contributed to the conception and design of the study and contributed to the drafting and revision of the manuscript and approved the final version. MAM and MSB conducted the experiments. MAM analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00074/full#supplementary-material>

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Octopamine Shifts the Behavioral Response From Indecision to Approach or Aversion in *Drosophila melanogaster*

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Animals must make constant decisions whether to respond to external sensory stimuli or not to respond. The activation of positive and/or negative reinforcers might bias the behavioral response towards approach or aversion. To analyze whether the activation of the octopaminergic neurotransmitter system can shift the decision between two identical odor sources, we active in *Drosophila melanogaster* different sets of octopaminergic neurons using optogenetics and analyze the choice of the flies using a binary odor trap assay. We show that the release of octopamine from a set of neurons and not acetylcholine acts as positive reinforcer for one food odor source resulting in attraction. The activation of a subset of these neurons causes the opposite behavior and results in aversion. This aversion is due to octopamine release and not tyramine, since in *Tyramine- β -hydroxylase* mutants (*T β h*) lacking octopamine, the aversion is suppressed. We show that when given the choice between two different attractive food odor sources the activation of the octopaminergic neurotransmitter system switches the attraction for ethanol-containing food odor to a less attractive food odor. Consistent with the requirement for octopamine in biasing the behavioral outcome, *T β h* mutants fail to switch their attraction. The execution of attraction does not require octopamine but rather initiation of the behavior or a switch of the behavioral response. The attraction to ethanol also depends on octopamine. Pharmacological increases in octopamine signaling in *T β h* mutants increase ethanol attraction and blocking octopamine receptor function reduces ethanol attraction. Taken together, octopamine in the central brain orchestrates behavioral outcomes by biasing the decision of the animal towards food odors. This finding might uncover a basic principle of how octopamine gates behavioral outcomes in the brain.

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INTRODUCTION

Animals must make constant decisions whether to respond to external sensory stimuli or not to respond. Depending on the internal condition of the animals, they might respond with approach, indifference or aversion. The observed indifference could be due to the inability to perceive the sensory information, the meaninglessness of the information, and/or the inability to decide to which information to respond. Other reasons for showing indifference include the inability of animals

to translate the decision into motor output and the inability to execute the locomotion required to fulfill the task. The activation of positive and/or negative reinforcers might bias the behavioral response towards approach or aversion.

In nature, *Drosophila melanogaster* is attracted to ethanol-enriched fermenting food sources (Dudley, 2002; Zhu et al., 2003). For example, female flies search for and prefer fermenting food sources as a substrate to lay their eggs (Richmond and Gerking, 1979; Azanchi et al., 2013). The search for food sources depends on olfactory cues (Chow and Frye, 2009). Given the choice between two similar attractive food odor sources, male and female *Drosophila* are more attracted to ethanol-enriched food odors than to a similar odor without ethanol (Ogueta et al., 2010). The low ethanol concentration that elicits approach functions as a key odorant in the food odor mixture (Giang et al., 2017). The approach depends on the positive reinforcing action of the octopaminergic/tyraminerbic neurotransmitter system, as neuronal activation of neurons expressing tyrosine decarboxylase 2 (dTdc2)—the enzyme required for tyramine and indirectly for octopamine synthesis (Cole et al., 2005)—results in attraction (Schneider et al., 2012). The voluntary movement to the site of neuronal activation resembles the voluntary self-administration of electric shocks into the rodent brain that are perceived by the animal as rewarding and uncover the function of reinforcing properties in the brain (Olds and Milner, 1954; Schneider et al., 2012). The optogenetic activating pattern used to stimulate reinforcing tyraminerbic/octopaminergic neurons in *Drosophila* brain resemble the high and low frequency of activity observed in the octopaminergic VUMmx1 neuron of the honey bee carrying information of the unconditioned stimulus in olfactory associative appetitive reward learning (Hammer, 1993; Schneider et al., 2012). Activation of the tyraminerbic/octopaminergic neurotransmitter system is not only sufficient to cause a bias, but it also might be required for the attraction. *Tyramine- β -hydroxylase* mutants (more specifically the *T β h^{nM18}* mutant)—lacking detectable level of octopamine and with increased TA levels—do not show ethanol-induced site attraction (Monastiriotti et al., 1996; Schneider et al., 2012). The loss of attraction is not due to the loss of odorant perception (Schneider et al., 2012), although octopamine might modulate sensory input. For example, octopamine has been shown to reduce the sensory response of bitter-sensing neurons (LeDue et al., 2016).

The octopaminergic neurotransmitter system acts also as a positive reinforcer in other behaviors, such as positive associative olfactory learning and memory in *Drosophila*, (Schwaerzel et al., 2003). However, there is also evidence that the octopaminergic neurotransmitter system might function as a negative reinforcer in the regulation of behavior, as the octopaminergic neurotransmitter system suppresses courtship conditioning (Zhou et al., 2012). Therefore, the octopaminergic neurotransmitter system might do both: function as a positive and negative reinforcer depending on the behavior or condition analyzed. In a more neutral/general way, the octopaminergic neurotransmitter system might function by selecting the right behavioral response or deciding what

stimulus to respond to when multiple types of information are presented.

Evidence that octopamine acts as positive and negative regulator is also derived from observations of other insects. In locusts, octopamine release at one site of the ventral nerve cord evokes bouts of rhythmic flight motor activity and at another site suppresses neuronal activity related to oviposition behavior (Sombati and Hoyle, 1984). These results led Sombati and Hoyle (1984) to propose the “orchestration hypothesis” to describe the function of octopamine. For every set of a behavior, a neuronal network exists that can be selectively activated or inhibited by the release of octopamine and therefore allow suppression of opposing behaviors, such as the initiation of flight and suppression of egg laying (Sombati and Hoyle, 1984). However, whether similar selection processes also work in the central brain to modify behavioral output has not been investigated.

To investigate whether the octopaminergic neurotransmitter system is also required in the selection of behavioral programs in the central brain of *Drosophila*, we first addressed whether octopamine can act as negative and positive reinforcer for an odor-guided behavior that involves decision making. To evaluate whether the fly makes a decision, we used a binary choice assay consisting of two odor traps filled with the same food odor to control for the same sensory input. We activated different sets of tyraminerbic/octopaminergic neurons using optogenetics and analyzed the consequences of this activation on the choice of the fly to enter a food odor trap. We next addressed whether indeed octopamine and not tyramine mediated these behavioral choices by introducing *T β h^{nM18}* mutants lacking octopamine to the same condition. We show that octopamine functions as a positive and negative reinforcer in odor-evoked behaviors depending on the sets of neurons that are activated. We provide evidence that the activation pattern used for optogenetic activation does not influence the function of the reinforcer. To analyze whether octopamine release shifts the behavioral outcome, we activated the octopaminergic neurons in the presence of a less attractive food odor in comparison to a more attractive ethanol-enriched food odor. We show that the release of octopamine is required to shift the attractiveness of an attractive food odor source to a less attractive food odor source. We further provide evidence that the attraction of ethanol-enriched food odors also requires the release of octopamine by pharmacologically altering octopamine levels and interfering with octopamine receptor function. Taken together, we provide evidence that octopamine is required to select behavioral responses and that the “orchestration of different behavioral circuits underlying behavioral elements” in the central brain is also mediated by octopamine.

MATERIALS AND METHODS

Fly Stocks

Flies were raised and kept on an ethanol-free standard cornmeal/molasses/yeast/agar medium on 12-h/12-h light/dark cycle at 25°C with 60% humidity. Flies carrying transposable elements were backcrossed for at least five generations to the *w¹¹¹⁸* maintained in the laboratory to isogenize the genetic

background. The following fly lines were used: w^{1118} , $T\beta h^{nM18}$ (Monastirioti et al., 1996), $norpA^-$; $UAS-ChR2$; $UAS-ChR2$ (Schneider et al., 2012), w^{1118} ; $UAS-ReaChR$; $UAS-ReaChR$ (Inagaki et al., 2014), w^{1118} ; $dTdc2-Gal4$ (Cole et al., 2005) and $ChAT-Gal80$ (Kitamoto, 2002) and w^{1118} ; $6.2_2-T\beta h-Gal4$ (Schneider et al., 2012).

Optogenetic Site Attraction Assay

Flies expressing channelrhodopsin (ChR) or red activatable ChR were raised on food with 200 μ l 250 mM all-*trans* retinal (ATR) dissolved in 100% ethanol or 200 μ l 100% ethanol in the dark. Fifty three- to five-day-old male flies were collected using CO₂ anesthesia and recovered in the dark for at least 24 h in the presence of food with either 100 μ l 100% ethanol or 100 μ l 250 mM ATR. The behavioral experiments were performed as previously described (Schneider et al., 2012). Briefly, flies were given the choice between two odor traps filled with 1.5 ml of apple-mango-juice (Alnatura, Germany GTIN: 4104420071841) for 19 h overnight in the dark at 25°C and 60% humidity.

Both traps were illuminated with two different LEDs with the same intensity ranging from 800 lux to 1700 lux. For the blue light-inducible ChRs, the following two diodes were used: a blue light LED (465–485 nm, Cree, Germany) and a warm white LED (Cree, XLAMP, XR_E LED with 2600–3700K CCT) combined with a blue light filter (high pass filter, 510 nm, HEBO, Aalen, Germany) resulting in yellow light. For the redshifted ChR, the following diodes were used: a blue light LED (465–485 nm, Cree, Germany) and an amber light LED (595 nm, Cree, XRCAMB-L1-0000-00J01). All LEDs repeated the activation pattern of 2 s 40 Hz, 16 s 8 Hz and 2 s 0 Hz for 19 h. The next day, the attraction index (AI) was calculated.

Olfactory Attraction Assay

The assay was performed as described previously (Ogueta et al., 2010). Briefly, 50 1- to 3-day-old male flies were collected using CO₂ anesthesia and recovered from the treatment for at least 24 h. The experiments were conducted for 19 h overnight at 25°C and 60% humidity on a cold white light plate or when using ChR on a dark surface. To determine ethanol attraction, flies choose between apple-mango juice and 5% ethanol-enriched apple-mango-juice. Prior to the test, flies were fed with different drugs as follows. The control group was fed on a solution containing 5% sucrose/5% red food color on filter paper. The experimental group received the same food with the drug. If not mentioned otherwise, the flies were starved for 3 h before treatment without any access to water. The concentration of 53 mM octopamine was fed for 1 h (300 μ l), 50 mM clonidine for 3 h (200 μ l), 200 nM naphazoline for 3 h (200 μ l) and 25 mM yohimbine for 2 h (300 μ l). Non-starved flies were fed with 3 mM epinastine for 2 days (300 μ l, daily remoistening of the filter paper) or with 345 mM tyramine (without 5% sucrose) for 18 h. For the ethanol pre-feeding experiments, flies were fed with 10% ethanol for 30 min (200 μ l) after they were starved overnight in the presence of moistened filter paper. Flies were recovered in the presence of moistened filter paper (200 μ l) to avoid intoxication during the behavioral experiments for 3.5 h.

Statistical Analysis

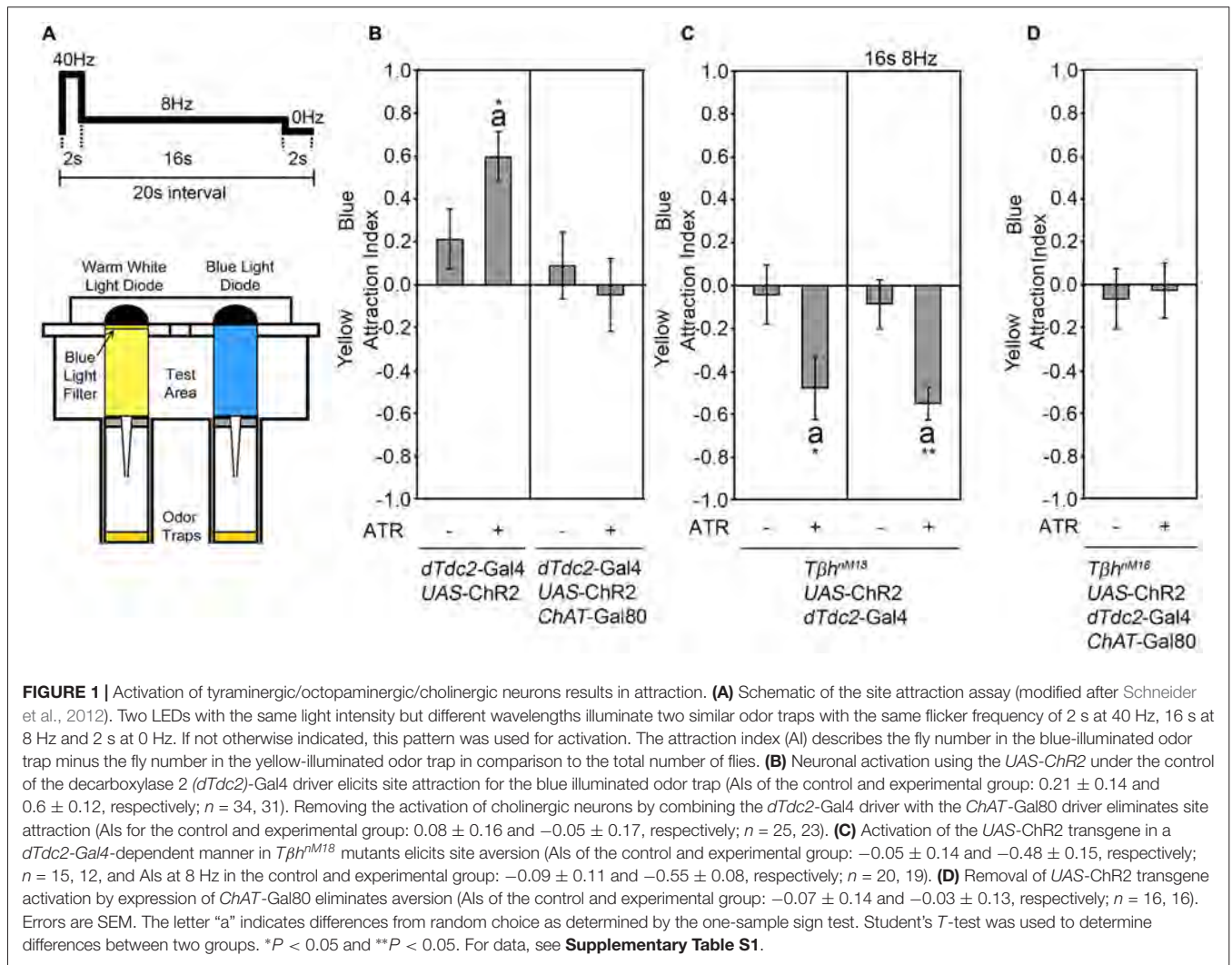
Errors represent the standard error of the mean (SEM). Bars labeled with the letter “a” indicate differences from random choice as determined with a one-sample sign test. Because the data were normally distributed for comparison of two experimental groups, Student’s *T*-test was used. For more than two groups, ANOVA and the Tukey-Kramer *post hoc* test were used. The significance levels are indicated as follows: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

RESULTS

In a binary choice assay, flies choose between two equal food odor traps (Ogueta et al., 2010). The presence of an attractive key odorant within the food odor biases the decision to move towards and enter the food odor trap containing the key odorant (Giang et al., 2017). The attraction for one odor trap in comparison to an equal second food odor trap can also be induced by activation of tyraminerpic/octopaminergic neurons using the *UAS-ChR2* transgene under the control of the *dTdc2-Gal4* driver (Schneider et al., 2012; **Figure 1A**). For these experiments, one odor trap is illuminated with blue light, resulting in activation of neurons expressing ChR2 in the presence of ATR, and the second odor trap is illuminated with yellow light with the same intensity to avoid a bias based on difference in light intensity (**Figure 1A**). To elicit the reinforcing properties of the tyraminerpic/octopaminergic neurons, we used a sequence of light stimulation of 40 Hz for 2 s, 8 Hz for 16 s followed by 2 s of no light. This activation pattern mimics the response of the reinforcing VUMmx1 neuron in honey bees (Hammer, 1993; Schneider et al., 2012).

The Activation of Octopaminergic Neurons Is Sufficient and Required for Attraction

The *dTdc2-Gal4* line drives transgene expression in at least 137 neurons (Busch et al., 2009). To narrow down the subset of neurons sufficient to induce site attraction, we restricted the expression of the *dTdc2-Gal4* driver using the *ChAT-Gal80* driver (Kitamoto, 2002; **Figure 1B**). The expression of the Gal80 repressor resulted in the expression of transgenes in 34 neurons in the brain, including 3–5 VUM neurons per segment and neurons within the G0b, G2b G3a/AL2, G3b, G5a and G5b, 5c clusters (see Supplementary Figure S1 in Schneider et al., 2012). Activation of these restricted sets of neurons using the *UAS-ChR2* transgene under the control of the *dTdc2-Gal4* driver combined with the *ChAT-Gal80* driver did not result in site attraction (**Figure 1B**). The *dTdc2* enzyme is required for the synthesis of tyramine and therefore indirectly required for octopamine synthesis (Cole et al., 2005). The *dTdc2-Gal4* line drives transgene expression in octopamine-expressing and 41 T β h-positive neurons in the brain (Busch et al., 2009; Schneider et al., 2012). To address whether octopamine elicits attraction, we performed a similar experiment in *T\beta h^{nM18}* mutants. The *T\beta h^{nM18}* mutants lack the T β h isoform that converts tyramine into octopamine and have no detectable levels of octopamine, but 8-fold significantly



increased levels of tyramine (Monastirioti et al., 1996). Activation of *dTdc2*-Gal4-targeted neurons in a *T β h^{NM18}* mutant background lacking octopamine resulted in loss of attraction and significant site aversion using two different activation frequencies (Figure 1C). This result suggests that octopamine release mediated attraction. The observed aversion might be due to increased tyramine levels or other neurotransmitter that were co-expressed in tyraminerbic/octopaminergic neurons. To test whether activation of acetylcholine transferase (ChAT)-positive neurons elicits aversion, expression of the *dTdc2*-Gal4 driver in the *T β h^{NM18}* mutant background was restricted using the *ChAT*-Gal80 driver (Figure 1D) to reduce transgene expression to 16 neurons in the brain, including a subset of VUM neurons and neurons of the G3a/AL2 and G3b cluster (Schneider et al., 2012; see Supplementary Figure S1). Repression of activation in cholinergic neurons resulted in a loss of aversion, indicating that activation of ChAT-positive neurons mediated the aversion. Taken together, the results suggest that octopamine release from acetylcholine co-expressing neurons mediates attraction.

Activation of a Subset of Octopaminergic Neurons Is Sufficient and Required for Aversion

We next addressed whether activation of a subset of neurons included in the expression pattern of the *dTdc2*-Gal4 driver line also resulted in attraction. Therefore, we expressed the ChR2 transgene in three VUMa4 neurons in the SOG using the *6.2-T β h*-Gal4 driver (Figure 2). The *6.2-T β h*-Gal4 driver targets transgene expression in the three VUMa4 neurons and also to non-*T β h* positive neurons (Schneider et al., 2012). In contrast to the whole set of VUM neurons included in the expression pattern of the *dTdc2*-Gal4 driver, the activation of neurons in *6.2-T β h*-Gal4 dependent manner resulted in significant aversion to the site of blue light activation (Figure 2A). Activation of a subset of dopaminergic neurons using the *TH*-Gal4 driver resulted in aversion (Schneider et al., 2012). To exclude that the observed aversion was due to activation of dopaminergic neurons, we combined the *6.2-T β h*-Gal4 with the *TH*-Gal80 driver to suppress expression in putative dopaminergic neurons (Figure 2A). Here, neuronal activation still resulted in significant

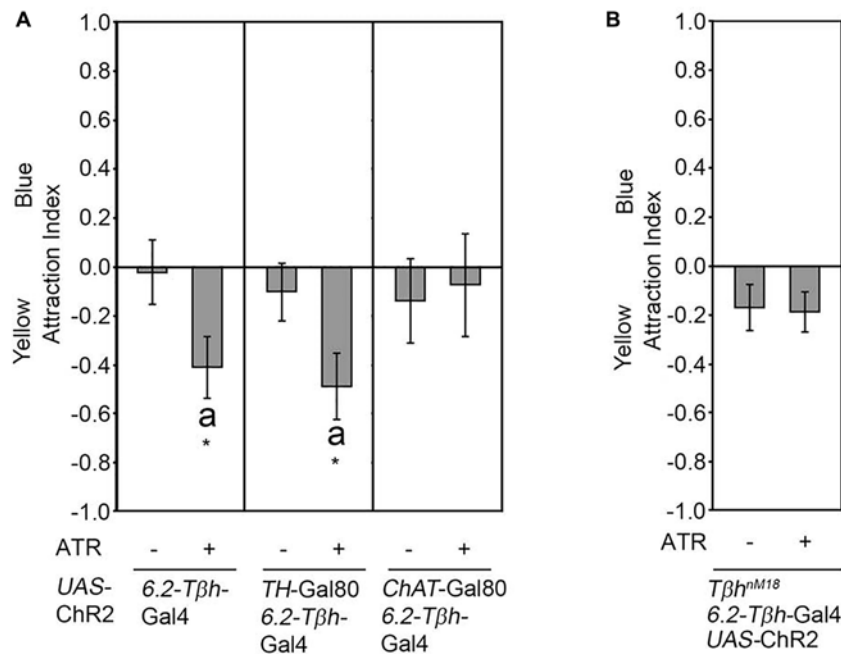


FIGURE 2 | Octopamine release causes aversion. **(A)** Neuronal activation under the control of the *6.2-Tβh-Gal4* driver elicits site aversion (AIs of the control and experimental group: 0.02 ± 0.14 and -0.41 ± 0.13 , respectively; $n = 22, 19$). Removal of the activation of dopaminergic neurons by combining the *6.2-Tβh-Gal4* driver with the *TH-Gal80* driver does not influence aversion (AIs for the control and experimental group: 0.1 ± 0.12 and -0.49 ± 0.14 , respectively; $n = 14, 14$). Removing ChAT positive neurons by using the *ChAT-Gal80* in combination with the *6.2-Tβh-Gal4* driver resulted in loss of aversion (AIs of control and experimental group: -0.14 ± 0.17 and -0.08 ± 0.21 respectively; $n = 13, 11$). **(B)** Activation of the *UAS-ChR2* transgene under the control of *6.2-Tβh-Gal4* in *Tβh^{M18}* mutants eliminates aversion (AIs of the control and experimental group: -0.17 ± 0.1 and -0.19 ± 0.08 , respectively; $n = 20, 19$). For activation, the flicker frequency of 2 s at 40 Hz, 16 s at 8 Hz and 2 s at 0 Hz was used. Errors are SEM. Differences from random choice were determined using the one-sample sign test and are indicated by the letter “a”. Student’s *T*-test was used to determine differences between two groups. * $P < 0.05$. For data, see **Supplementary Table S2**.

aversion (**Figure 2A**). Restriction of the Gal4 expression pattern of the *6.2-Tβh-Gal4* driver to non-cholinergic neurons using the *ChAT-Gal80* driver resulted in loss of aversion showing that activation of ChAT positive neurons mediate aversion (**Figure 2A**). To investigate whether the observed aversion was also regulated by octopamine release, we activated neurons targeted by the *6.2-Tβh-GAL4* driver in *Tβh^{M18}* mutants lacking octopamine (**Figure 2B**). Here, the activation did not elicit site aversion or attraction. Therefore, the results suggests that octopamine can also mediate aversion depending on the sets of octopaminergic neurons activated.

The Frequency and Intensity of Light Activation Influences the Behavioral Outcome

The frequency used to activate the *UAS-ChR2* transgene mimicked the frequency used to depolarize the VUMmx1 neuron in honey bees (Hammer, 1993; Schneider et al., 2012). The depolarization with electrodes in honey bees and the activation of the *UAS-ChR2* transgene in *Drosophila* using optogenetics with this frequency was sufficient to elicit the reinforcer (Hammer, 1993; Schneider et al., 2012). To further investigate whether the observed site attraction or site aversion was caused by a specific activation frequency, we used different light flicker frequencies

with a constant intensity to activate the *UAS-ChR2* transgene in a *dTdc2-GAL4*-dependent manner and analyzed the consequence of this activation on site attraction (**Figure 3**). Neither the short pulse of 40 Hz nor the long pulse of 8 Hz was sufficient to result in site attraction (**Figure 3A**). We next tested the average frequency of 11.5 Hz. The flicker pattern still did not result in site attraction. A reduction of 40–20 Hz within the 40–8 Hz pattern was sufficient to elicit a significant attraction. The observed frequency-dependent site attraction suggested that either a specific frequency or the kinetics of the transgene influenced the behavioral outcome. We next tested whether there was a similar frequency dependence behavioral outcome when using the *6.2-Tβh-Gal4* driver (**Figure 3B**). Activation of the *UAS-ChR2* transgene with an 8-Hz pulse followed by 4 s in the dark in a *6.2-Tβh-Gal4*-dependent manner was sufficient to elicit site aversion. However, neither the reduction to 20 Hz for 2 s followed by 8 Hz for 16 s nor the activation by only 40 Hz resulted in site aversion. Next, we addressed whether the intensity of the light influenced the activation of the transgene (**Supplementary Figure S1, Table S6**). The flicker sequence used for activation was kept constant with a pattern of 40 Hz for 2 s followed by 8 Hz for 16 s. Changing the light intensity for neuron activation resulted in site attraction in a *dTdc2-GAL4*-dependent manner; however, the degree of attraction as measured in AI varied from 0.3 to 0.6. Changing the light

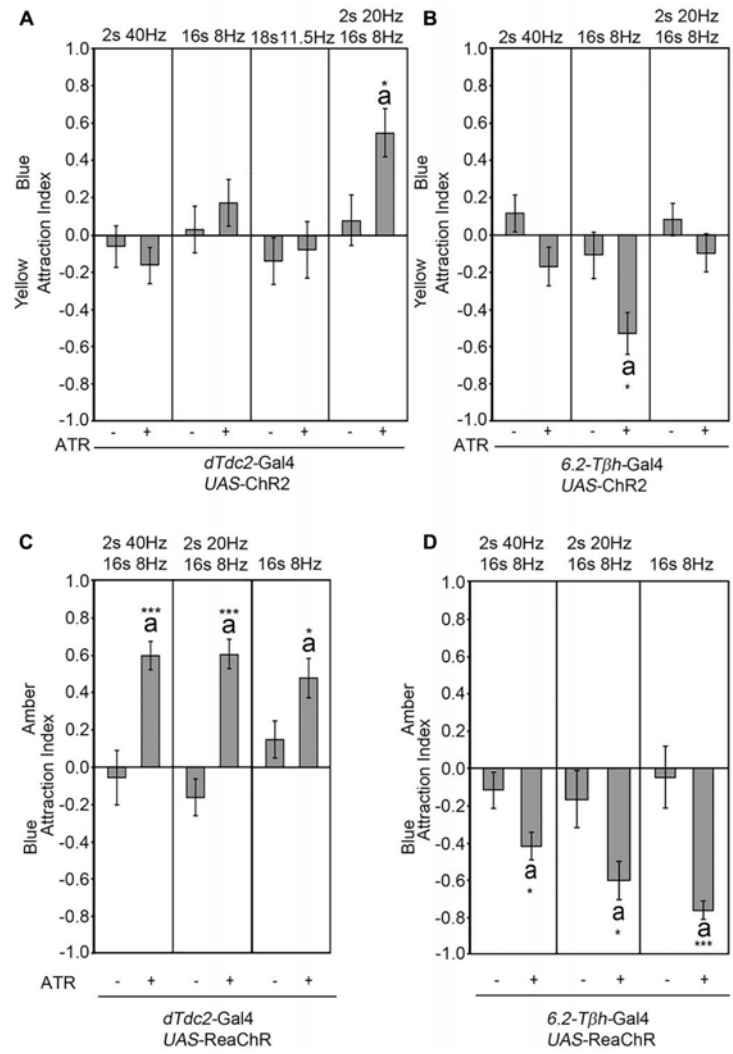


FIGURE 3 | Frequency-dependent activation results in attraction. Different flicker patterns indicated above the panel were used to activate neurons in a *dTdc2-Gal4*-dependent manner (**A,C**) and *6.2-Tβh-Gal4*-dependent manner (**B,D**). Only activation with a flicker of 2 s at 20 Hz, 16 s at 8 Hz followed by 2 s of silence continues to elicit site attraction (A, Als of the control and experimental group: -0.08 ± 0.14 and 0.55 ± 0.13 , respectively; $n = 27, 23$). (**B**) The 8 Hz pattern is sufficient to elicit site aversion when used to activate neurons (Als of the control and experimental group: -0.11 ± 0.12 and -0.53 ± 0.11 , respectively; $n = 20, 14$). (**C**) Activation with *UAS-ReaChR* in a *dTdc2-Gal4* dependent manner leads to site attraction (2 s at 40 Hz, 16 s at 8 Hz activation pattern: Als of the control and experimental group: -0.06 ± 0.15 and 0.6 ± 0.08 , respectively; $n = 18, 18$; 2 s at 20 Hz, 16 s at 8 Hz activation pattern: Als of the control and experimental group: -0.17 ± 0.1 and 0.6 ± 0.08 , respectively; $n = 13, 7$; 16 s at 8 Hz activation pattern: Als of the control and experimental group: 0.15 ± 0.1 and 0.47 ± 0.11 , respectively; $n = 12, 8$). (**D**) Activation with *UAS-ReaChR* in a *6.2-Tβh-Gal4*-dependent manner results in site aversion (2 s at 40 Hz, 16 s at 8 Hz activation pattern: Als of the control and experimental group: -0.12 ± 0.1 and -0.42 ± 0.08 , respectively; $n = 23, 20$; 2 s at 20 Hz, 16 s at 8 Hz activation pattern: Als of the control and experimental group: -0.17 ± 0.2 and -0.61 ± 0.1 , respectively; $n = 23, 16$; 16 s at 8 Hz activation pattern: Als of the control and experimental group: -0.05 ± 0.17 and -0.77 ± 0.05 , respectively; $n = 18, 17$). Errors are SEM. Differences from random choice as determined by the one-sample sign test are labeled with the letter “a”. Student’s *T*-test was used to determine differences between two groups with significance levels as follows: $*P < 0.05$, $***P < 0.001$. For data, see **Supplementary Table S3**.

intensity for neuron activation in a *6.2-Tβh-Gal4*-dependent manner only resulted in differences between the control and experimental group when neurons were activated with 1200 lux (**Supplementary Figure S1B**). Thus, the activation frequencies of the *UAS-ChR2* transgene and light intensities influence the behavioral outcome.

To address, independent of the kinetics of the blue light sensitivity of the *ChR2* transgene, whether neuronal activation

of tyraminerpic/octopaminergic neurons induced site attraction, we used the *UAS-ReaChR* transgene for neuronal activation (**Figure 3C**; Inagaki et al., 2014). The *ReaChR* encodes a redshifted channel rhodopsin variant with an activation light spectrum between orange and red, higher photocurrents and faster kinetics (Lin et al., 2013). Activation of neurons using the *UAS-ReaChR* transgene in a *dTdc2-Gal4*-dependent manner was sufficient to elicit site attraction, when the

tyramine/octopamine-positive neurons were activated by amber light (Figure 3C). All tested activation patterns resulted in attraction. In addition, activation of neurons with three different flicker frequencies in a *6.2-Tβh-Gal4*-dependent manner caused site aversion (Figure 3D). Therefore, the activation pattern of the neurons did not appear to be frequency-dependent but rather based on the kinetic of the transgene. Independent of the transgene, the activation of neurons in a *dTdc2-Gal4*-dependent manner was sufficient to elicit site attraction, and activation of neurons in a *6.2-Tβh-Gal4*-dependent manner was sufficient to elicit site aversion.

Octopaminergic Neurons Are Required to Switch the Behavioral Response

The release of octopamine from acetylcholine co-expressing neurons mediates attraction for the site of neuronal activation. The site attraction could be due to reinforcement or due to a behavioral switch. To test whether the octopamine release reinforced the positive association with one site of the behavioral choice paradigm, we wanted to analyze whether octopamine release could increase a pre-existing attraction. Normally flies show attraction to 5% ethanol-enriched food odors when given the choice between food odor and ethanol-enriched food

odor (Schneider et al., 2012). To analyze whether octopamine release increased this attraction, flies expressing the *UAS-ChR2* transgene under the control of the *dTdc2-Gal4* driver were offered a choice between a food odor trap and a 5% ethanol-enriched food odor trap that was illuminated with neuron-activating flickering blue light (Figure 4A). Activation of tyraminerbic/octopaminergic neurons did not significantly increase the attraction for the ethanol-enriched food odor trap. To test whether the activation of tyraminerbic/octopaminergic neurons would switch a preexisting ethanol attraction to the site of blue light activation, we enriched the yellow light-illuminated food odor trap with ethanol (Figure 4B). Here, as expected, control flies were significantly more attracted to the ethanol-enriched yellow-illuminated food odor trap. The activation of tyraminerbic/octopaminergic neurons significantly suppressed this ethanol attraction, indicating that tyraminerbic/octopaminergic neurons are not directly involved in attraction rather mediating the switch between two choices.

To independently address whether octopamine release mediated switches between behavioral elements rather than being directly involved in the execution of behavior, we reanalyzed the choice behavior of *Tβh^{nM18}* mutants (Figure 4C). In contrast to control flies, *Tβh^{nM18}* mutants failed to show attraction to 5% ethanol-enriched food odors (Schneider et al.,

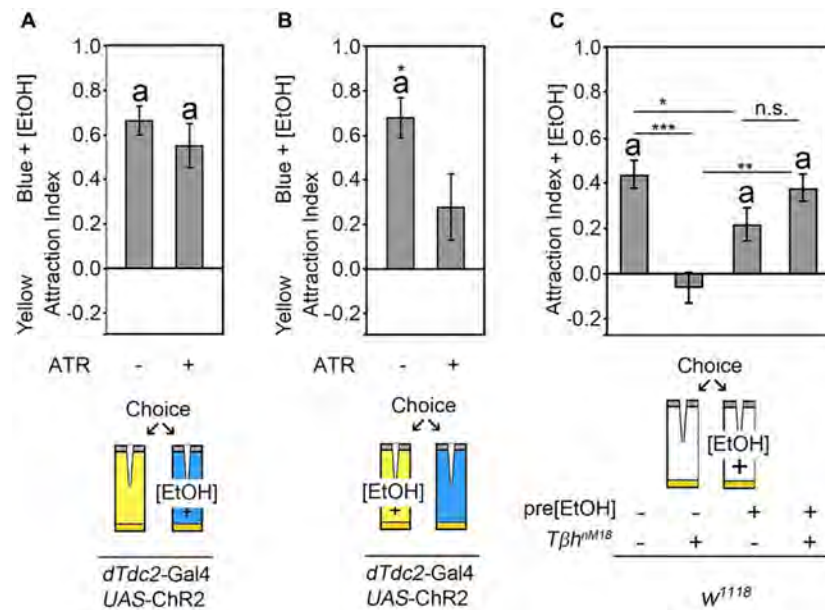
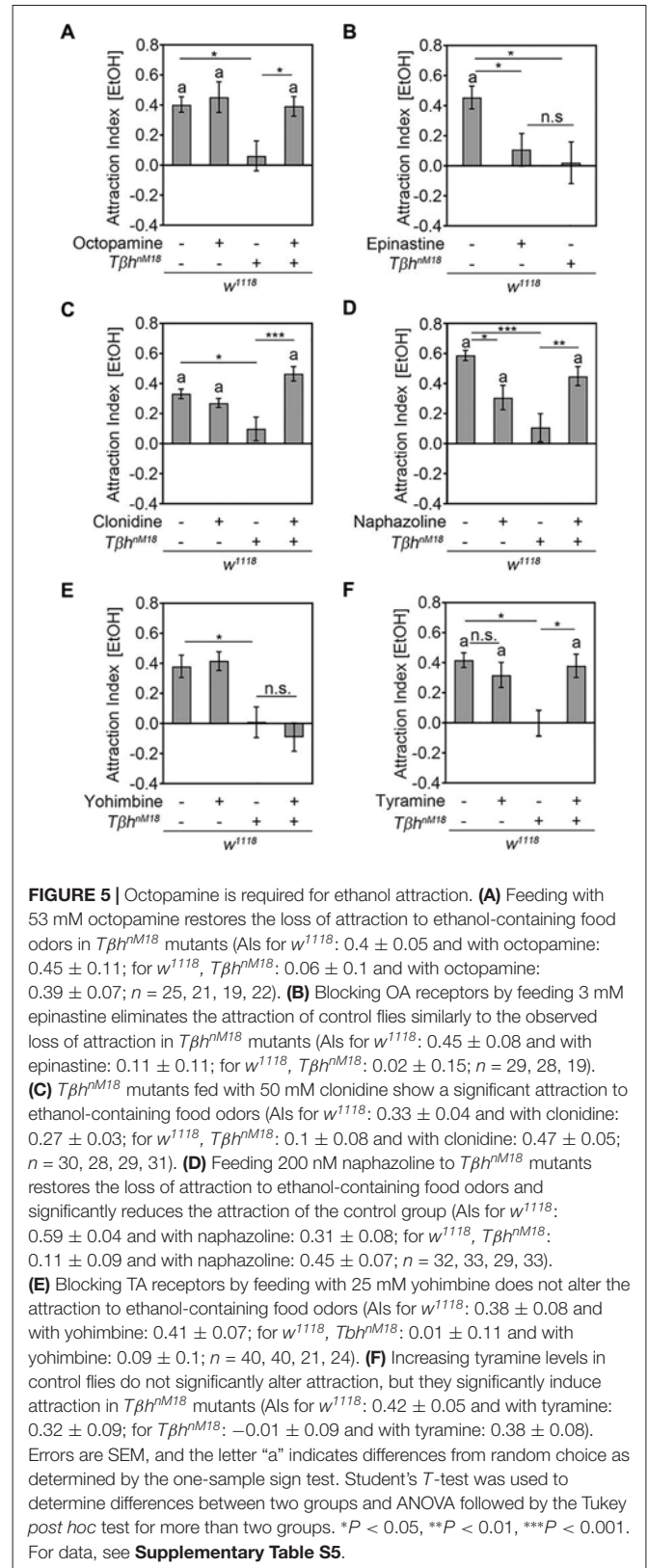


FIGURE 4 | Octopaminergic neurotransmitter shifts behavioral outcomes. **(A)** Neuronal activation using the blue light-sensitive *UAS-ChR2* transgene in a *dTdc2-Gal4*-dependent manner results in attraction to the blue light-illuminated odor trap. Addition of 5% ethanol to the blue light-illuminated trap did not change this attraction significantly (AIs of the control and experimental group: 0.66 ± 0.07 and 0.55 ± 0.1 , respectively, $n = 25, 17$). **(B)** Addition of 5% ethanol to the yellow light-illuminated trap results in a significant reduction of the attraction to the blue light-illuminated food odor trap (AIs of the control and experimental group: 0.68 ± 0.09 and 0.28 ± 0.15 , respectively, $n = 20, 18$). **(C)** Flies normally prefer the 5% ethanol-enriched food odor trap over the plain food odor trap, and *Tβh^{nM18}* mutants differ significantly from the control in their behavior and do not show attraction. Pre-feeding control flies with 10% ethanol-enriched sucrose solution results in a similar degree of attraction to the 5% ethanol-containing food odor trap. Ethanol pre-feeding in *Tβh^{nM18}* mutants results in a significant attraction to the 5% ethanol-containing food odor trap (AIs for *w¹¹¹⁸*: 0.44 ± 0.06 and with EtOH: 0.22 ± 0.08 ; for *w¹¹¹⁸*, *Tβh^{nM18}*: -0.07 ± 0.07 and with EtOH: 0.38 ± 0.06 ; $n = 33, 31, 17, 20$). The errors are SEM. The differences from random choice were determined using the one sample- sign test, and significance is indicated by the letter “a”. Student’s *T*-test was applied to determine differences between two groups, and ANOVA followed by Tukey *post hoc* analyses were used for comparisons among more than two groups. Significant differences are indicated as follows: * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. For data, see **Supplementary Table S4**.

2012; **Figure 4C**). To address whether $T\beta h^{nM18}$ were unable to show attraction *per se*, we pre-exposed flies to ethanol by feeding a 10% ethanol-enriched 5% sucrose solution for 30 min. After 3.5 h of recovery, the attraction to ethanol-enriched food odors was analyzed in the binary choice assay (**Figure 4C**). Ethanol pre-fed control flies showed an attraction to ethanol-enriched food odors, and ethanol pre-feeding of $T\beta h^{nM18}$ mutants resulted in a similar significant ethanol attraction. These results showed that ethanol pre-feeding to $T\beta h^{nM18}$ mutants restored the attraction to ethanol-containing food odors. Thus, $T\beta h^{nM18}$ mutants were able to develop the attraction but failed to choose ethanol-enriched food odors in an ethanol naïve choice situation over non-ethanol-containing food odors. Taken together, activation of the octopaminergic/tyraminergeric neurotransmitter system can shift the attraction, and $T\beta h^{nM18}$ fail to show ethanol attraction because they cannot shift their behavioral response.

Octopamine Is Required for Ethanol Attraction

To determine whether the loss of ethanol attraction in ethanol naïve $T\beta h^{nM18}$ was due to the loss of octopamine, we performed pharmacological experiments (**Figure 5**). First, we fed $T\beta h^{nM18}$ mutants with 53 mM octopamine—a concentration that restored the egg laying defect of the $T\beta h^{nM18}$ mutants (Monastirioti et al., 1996)—and analyzed their ethanol attraction (**Figure 5A**). In addition, we fed control flies with octopamine to investigate whether increased octopamine levels could increase attraction. Feeding with OA restored the loss of ethanol attraction in $T\beta h^{nM18}$ mutants to control levels, but it did not alter the attraction of w^{1118} flies (**Figure 5A**). To independently confirm that octopamine signaling was required for attraction, w^{1118} flies were fed with 3 mM of the antagonist epinastine for octopamine receptors, a concentration that has been shown to effectively interrupt TfAP-2-induced hyperactivity in *Drosophila* (Williams et al., 2014). Epinastine-fed control flies showed a similar significant loss of ethanol attraction to $T\beta h^{nM18}$ mutants (**Figure 5B**). To confirm that octopamine receptor signaling was required for ethanol attraction, we fed βh^{nM18} mutant and control flies with two octopamine receptor agonists—50 mM clonidine or alternatively 200 nM naphazoline (Evans, 1981; Evans and Maqueira, 2005)—and investigated the effects on attraction (**Figures 5C,D**). The octopamine receptor agonist naphazoline and clonidine should restore octopamine receptor signaling in $T\beta h^{nM18}$ mutants. Clonidine turned the loss of ethanol attraction of $T\beta h^{nM18}$ mutants into attraction, but it did not influence the attraction of control flies (**Figure 5C**). Naphazoline also turned the loss of attraction into ethanol attraction in $T\beta h^{nM18}$ mutants (**Figure 5D**). However, naphazoline also altered the attraction in control animals by reducing the attraction, suggesting that too much activation of octopamine receptors might result in aversion or that naphazoline activates additional receptors that mediate aversion. To address the putative function of increased tyramine levels on ethanol attraction, tyramine receptor signaling was blocked in control flies and $T\beta h^{nM18}$ mutants by feeding with the tyramine receptor antagonist yohimbine. Feeding with



25 mM yohimbine—a concentration that rescues the $T\beta h^{nM18}$ mutant phenotype in flight initiation and maintenance (Brembs

et al., 2007)—did not alter the attraction to ethanol in control or in mutant flies (**Figure 5E**). To independently address whether increased tyramine levels altered ethanol attraction, 345 mM tyramine was fed to control flies, and the effects on attraction to ethanol were analyzed (**Figure 5F**). Control flies fed with tyramine did not show a significantly reduced attraction to ethanol-containing food odors. Feeding tyramine to the *Tβh^{nM18}* mutant significantly induced attraction to ethanol-containing food odors, suggesting that feeding with tyramine was effective and might function at high levels as an agonist for octopamine receptors, or alternatively, activation of tyramine receptors might also promote the induction of attraction to ethanol-containing food odors. Taken together, octopamine is required for the attraction to ethanol, and increased TA levels in *Tβh^{nM18}* mutants are not responsible for the loss of attraction.

DISCUSSION

The release of octopamine from acetylcholine co-expressing tyraminerpic/octopaminergic neurons is necessary to induce a bias in a behavioral choice between two identical food odor traps, resulting in attraction for one odor trap. The release of tyramine or acetylcholine from the same set of cholinergic neurons results in aversion. In addition, the release of octopamine from VUMa4 neurons acts as a negative reinforcer when flies choose between two identical food odor traps and causes aversion. Therefore, octopamine mediates aversion and attraction depending on the set of neurons activated. In **Figure 6** we provide an overview summarizing these results. The behavioral outcome of neuronal activation depends on the kinetics of the channelrhodopsin transgene used for activation. Nevertheless, independent of the transgene, the activation of tyraminerpic/octopaminergic neurons is sufficient to elicit site attraction, and the activation of VUMa4 neurons is sufficient to elicit site aversion. Given the choice between an attractive ethanol-enriched food odor and a less attractive food odor, the activation of tyraminerpic/octopaminergic neurons is able to shift the attraction to the less attractive food odor. The induction of attraction for ethanol-enriched food odors in a binary choice also requires the function of octopamine, supporting the requirement for octopamine to trigger the behavior towards approach, e.g., ethanol attraction.

Octopamine Acts as Positive and Negative Reinforcer

In *Drosophila*, octopamine has been shown to function as a positive reinforcer in several behaviors that involve the processing, evaluation and behavioral response of olfactory information. Innate attraction to ethanol-containing food odors requires the activation of tyraminerpic/octopaminergic neurons, and mutants lacking octopamine fail to show attraction (Schneider et al., 2012). In more complex behaviors such as olfactory learning and memory, octopamine functions as a positive reinforcer, since *Tβh^{nM18}* mutants fail to show a positive association or attraction to a previous sucrose rewarded odor (Schwaerzel et al., 2003). Similar to the adult nervous system, in the developing nervous system of 3rd

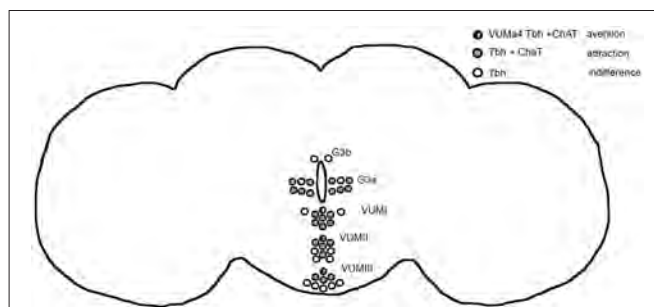


FIGURE 6 | Model for octopamine induced attraction and aversion. The circles highlight the Tbh positive neurons targeted by the *dTdc2-Gal4* driver in the adult brain (Schneider et al., 2012). The Tbh is the rate limiting enzyme for octopamine synthesis (Monastirioti et al., 1996). Activation of these neurons using *UAS-ChR2* elicits site attraction (**Figure 1**). When the Gal4 expression of the driver is restricted by co-expression of *ChAT-GAL80* driver the flies choose both odor traps equally (**Figure 1**). The neurons that are not affected by the *ChAT-GAL80* repressor are indicated in open circles. They only express Tbh and activation of these neurons results in indifferences (**Figure 1**). The ChAT and Tbh expressing neurons are indicated by gray circles and they are responsible—when activated—to mediate attraction. Within these set of positive neurons, the activation of the VUMa4 neuron (indicated by a circle that is half black and half gray) results in aversion (**Figure 2**). The VUMa4 neurons are Tbh positive (Schneider et al., 2012). Since the *ChAT-GAL80* repressor eliminates the aversion caused by activation of the 6.2 *Tbh-Gal4* driver, the VUMa4 neuron is also ChAT positive (**Figure 2**). The activation of the VUMa4 neuron results in aversion, but when the VUMa4 neuron is activated together with other octopaminergic neurons, attraction occurs. This result is consistent with the idea that the activation of a second set of octopaminergic neurons can overrule the octopamine-induced aversion by the VUMa4 neuron. For data, see **Supplementary Table S6**.

instar larvae, activation of tyraminerpic/octopaminergic neurons substitutes for reward in olfactory larval learning, and the activity of tyraminerpic/octopaminergic neurons is required for olfactory appetitive learning and memory (Schroll et al., 2006; Selcho et al., 2014). Here, we show that activation of tyraminerpic/octopaminergic neurons is sufficient to bias the decision toward one of two similar odor traps and that *Tβh^{nM18}* mutants fail to show attraction upon activation of the same set of neurons (**Figure 1**). Octopamine release from VUMa4 neurons results in aversion, indicating that octopamine functions as negative reinforcer in an olfactory choice situation (**Figure 2**). Eliminating octopamine release from 6.2-*Tβh-Gal4*-targeted neurons by introducing *Tβh^{nM18}* mutants clearly eliminated aversion, showing that octopamine release is required for aversion. Another observation supporting that octopamine acts as a negative reinforcer was derived from the observation that the octopamine receptor agonist naphazoline reduced attraction in control flies (**Figure 5D**). There is evidence that the octopaminergic system might also function as negative reinforcer in other behaviors. In olfactory aversive learning and memory, when an odor is paired with an electric shock, *Tβh^{nM18}* mutants show reduced learning abilities, indicating an impairment of the negative reinforcing function of the electric shock (Iliadi et al., 2017). Furthermore, activation of tyraminerpic/octopaminergic neurons suppresses courtship conditioning, another form of aversive associative learning (Zhou et al., 2012).

On the cellular level, opposing neuronal functions for octopamine have been previously observed. For example, in the ventral nerve cord of locusts, octopamine release causes, at one site, bouts of rhythmic flight motor activity and, at another site, suppression of neuronal activity related to egg laying (Sombati and Hoyle, 1984). The opposing roles of octopamine might also be explained by the activation of different classes of octopamine receptors that either activate or reduce neuronal activity, both of which have been identified in *Drosophila melanogaster* (Evans and Maqueira, 2005). In addition to the negative reinforcing function of octopamine, an additional neurotransmitter mediates aversion in the *dTdc2*-Gal4-targeted neurons, since the introduction of *Tβh^{nM18}* mutants resulted in significant aversion. One possible candidate for this is tyramine. For example, feeding the tyramine receptor antagonist yohimbine to *Tβh^{nM18}* mutant larvae partially restored the observed reduced locomotion in the mutants, suggesting that the reduced locomotion was due to an increased tyramine level and that tyramine might counteract the function of octopamine (Saraswati et al., 2004). However, feeding with the tyramine receptor antagonist yohimbine did not interfere with attraction to ethanol in control flies, nor did it alter the loss of attraction in *Tβh^{nM18}* mutants, suggesting that tyramine is not responsible for the observed aversion. Furthermore, feeding tyramine to control flies did not suppress the attraction to ethanol-enriched food odors (Figure 5F). Rather, feeding tyramine to *Tβh^{nM18}* mutants resulted in attraction, suggesting that high levels of tyramine might also function also as a positive reinforcer. Another candidate is acetylcholine. Neuronal activation of neurons in a *dTdc2*-Gal4-dependent manner might also co-release other neurotransmitters that negatively regulate attraction. Not all neurons targeted by the *dTdc2*-Gal4 line are octopaminergic or Tβh-positive (Busch et al., 2009; Schneider et al., 2012), and *ChAT*-Gal80 represses *dTdc2*-Gal4-dependent transgene expression (Schneider et al., 2012). Consistent with this observation, some of the targeted VUM neurons express ChAT (Sayin et al., 2018). However, activation of VUMa4 neurons in *Tβh^{nM18}* mutants eliminates the aversion, clearly indicating that octopamine, in addition to possible other neurotransmitter systems, might induce aversion. Taken together, octopamine functions as a positive and negative reinforcer in response to olfactory cues such as food odors.

Octopamine Biases Behavioral Outcomes

How does a neurotransmitter mediate both negative and positive reinforcement to an olfactory cue? In associative olfactory short-term learning and memory, dopamine mediates two different sets of neurons: positive and negative reinforcing properties (Claridge-Chang et al., 2009; Aso et al., 2010; Liu et al., 2012). The same phenomenon might also be true for octopaminergic neurons. Octopamine-dependent activation of the VUMa4 neuron results in aversion, but when the VUMa4 neuron is activated together with other octopaminergic neurons, attraction occurs. This result indicates that the activation of a second set of octopaminergic neurons can overrule the octopamine-induced aversion by the VUMa4 neuron. Such an attraction might be the primary behavioral outcome when

octopamine release is depleted, as supported by the observation that *Tβh* mutants with no detectable levels of octopamine fail to show attraction and feeding control flies with the octopamine receptor antagonist epinastine results in a reduction of ethanol-induced attraction rather than increased attraction. Feeding the agonist of octopamine—naphazoline—to control flies also reduces the attraction to ethanol enriched food odor (Figure 5D). This could be due to the activation of octopamine dependent negative reinforcement resulting in aversion.

If octopamine mediates both attraction and aversion to olfactory cues, then a situation must arise where octopamine-deficient flies respond with indecision in a choice situation, which is indeed the case. *Tβh^{nM18}* mutants fail to show attraction to ethanol-enriched food sources. The indecision to choose between the two odor sources might depend on the inability to detect ethanol within food odors, the lack of a positive reinforcer, inability to make a decision or failure to perform the motor skills required for the task. *Tβh^{nM18}* mutants sense ethanol and food odors and can distinguish between them (Schneider et al., 2012), eliminating the possibility that failures in odor perception account for the indifference. During olfactory learning and memory, odor ethanol functions as a positive reinforcer (Kaun et al., 2011), and *Tβh^{nM18}* mutants show attraction to ethanol-containing food odors when they are pre-exposed to ethanol (Figure 4C), showing that the mechanism resulting in positive reinforcement is still functional in *Tβh^{nM18}* mutants. Furthermore, *Tβh^{nM18}* mutants still enter food odor traps, showing that the motor ability of the flies and the motivation to seek food is still intact. Consistent with a function in decision making, the activation of octopaminergic/tyraminergeric neurons shifts the attraction from the ethanol-enriched food odor to a less attractive odor (Figure 4B) and therefore biases the behavioral outcome.

Are there other behaviors where flies normally must decide to respond to odor information and where *Tβh^{nM18}* mutants fail to make the right decision or correct response? After exposure to a novel olfactory stimulus such as ethanol, normal flies show an increased startle response as measured by an increase in locomotion (Scholz, 2005). The *Tβh^{nM18}* mutants not only fail to show a startle response but also show a repression of locomotor activity that can be contributed to the lack of octopamine, mimicking a freezing response. The immobility is turned into activity when the ethanol stimulus is sufficiently strong (Scholz, 2005). Another example that involves a behavioral choice is the associative appetitive and aversive olfactory learning paradigm that is analyzed in the Tully-Quinn paradigm (Tully and Quinn, 1985). In this paradigm, flies must demonstrate what they learned in a binary choice between two odors. Before the experiments, the odor concentrations are balanced so that they evoke a similar behavioral response to ensure that the strength of the presented odor stimuli is comparable. *Tβh^{nM18}* mutants fail to respond to the positive reinforced odor with attraction within the given time, but they respond with aversion to the negative reinforced odor (Schwaerzel et al., 2003) or only respond in a reduced manner (Iliadi et al., 2017).

The difference between appetitive and aversive learning defects in *Tβh^{nM18}* mutants might be due to the strength of the reinforcer. The lack of comparability of the strength of the reinforcer between aversive and appetitive olfactory learning and memory is also observed in the degree of association formed between the odor and the reinforcer, as reflected in differences in the learning score. Flies show lower learning scores when a positive reinforcer, such as sucrose, is paired with an odor compared with when a negative reinforcer, such as an electric shock, is paired (Schwaerzel et al., 2003). The influence of the threshold of the reinforcing stimulus is also observed in the behavioral response of *Tβh^{nM18}* mutants when they are pre-exposed to ethanol and respond with ethanol attraction in the binary choice paradigm (Figure 4C). Here, the pre-exposure to ethanol odor is sufficiently strong to elicit attraction. In summary, octopamine orchestrates the behavioral response to odors. It remains to be investigated whether this finding holds true for other sensory information, such as taste and vision, and how octopamine integrates different external and/or internal information to bias the behavioral outcome.

ETHICS STATEMENT

The animal studies including the model organism *Drosophila melanogaster* were conducted in agreement with the regulations of the DFG and the land North Rhine Westphalia.

AUTHOR CONTRIBUTIONS

GC and HS: conception and design of the work; acquisition, data analysis and interpretation. GC: original draft—writing. HS: supervision, project administration and funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00131/full#supplementary-material>

FIGURE S1 | Effect of light intensity on behavioral outcome. The same flicker frequency of 2 s at 40 Hz, 16 s at 8 Hz was used for all experiments, but the intensity of both LEDs was changed simultaneously. **(A)** Different intensities indicated above the panel were used to activate neurons with a blue light-sensitive *UAS-ChR2* under the control of the *dTdc2-Gal4* driver. All the used intensities resulted in attraction for the site of activation (Als for the 1700 lux control and experimental group: -0.07 ± 0.12 and 0.32 ± 0.1 , $n = 20$, 20; for 1000 lux: 0.21 ± 0.14 and 0.6 ± 0.12 , $n = 34$, 31; for 800 lux: 0.02 ± 0.09 and 0.43 ± 0.1 , respectively, $n = 39$, 33). **(B)** Neurons activated with a blue light-sensitive *UAS-ChR2* under the control of the *6.2-Tβh-Gal4* driver with different intensities showed significant aversion when illuminated with 1200 lux, but not 1700 lux or 1000 lux (Als for the 1700 lux control and experimental group: -0.31 ± 0.14 and -0.62 ± 0.11 , $n = 20$, 16; for 1200 lux: -0.02 ± 0.13 and -0.41 ± 0.13 ; $n = 22$, 19 and for 1000 lux: 0.12 ± 0.13 and -0.02 ± 0.12 , respectively; $n = 21$, 19). **(C)** Activation with the amber-sensitive *UAS-ReaChR* in a *dTdc2-Gal4*-dependent manner leads to site attraction for the amber-illuminated site (Als for the 1200 lux Als of control and experimental group: -0.06 ± 0.15 and 0.6 ± 0.08 , $n = 18$, 18; Als for the 800 lux 0.21 ± 0.11 and 0.1 ± 0.14 , respectively, $n = 14$, 5). Errors bars are SEM. The one-sample sign test was used to determine differences from random choice, and significant differences are labeled with the letter “a”. Student’s *T*-test was used to determine differences between two groups with significance levels as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data for **Supplementary Figure S1**, see **Supplementary Table S6**.

TABLE S1 | Data for **Figure 1**.

TABLE S2 | Data for **Figure 2**.

TABLE S3 | Data for **Figure 3**.

TABLE S4 | Data for **Figure 4**.

TABLE S5 | Data for **Figure 5**.

TABLE S6 | Data for **Supplementary Figure S1**.

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Attractant or Repellent? Behavioral Responses to Mammalian Blood Odor and to a Blood Odor Component in a Mesopredator, the Meerkat (*Suricata suricatta*)

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It is well-established that the odor of mammalian blood is attractive to top predators such as tigers and wolves and aversive to prey species such as mice and rats. Recent studies have shown that the mammalian blood odor component *trans*-4,5-epoxy-(E)-2-decenal (TED) elicits corresponding behavioral responses in these two groups of mammals. Here we assess whether a mesopredator, that is, a small-bodied carnivorous mammal that is both predator and prey, is attracted to or repelled by the odor of mammalian blood and TED. To this end, we assessed the behavior of a group of 15 captive meerkats (*Suricata suricatta*) when presented with wooden logs that were impregnated either with horse blood or with TED, and compared it to their behavior toward a fruity odor (*iso*-pentyl acetate) and a near-odorless solvent (diethyl phthalate). We found that the meerkats displayed significantly more interactions with the odorized wooden logs such as sniffing and pawing when these were impregnated with the two prey-associated odors compared to the two non-prey-associated odors. Most importantly, no significant difference was found in the number of interactions with the wooden logs impregnated with horse blood and TED, respectively. These results demonstrate that meerkats, despite being small-bodied mesopredators, are clearly attracted to the odor of mammalian blood. Further, the results suggest that a single blood odor component can be as efficient as the odor of real blood in eliciting behavioral responses in this herpestid mammal, similar to previous findings in feline and canine top predators.

Keywords: blood odor, epoxydecenal, behavior, mesopredator, meerkats, *Suricata suricatta*

INTRODUCTION

The vast majority of naturally occurring odor stimuli are highly complex mixtures composed of dozens or even hundreds of volatile compounds (Ohloff, 1994; Sell, 2014). Olfactory systems are therefore faced with the problem to recognize the identity of an odor stimulus despite permanent fluctuations in its composition or intensity due to changes in the odor source itself (e.g., ripening of a fruit) or the environment (e.g., convection of volatiles in air currents) (Riffell et al., 2009). How the olfactory system achieves and maintains stimulus identity of complex odor mixtures is still not fully understood (Thomas-Danguin et al., 2014).

One possible strategy for an animal to recognize a complex odor mixture is to rely on only one or a few “key” or “character impact” compound(s) which determine its odor identity (Dunkel et al., 2014). This strategy, of course, requires that the compound in question is reliably present in the odor mixture and that it can be reliably detected against the noise of the other compounds (Nehring et al., 2013). Thus, a high olfactory sensitivity for such a “key” or “character impact” compound should be expected in species for which the corresponding odor mixture is behaviorally relevant (Laska et al., 2005; Sarrafchi et al., 2013).

Behavioral tests assessing the ability of animals to recognize and to properly respond to the presentation of behaviorally relevant odors yielded rather mixed results concerning the efficiency of single compounds that are part of a complex odor mixture to elicit such adaptive behavioral responses. With regard to food odors, for example, recent studies found that frugivorous mammals do not seem to rely on single compounds to assess the degree of ripeness of a fruit but rather on the relative abundance of several compounds (Hodgkison et al., 2013; Nevo et al., 2015). Concerning the olfactory recognition of predators by prey species via body-borne odors, some studies found that single compounds are sufficient to elicit avoidance responses whereas other studies failed to find such effects or reported the behavioral responses to be weaker compared to those elicited by the full odor mixture (for a review, see Apfelbach et al., 2017).

The odor of blood has been shown to be attractive to mammalian top predators such as tigers (Nilsson et al., 2014) and wolves (Arshamian et al., 2017) and to be aversive to mammalian prey species such as the mouse (Sandnabba, 1997; Lahger and Laska, 2018) and the rat (Stevens and Saplikoski, 1973; Hornbuckle and Beal, 1974; Mackay-Sim and Laing, 1981). Interestingly, the tigers and wolves were equally attracted to and the mice were equally repelled by *trans*-4,5-epoxy-(E)-2-decenal (TED), a single component of mammalian blood odor which has been described by humans as having a typical “metallic, blood-like” odor quality (Buettner and Schieberle, 2001). These findings suggest that TED might indeed be a “key” or “character impact” compound which determines or at least contributes to the reliable recognition of blood odor. This notion is further supported by the finding that mice are extraordinarily sensitive to TED with olfactory detection thresholds in the ppt (parts per trillion) range (Sarrafchi and Laska, 2017).

Meerkats (*Suricata suricatta*) are carnivorous mammals belonging to the mongoose family (Herpestidae). They have well-developed olfactory brain structures (Gittleman, 1991; van Valkenburgh et al., 2014) and are known to strongly rely on olfactory cues in the context of social communication (Jordan, 2007; Mares et al., 2011; Leclaire et al., 2013). Similarly, meerkats have been reported to use their sense of smell for predator avoidance (Hollén and Manser, 2007; Zöttl et al., 2013) and for foraging and food selection (Leclaire, 2017). Due to their small body mass of 0.7–1.2 kg (van Staaden, 1994), they are typical mesopredators, meaning that they are both predators of smaller prey species and, at the same time, prey to larger predators. Their diet, although primarily based on arthropods, includes up to 20% (by volume) of small-bodied mammals and other vertebrates such as birds, eggs, lizards, and snakes (van Staaden, 1994; Doolan and

Macdonald, 1996). Therefore, it should be interesting to assess whether meerkats are attracted to or repelled by the odor of blood and if they also display the same behavior toward TED.

It was therefore the aim of the present study to (1) assess behavioral responses of meerkats to mammalian blood odor and to the mammalian blood odor component *trans*-4,5-epoxy-(E)-2-decenal, (2) to compare their behavioral responses to those toward a fruity odor and a near-odorless control, and (3) to compare their behavioral responses to those of top predators and prey species tested previously on the same odor stimuli.

MATERIALS AND METHODS

Ethics Statement

The experiments reported here comply with the *Guide for the Care and Use of Laboratory Animals* (8th edition, National Research Council, 2011) and also with current Swedish laws. They were performed according to a protocol approved by the ethical board of the Swedish Board of Agriculture (Jordbruksverket, protocol # 31-2647/10).

Animals

The study was conducted at Kolmården Wildlife Park, near Norrköping, Sweden. A group of 15 meerkats (*S. suricatta*), comprising twelve males and three females ranging from a few months to 10 years of age, was observed. All animals were born in captivity. The enclosure of the meerkats was composed of an indoor and an outdoor part, connected by a sliding door. The indoor enclosure was 40 m² and had a ground substrate of sand. It contained standing brush material, tree stumps of a varied height, and different hiding places such as wooden nest boxes. The outdoor enclosure was 330 m² with mainly earth as ground substrate. In addition to tree stumps, the outdoor enclosure also had a grassy area, coniferous trees, rocks and bushes scattered throughout the area. The ground substrate of both the indoor and the outdoor enclosure allowed the animals to dig burrows and tunnels. The meerkats could freely choose between the indoor and the outdoor enclosure during the daytime, but were kept indoors over night. They were provided with food three times per day (in the morning, that is, about 60 min prior to the start of the day's observations; around noon, that is, during the hour between the morning and the afternoon observations; and in the afternoon, that is, after the end of the day's observations). Their food consisted of mice, baby chicken, pieces of meat from different even-toed ungulate species (such as deer and antelope, but not from odd-toed ungulate species such as horses), cat food pellets (Four Friends Senior, Västerås, Sweden), fruit (banana, apple, different berries), crickets, mealworms, chicken eggs.

Odor Stimuli

The four odor stimuli used were:

Blood from a domestic horse (*Equus ferus caballus*). The blood was collected directly after the horse was euthanized and immediately deep-frozen in aliquots of 0.5 ml at –20°C. On the morning of each testing day, five aliquots of

horse blood were thawed and warmed up to approximately 25°C.

trans-4,5-epoxy-(E)-2-decenal (CAS# 134454-31-2), henceforth abbreviated as TED. This odorant has been identified as a volatile component in mammalian blood and evokes a typical “metallic, blood-like” odor quality in humans (Buettner and Schieberle, 2001). It was presented at a dilution of 1:100 (in diethyl phthalate) from a stock solution of 5 mg/ml.

The rationale for using these two odor stimuli was that horse blood and *trans*-4,5-epoxy-(E)-2-decenal had also been used in previous studies assessing behavioral responses of top predators (Nilsson et al., 2014; Arshamian et al., 2017) and a prey species (Lahger and Laska, 2018) to blood odor.

iso-pentyl acetate (CAS# 123-92-2). This odorant has been identified as a volatile component in a variety of fruits and evokes a typical “banana-like” odor quality in humans (Burdock, 2009). It was presented at a dilution of 1:1,000 (in diethyl phthalate).

Diethyl phthalate (CAS# 84-66-2). This organic solvent is near-odorless and was used both for diluting the two monomolecular odorants mentioned above and as a blank stimulus.

The concentrations mentioned above for the blood odor component TED and for the fruity odor (*iso*-pentyl acetate) were chosen in order to provide stimuli that were clearly detectable for humans, but not overwhelmingly strong so that a relatively close contact to the odor source was necessary to detect them.

Experimental Procedure

The four different odor stimuli were presented to the animals using wooden (spruce) logs of 48 cm × 4.5 cm × 4.5 cm. Each log was impregnated with 0.5 ml of a given odor stimulus immediately prior to each presentation. The odor stimulus was applied on the two largest surfaces of a log using a micropipette and then spread over the surface using a paint brush. Plastic gloves were used whenever the logs were handled to avoid that they were contaminated with human odor. Five logs, impregnated with the same odor stimulus, were used during a given experimental day.

At the start of each experimental day, five freshly odorized wooden logs were placed into the outdoor enclosure of the meerkats. Care was taken to present the wooden logs to the animals at positions that differed between experimental days and, at the same time, allowed the experimenter to see all five logs at least at the start of the observation. Immediately after the logs were put in place, the animals were allowed access into the outdoor enclosure and were observed for 3 h in the morning and 3 h in the afternoon (between 08:00 a.m. and 04:00 p.m.). At the end of each experimental day the wooden logs were removed from the enclosure. Experiments were only performed on non-rainy days to prevent the odor stimuli from being washed away by the rain. At least 1 day was interspersed between consecutive presentations of odorized wooden logs. Each of the four odor stimuli was presented for a total of five times in a pseudo-randomized order which resulted in a total of 20 experimental days.

Continuous sampling was used to record the occurrence of each interaction with a wooden log which was visible to the experimenter. A total of twelve behaviors involving different kinds of interaction with or immediate behavioral responses to the inspection of an odorized wooden log were considered (Table 1 and Figure 1). During one experimental day per odor stimulus, the duration of the behaviors was also recorded using a stopwatch.

Data Analysis

Differences in the frequency of occurrence of behavioral responses between odor stimuli were assessed using the Chi-square test. Within-species comparisons of the duration of behavioral responses were performed using the Friedman test. Correlational analyses were performed by calculating Spearman rank-order coefficients r_s which were tested for significance by computing z -scores. As the number of animals differed between the species that were compared, we calculated the number of interactions *per animal* by dividing the total number of interactions observed in a given species by the number of animals. All statistics were performed using SPSS, version 22.0.

RESULTS

Number of Interactions

Across all 20 observation days with the four different odor stimuli combined the meerkats interacted in total 2850 times with the odorized wooden logs. Ten out of the twelve behaviors described in the ethogram were observed (Table 2). Sniffing was by far the most frequently displayed behavior with 1970 interactions, representing 69% of all observations. Pawing was another frequently observed behavior with 834 occurrences, accounting for 29% of all observations. Thus, sniffing and pawing combined accounted for >98% of all interactions with the odorized wooden logs that the meerkats displayed. Licking

TABLE 1 | Ethogram of all behaviors considered in the present study.

Functional term	Description
Sniffing	Using the nose to investigate a wooden log
Pawing	Using the paw or claws to scratch a wooden log
Licking	Using the tongue to investigate a wooden log
Biting	Using the teeth to investigate a wooden log
Toying	Moving or otherwise manipulating a wooden log
Flehmen	Curling of the upper lip and “grimacing” when investigating a wooden log
Self-impregnating	Rubbing the face or other body part at a wooden log
Scent-marking	Depositing body-borne odors onto a wooden log
Orientating	Turning head, ears, or eyes following an interaction with a wooden log
Guarding	Resting close to or on top of a wooden log
Vocalizing	Producing sounds during or following an interaction with a wooden log
Fleeing	Rapidly moving away following an interaction with a wooden log



FIGURE 1 | The two behaviors performed most often by the meerkats. **(Upper)** A meerkat sniffing at an odorized wooden log. **(Lower)** A meerkat pawing at an odorized wooden log.

and flehmen were never displayed by the meerkats with any of the four odor stimuli.

Comparison Between Odor Stimuli

A comparison between the four odor stimuli showed that the meerkats displayed a significantly higher number of interactions with the wooden logs when these were odorized with horse

blood compared to when they were odorized with the fruity odor ($\chi^2 = 83.701$, $P < 0.0001$) and the blank control ($\chi^2 = 27.597$, $P < 0.0001$). Similarly, the meerkats interacted significantly more often with the wooden logs when these were odorized with the blood odor component TED compared to the the fruity odor ($\chi^2 = 70.689$, $P < 0.0001$) and the blank control ($\chi^2 = 20.263$, $P < 0.0001$). In contrast, the number of interactions with the wooden logs did not differ significantly between the horse blood and the blood odor component TED ($\chi^2 = 0.572$, $P = 0.450$).

Duration of Interactions

The mean duration of interactions with the odorized wooden logs was 3.1 ± 5.5 s for the four different odor stimuli combined (Table 3).

No significant differences in the mean duration of interactions with the wooden logs were found between any of the four odor stimuli (Friedman ANOVA: $\chi = 6.01$, $P = 0.111$).

Variability Between Sessions

No significant correlation between the number of interactions with the wooden logs across the five sessions was found with any of the four odor stimuli (Spearman test, horse blood: $r_s = -0.70$, $P > 0.05$; blood odor component: $r_s = -0.60$, $P > 0.05$; fruity odor: $r_s = -0.70$, $P > 0.05$; solvent: $r_s = -0.60$, $P > 0.05$). However, with all four odor stimuli the number of interactions with the wooden logs was higher in the first compared to the fifth session (Figure 2). Accordingly, a non-significant trend for a decrease in the animals' interest in the wooden logs across sessions was found as indicated by the negative correlation coefficients.

DISCUSSION

The results of the present study demonstrate that meerkats, small-bodied mesopredators, are clearly attracted to, and not repelled by, the odor of blood. Further, they show that the blood odor component *trans*-4,5-epoxy-(E)-2-decenal is as efficient in

TABLE 2 | Number of interactions with the odorized wooden logs in the meerkats ($n = 15$).

Behavior	Horse blood	Blood component	Fruity odor	Blank control	Total
Sniffing	598	551	378	443	1970
Pawing	252	258	126	198	834
Licking	0	0	0	0	0
Biting	0	2	2	3	7
Toying	1	6	0	0	7
Flehmen	0	0	0	0	0
Self-impregnating	0	1	1	0	2
Scent-marking	0	0	0	4	4
Orientating	4	2	7	3	16
Guarding	1	0	2	1	4
Vocalizing	0	1	0	0	1
Fleeing	0	4	1	0	5
Total	856	825	517	652	2850

TABLE 3 | Duration of interactions with the odorized wooden logs in the meerkats ($n = 15$).

Horse blood	Blood component	Fruity odor	Blank control	Total
2.2 ± 1.9	3.1 ± 6.3	4.5 ± 8.0	2.7 ± 4.0	3.1 ± 5.5

Given are mean (±SD) values in seconds.

eliciting behavioral attraction responses in *S. suricatta* as the odor of real blood.

Comparison Between Species

Our finding that the meerkats were clearly attracted to the odor of blood and to the blood odor component TED is not trivial considering that they are small-bodied mammals which are prey to a variety of larger-bodied predators (van Staaden, 1994). Thus, it should not have been surprising if the meerkats in our study would have behaved like mice and rats, that is, like a prey species and accordingly would have avoided the odor of blood (Hornbuckle and Beal, 1974; Lahger and Laska, 2018). If we further consider that the diet of meerkats is not exclusively based on smaller vertebrates but also includes a variety of non-vertebrate food items such as arthropods and plant material (van Staaden, 1994), it is actually somewhat unexpected that the meerkats behaved like top predators such as tigers and wolves (Nilsson et al., 2014; Arshamian et al., 2017) and were attracted to blood odor. This, in turn, suggests that the attraction to blood odor displayed by carnivorous mammals of the feline and canine families may be an evolutionarily old trait as the mongoose family (Herpestidae) to which the meerkats belong split from the other carnivore families about 25 million years ago (Nyakatura and Bininda-Emonds, 2012).

A comparison of the behavioral responses of the meerkats in the present study to those of several species of top predators tested previously with the same method and odor stimuli (Nilsson et al., 2014; Arshamian et al., 2017) shows that the

meerkats displayed the highest number of interactions *per animal* with the odorized wooden logs (Table 4). This was true both when considering all four odor stimuli combined and separately.

The total number of interactions with the odorized wooden logs *per animal* displayed by the meerkats was almost five times higher than that of the Asian wild dogs, and more than three times higher compared to the African wild dogs and Siberian tigers, respectively. The South American bush dogs and the Eurasian wolves displayed a higher interest *per animal* to all four odor stimuli than the other two top predators, but still lower numbers compared to the meerkats. This raises the question as to possible reasons underlying these between-species differences in their degree of interest toward the odorized wooden logs. Several possible explanations should be considered:

Although the possibility that differences in enclosure size might have affected the likelihood of an animal to encounter and interact with the odorized wooden logs cannot be ruled out completely, it seems unlikely to explain the above-mentioned between-species differences in interest as correlations between enclosure size ($m^2/animal$) and interest in the odorized wooden logs (interactions/animal) were not significant for any of the four odor stimuli considered separately (Spearman test: real blood odor: $r_s = -0.49, p = 0.33$; TED: $r_s = -0.43, p = 0.40$; fruity odor: $r_s = -0.60, p = 0.21$; solvent: $r_s = -0.49, p = 0.33$) or combined ($r_s = -0.43, p = 0.40$). Similarly, although subadult mammals tend to be more reactive to novel objects and odor stimuli than adult conspecifics (Glickman and Sroges, 1966), differences in the age composition of the studied carnivore species are also unlikely to explain the observed between-species differences in interest toward the odorized wooden logs as with all six species under consideration >90% of all individuals were adults. Whether between-species differences in overall activity level may account for the finding that the meerkats displayed the highest interest in the odor stimuli used here needs further investigation. Although we did not systematically record the overall activity of the meerkats across the 6 h of observation per experimental day, it

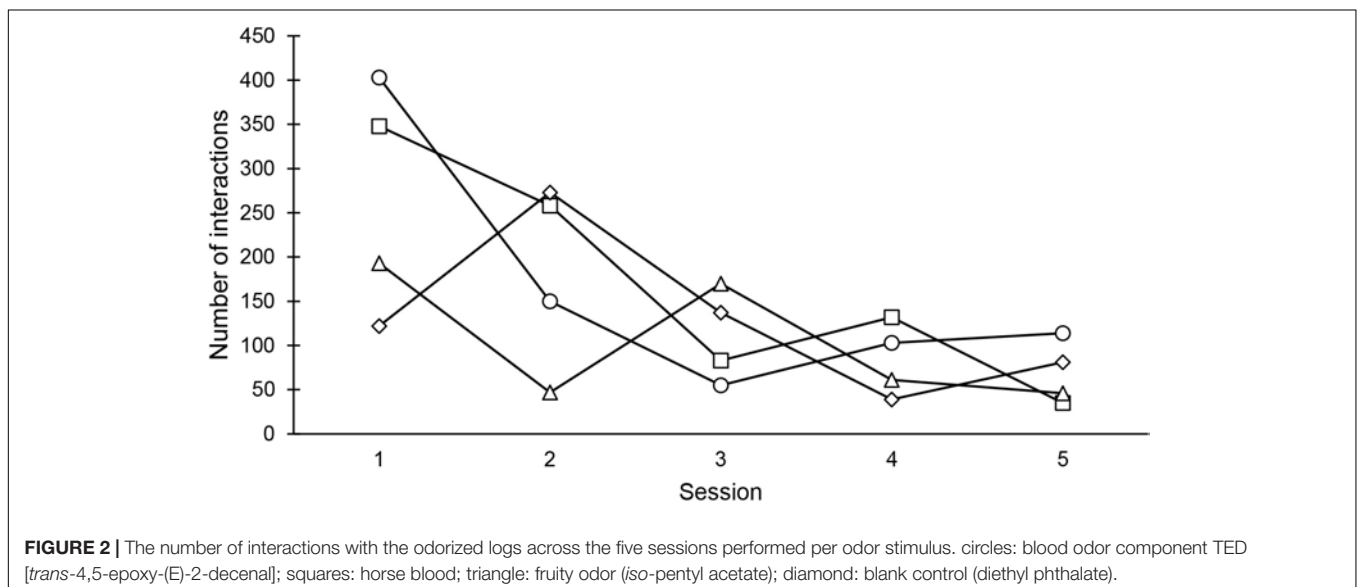


TABLE 4 | Number of interactions per animal with the odorized wooden logs.

Species	Horse blood	Blood component	Fruity odor	Blank control	Total
Meerkats ¹	57.1	55.0	34.5	43.5	190.0
Eurasian wolves ²	48.1	45.0	17.6	14.4	125.1
African wild dogs ³	11.8	23.6	10.6	7.9	53.9
Asian wild dogs ³	13.4	14.2	6.3	4.3	38.2
South American Bush dogs ³	54.0	52.1	29.7	22.4	158.2
Siberian tigers ³	27.7	27.2	5.7	4.8	65.3

(Data from ¹present study, ²Arshamian et al., 2017, ³Nilsson et al., 2014).

was obvious that they, similar to the other carnivore species tested in previous studies, alternated between phases of rest and phases of activity. Thus, there were at least no obvious between-species differences in this parameter.

Whether the observed between-species differences in interest toward the odorized wooden logs might reflect generic differences in their use of the sense of smell for exploring objects and/or odors is hard to decide. All six carnivore species are known to possess well-developed olfactory brain structures (Gittleman, 1991; van Valkenburgh et al., 2014) and all have been reported to use olfactory cues for social communication and foraging (Nowak, 2005). However, this does not exclude the possibility that the importance and use of the sense of smell may indeed differ among these carnivores. Thus, at this point no conclusive answer can be given as to why the meerkats of the present study displayed a markedly higher interest toward all four odor stimuli compared to the other carnivore species.

Recognition of the Odor Identity of a Complex Odor Mixture

With regard to the reliable recognition of behaviorally relevant odors which are almost always complex mixtures of volatiles the olfactory system can adopt two different strategies:

The first strategy implies that the olfactory system relies on only one or a few “key” or “character impact” compounds which are part of a complex mixture and are used for the recognition of an odor. Accordingly, this strategy requires olfactory receptors that are highly specific to a given ligand and central circuits which allow for a quick and hard-wired translation of a chemical stimulus into a specific behavioral response (Wilson and Stevenson, 2006). Further, this strategy requires that the “key” or “character impact” compound in question is reliably present in the odor mixture and that it can be reliably detected against the noise of the other compounds that are part of the odor mixture as well as against the chemical background noise in the environment (Nehring et al., 2013). Thus, this strategy also requires a high olfactory sensitivity for such a compound in species for which the corresponding odor is behaviorally relevant.

The second strategy implies that the olfactory system relies on either the full bouquet of volatiles that comprise an odor mixture or at least on a larger proportion of its components for the recognition of an odor. Accordingly, this strategy does not require olfactory receptors to be highly specific but instead requires central circuits to create a unitary percept or “odor object” from a complex mixture of volatiles which is stable against

fluctuations in the composition and intensity of the mixture components (Wilson and Stevenson, 2006). Thus, this strategy requires the olfactory system to generate patterns of activation which allow for fine discrimination of similar odor mixtures and a cut-off criterion which allows for distinguishing the odor object in question from similar odor objects. An example for the first strategy, in which the olfactory system relies on “key” or “character impact” compounds for odor recognition, would be the perception of certain pheromones, e.g., the rabbit mammary pheromone 2-methylbut-2-enal which has been identified as a component of rabbit milk and elicits a hard-wired behavioral response in rabbit pups (Coureaud et al., 2003; Schaal et al., 2003). This strategy is often connected to innate behavioral responses. An example for the second strategy, in which the olfactory system relies on a complex mixture of volatiles for odor recognition, would be the perception of individual body odors (Beauchamp and Yamazaki, 2003). This strategy is often connected to learned behavioral responses.

To which of these two strategies do our findings of the present study as well as those of previous studies on behavioral responses to blood odor and to the blood odor component *trans*-4,5-epoxy-(E)-2-decenal fit?

Our finding that the blood odor component TED elicited the same high degree of interest in the meerkats as the odor of real blood suggests that this monomolecular compound may serve as a “key” or “character impact” compound for the odor of blood in this and other carnivore species. However, we would like to emphasize that we do not know whether TED is “the” or just “a” component of blood odor that induces a behavioral attraction response. Considering the high number of volatiles that comprise the odor of blood (Kusano et al., 2013) it is not feasible to test all of them with a given species of animal. However, gas chromatography-olfactometry showed that TED was the only blood odor component described as having the typical “metallic, blood-like” quality that is characteristic for the odor of blood as perceived by humans (Rachamadugu, 2012).

Trans-4,5-epoxy-(E)-2-decenal is a product of lipid peroxidation (Buettner and Schieberle, 2001). Considering that this biochemical process is ubiquitous in the metabolism of mammals, it is very likely that TED is present in the blood odor of all mammals. Thus TED is likely to fulfill the prerequisite of the first strategy mentioned above of being reliably present in the odor mixture. Unfortunately, the olfactory detection threshold for TED is not known for any of the predator species that are attracted by this blood odor component. However, human

subjects and mice which are both repelled by the odor of TED (Arshamian et al., 2017; Lahger and Laska, 2018) have been shown to be extraordinarily sensitive to this compound with threshold values in the ppt (parts per trillion) range (Buettnner and Schieberle, 2001; Sarrafchi and Laska, 2017). Thus, there is at least circumstantial evidence that TED may fulfill the prerequisite of a “key” or “character impact” compound of being detectable at low concentrations.

To the best of our knowledge, no olfactory receptor has been identified so far as having TED as its specific ligand in any species. Thus, we do not know yet if TED may fulfill the prerequisite of the first strategy mentioned above of having an olfactory receptor that is highly specific to this ligand. Future studies should therefore aim at testing the specificity and sensitivity of olfactory receptors for TED.

Finally, we do not know whether the attraction response to both blood odor and TED observed in mammalian predators is innate, which would support the notion that the first strategy mentioned above might apply, or whether the attraction response is acquired. Thus, future studies should assess behavioral responses of newborn predators to these odors. The finding that laboratory-born mice that were naïve with regard to the odors of blood and TED clearly avoided both odors (Lahger and Laska, 2018) suggests that behavioral responses to TED might indeed be innate rather than acquired.

The Bipolar Behavioral Effect of Blood Odor and TED

Most of the behaviorally relevant odors studied so far have one thing in common: they elicit a certain behavioral response in a given species, or in a given group of species, and they fail to elicit any response in other species, or other groups of species. It is a hallmark of pheromones, for example, to be species-specific and thus to elicit adaptive behavioral responses, e.g., mating or aggregation, in conspecifics but not in heterospecifics (Wyatt, 2014). Similarly, food odors usually elicit adaptive behavioral responses, e.g., foraging for or inspection of a food item, in those species that feed on a given type of food but are usually ignored

by species that do not include this type of food into their diet (Stoddart, 1980). Predators are attracted by the odor of their prey whereas non-predators are usually indifferent to the odor of the prey species in question (Conover, 2007). Accordingly, prey species are repelled by the odor of their natural predator whereas non-prey species usually are not (Parsons et al., 2017).

The odors of blood and of its component *trans*-4,5-epoxy-(E)-2-decenal appear to be rather unique in the sense that they elicit a bipolar behavioral effect: an attraction response in predators (Nilsson et al., 2014; Arshamian et al., 2017) and an avoidance response in prey species (Hornbuckle and Beal, 1974; Lahger and Laska, 2018). In this context it is interesting to note that the avoidance response to both blood odor and TED displayed by mice suggests that these odors are likely to contain a warning cue rather than a fear cue as the animals significantly avoided these odor stimuli without showing behavioral indicators of fear such as freezing or defecation (Lahger and Laska, 2018). Similar behavioral responses indicative of a warning cue, but not a fear cue, have been reported in rats when presented with the odor of sick conspecifics (Arakawa et al., 2010). Future studies should therefore aim at elucidating the neural basis of these opposing behavioral responses, for example whether there are “labeled line”-like neural pathways mediating the connection between the perception of blood odor or of the blood odor component TED and behavioral attraction or avoidance responses.

AUTHOR CONTRIBUTIONS

HP, MA, and ML conceived the study, analyzed the data, and wrote the manuscript. HP collected the data. All authors critically revised the manuscript, approved the final version, and agree to be accountable for its content.

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fMRI-Based Brain Responses to Quinine and Sucrose Gustatory Stimulation for Nutrition Research in the Minipig Model: A Proof-of-Concept Study

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The minipig model is of high interest for brain research in nutrition and associated pathologies considering the similarities to human nutritional physiology, brain structures, and functions. In the context of a gustatory stimulation paradigm, fMRI can provide crucial information about the sensory, cognitive, and hedonic integration of exteroceptive stimuli in healthy and pathological nutritional conditions. Our aims were (i) to validate the experimental setup, i.e., fMRI acquisition and SPM-based statistical analysis, with a visual stimulation; (ii) to implement the fMRI procedure in order to map the brain responses to different gustatory stimulations, i.e., sucrose (5%) and quinine (10 mM), and (iii) to investigate the differential effects of potentially aversive (quinine) and appetitive/pleasant (sucrose) oral stimulation on brain responses, especially in the limbic and reward circuits. Six Yucatan minipigs were imaged on an Avanto 1.5-T MRI under isoflurane anesthesia and mechanical ventilation. BOLD signal was recorded during visual or gustatory (artificial saliva, sucrose, or quinine) stimulation with a block paradigm. With the visual stimulation, brain responses were detected in the visual cortex, thus validating our experimental and statistical setup. Quinine and sucrose stimulation promoted different cerebral activation patterns that were concordant, to some extent, to results from human studies. The insular cortex (i.e., gustatory cortex) was activated with both sucrose and quinine, but other regions were specifically activated by one or the other stimulation. Gustatory stimulation combined with fMRI analysis in large animals such as minipigs is a promising approach to investigate the integration of gustatory stimulation in healthy or pathological conditions such as obesity, eating disorders, or dysgeusia. To date, this is the first intent to describe gustatory stimulation in minipigs using fMRI.

Keywords: fMRI, brain, pig, nutrition, gustatory stimulation, visual stimulation, hedonism

INTRODUCTION

The pig and minipig models are now recognized as one of the most prominent large animal model for human nutritional physiology (Roura et al., 2016; Roura and Fu, 2017) and neuroimaging studies (Sauleau et al., 2009; Clouard et al., 2012b; Val-Laillet et al., 2015). Given the exponential use of magnetic resonance imaging in human, many efforts have to be made in order to adapt MRI setup to the pig and minipig specificities. Regarding brain investigation with functional MRI (fMRI) and, to our knowledge, only few studies were performed in pigs and minipigs, including different kinds of stimulation paradigms and stimuli, such as visual (Fang et al., 2006; Gizewski et al., 2007), somatosensory (Fang et al., 2005; Duhaime et al., 2006), pharmacological (Mäkirananta et al., 2002), and deep-brain stimulations (Min et al., 2012; Knight et al., 2013; Paek et al., 2015; Gibson et al., 2016; Settell et al., 2017). To date, no study has been performed with fMRI to explore the brain responses to gustatory stimulations for nutrition research.

Olfactory and gustatory stimulations have already been widely investigated in the pig and minipig models. Roura and Fu (2017) and Val-Laillet (2018) have recently provided complete reviews on this topic and the scope of the methodologies used for this purpose come from ethology (Clouard et al., 2012a,c; Clouard and Val-Laillet, 2014) to electrophysiology (Danilova et al., 1999) and nuclear imaging, such as single photon emission computed tomography (SPECT) or positron emission tomography (PET; Clouard et al., 2012a, 2014b; Val-Laillet et al., 2015; Val-Laillet, 2018). These approaches provide either information about a long-term integration of the stimulation, i.e., behavior or nuclear imaging, or a direct but local impact, i.e., electrophysiology. Furthermore, nuclear imaging, usually performed with an acquisition time frame of several minutes, can only inspect the global brain changes, such as in brain perfusion for SPECT with hexa-methyl-propyl-amineoxime (HMPAO) detection, or glucose metabolism for PET with fluoro-deoxyglucose detection. fMRI solely can provide information on the acute response (15–20 s) to a repeated single and short stimulation in a global brain analysis, making this methodology of high interest for nutritional studies. Moreover, different kinds of stimulations and their controls can be investigated during the same imaging session, contrary to paradigms using nuclear imaging, which significantly increases the methodological strength and statistical comparison power between stimulations, in addition to temporal resolution, and decreases the costs and constraints for animal experimentation on large animals.

The couple pleasant vs. aversive stimulation is currently gaining more attention for nutritional studies in human. Indeed, this approach is being used to decipher the organization of the gustatory cortex, e.g., the insular cortex (Rudenga et al., 2010; Dalenberg et al., 2015) and the associated brain areas (Zald et al., 2002; van den Bosch et al., 2014; Field et al., 2015). This approach is also used to investigate the impact of aging (Hoogeveen et al., 2015) or pathologies, such as obesity (Szalay et al., 2012), on those brain areas. Besides species differences in terms of taste detection and integration between human and the pig or minipig

model (Roura and Fu, 2017), the pleasant vs. aversive impact of compounds have been already investigated with behavioral exploration and nuclear imaging in pigs (Clouard et al., 2012b,c) but not with fMRI.

In this study, we aim to validate in the minipig model a fMRI-based analysis of brain responses to sucrose vs. quinine stimulation as a model of pleasant vs. aversive stimulation paradigm. For this purpose, we first validate the experimental and analysis setup with a visual stimulation based on a previous study (Gizewski et al., 2007). This first attempt of fMRI analysis of gustatory stimulation should be of high interest for nutritional studies for basic research and pre-clinical purposes.

MATERIALS AND METHODS

Animals

Experiments were conducted in accordance with the current ethical standards of the European Community (Directive 2010/63/EU), Agreement No. C35-275-32, and Authorization No. 35-88. The Regional Ethics Committee in Animal Experiment of Brittany has validated and approved the entire procedure described in this paper (Project No. 2015051312053879). A total of six 1-year-old 30-kg male Yucatan minipigs were used in this study. The pigs were housed in individual pens (150 cm × 60 cm × 80 cm) and had free access to water. A chain was suspended in each pen to enrich the environment of the animals and fulfill their natural disposition to play. The room was maintained at ~24°C with a 13:11-h light–dark cycle.

Anesthesia

Pre-anesthesia was performed with an intramuscular injection of ketamine (5 mg/kg – Imalgene 1000, Merial, Lyon, France) in overnight-fasted animals. Isoflurane inhalation (Aerane 100 ml, Baxter SAS, France) was used to suppress the pharyngotracheal reflex and then establish a surgical level of anesthesia, 3–5 and 2–3% v/v, respectively. After intubation, anesthesia was maintained with 2.5% v/v isoflurane and mechanical respiration allowed adjustment of respiratory frequency at 20 breathing/minute with a tidal volume of 450 ml. Cotton wool with an additional headset were used to conceal the animal's ears.

Visual and Gustatory Stimulation

For visual and gustatory stimulation, we used a custom-made stimulation apparatus, which was located outside the magnet-shielded room (5-m distance) and used to deliver both visual and gustatory stimulations upon synchronization with the MRI system.

Visual Stimulation

Both eyes were entirely covered by an opaque cap. The stimulation apparatus produced the visual stimulation that was conducted to the animal's right eye cap with an optical fiber. Visual stimulation consisted of flashlight at a frequency of 8 Hz. A block paradigm was used: 20-s stimulation ON,

20-s stimulation OFF, repeated 15 times. The entire stimulation protocol duration was about 10 min.

Gustatory Stimulation

Animals were equipped with an oral apparatus allowing gustatory stimulation as previously described (Clouard et al., 2012a) and consisting of three tubes, one for each solution, with an additional circular tube for continuous aspiration of liquid. Quinine (10 mM, Q1125-25G, Sigma-Aldrich, St. Quentin Fallavier, France) and sucrose (5%, S/8560/65, Fisher Chemical, Leics, United Kingdom) were solubilized in artificial saliva (Hellekant et al., 1997). In order to obtain the highest brain responses for each stimulation, three blocks of stimulation were performed in the following order, from the least to the most persistent solution in mouth: control stimulation with artificial saliva, sucrose stimulation, and quinine stimulation, each stimulation being repeated 15 times. Each stimulation consisted of oral stimulation (5 s, 24 mL/min), 25-s pause, rinse with artificial saliva (15 s, 24 mL/min), and pause (15 s). The entire stimulation protocol duration was about 45 min.

MRI Image Acquisition

Image acquisition was performed on a 1.5-T magnet (Siemens Avanto) at the Rennes Platform for Multimodal Imaging and Spectroscopy. Acquisitions were performed using a combination of coils (Body and Spine matrix coils) for optimized signal to noise ratio acquisition. *T1-weighted anatomical image acquisition*: a MP-RAGE sequence was adapted for adult minipig anatomy (1.2 mm × 1.2 mm × 1.2 mm, NA = 2, TR = 2400 ms, TE = 3.62 ms, TI = 854 ms, FA = 8°, acquisition duration 15 min). *BOLD signal acquisition*: an echo planar imaging sequence was adapted for minipig head geometry (TR/TE: 2500/40 ms, FA: 90°, voxel size: 2.5 mm × 2.5 mm × 2.5 mm). The first four acquired volumes were excluded for the data analysis, meaning that no stimulation was performed during this period.

Data Analysis and Statistical Image Analysis

Data analysis was performed with SPM12 (version 6906, Wellcome Department of Cognitive Neurology, London, United Kingdom). After slice timing correction, realignment, and spatial normalization on a pig brain atlas (Saikali et al., 2010), images were smoothed with a Gaussian kernel of 4 mm. *Voxel-based statistic*: first-level (within-individual contrast) and second-level (within-group contrast) statistics were assessed with a threshold set at $p < 0.05$ to produce the brain maps of activation. No suprathreshold voxels were detected with FDR correction at $p < 0.05$. *ROI-based statistic*: anatomical ROIs from the Saikali pig atlas (Saikali et al., 2010) were extracted and ROI-based statistic was performed using the Marsbar toolbox with a uncorrected p -value threshold set at 0.05 for the whole ROI. Statistical analysis was performed either on single stimulation, e.g., changes compared to baseline, or between stimulations differences. Data from only five minipigs were used for visual analysis due to a

complication during acquisition of the visual stimulation in one animal.

RESULTS

fMRI Setup and Statistical Analysis Validation With Visual Stimulation

We first aimed to validate the experimental setup, the acquisition, and the statistical analysis pipeline with a visual stimulation paradigm such as previously described (Fang et al., 2006; Gizewski et al., 2007). We could easily distinguish a significant BOLD response in the left occipital lobe (**Figure 1A**). This cluster was mostly found in the primary visual system V1. The ROI-based statistical analysis confirmed a significant BOLD response in V1 but also in V2 (**Figure 1B**).

Brain Responses to Artificial Saliva Stimulation

With artificial saliva stimulation, we could detect a large activation pattern in olfacto-gustatory centers (Rolls, 2005) such as the insular cortex, the prepyriform area, and thalamic regions, but also in other brain areas (**Figure 2A**). The ROI-based statistical analysis validated the increased BOLD responses within these brain regions (**Figure 2B**).

All stimulations (saliva, sucrose, and quinine) were able to activate, but in different subparts, the primary somatosensory

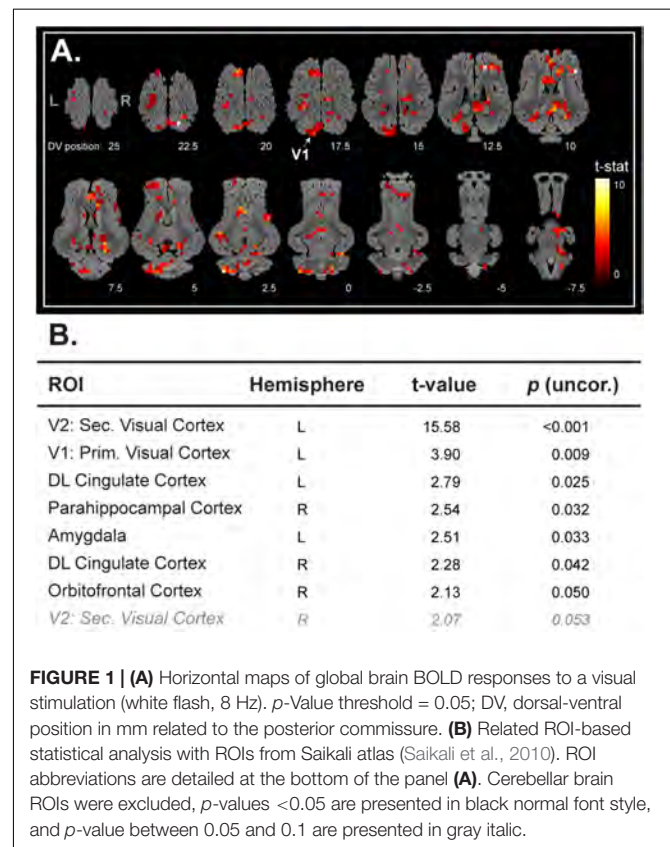


FIGURE 1 | (A) Horizontal maps of global brain BOLD responses to a visual stimulation (white flash, 8 Hz). p -Value threshold = 0.05; DV, dorsal-ventral position in mm related to the posterior commissure. **(B)** Related ROI-based statistical analysis with ROIs from Saikali atlas (Saikali et al., 2010). ROI abbreviations are detailed at the bottom of the panel **(A)**. Cerebellar brain ROIs were excluded, p -values <0.05 are presented in black normal font style, and p -value between 0.05 and 0.1 are presented in gray italic.

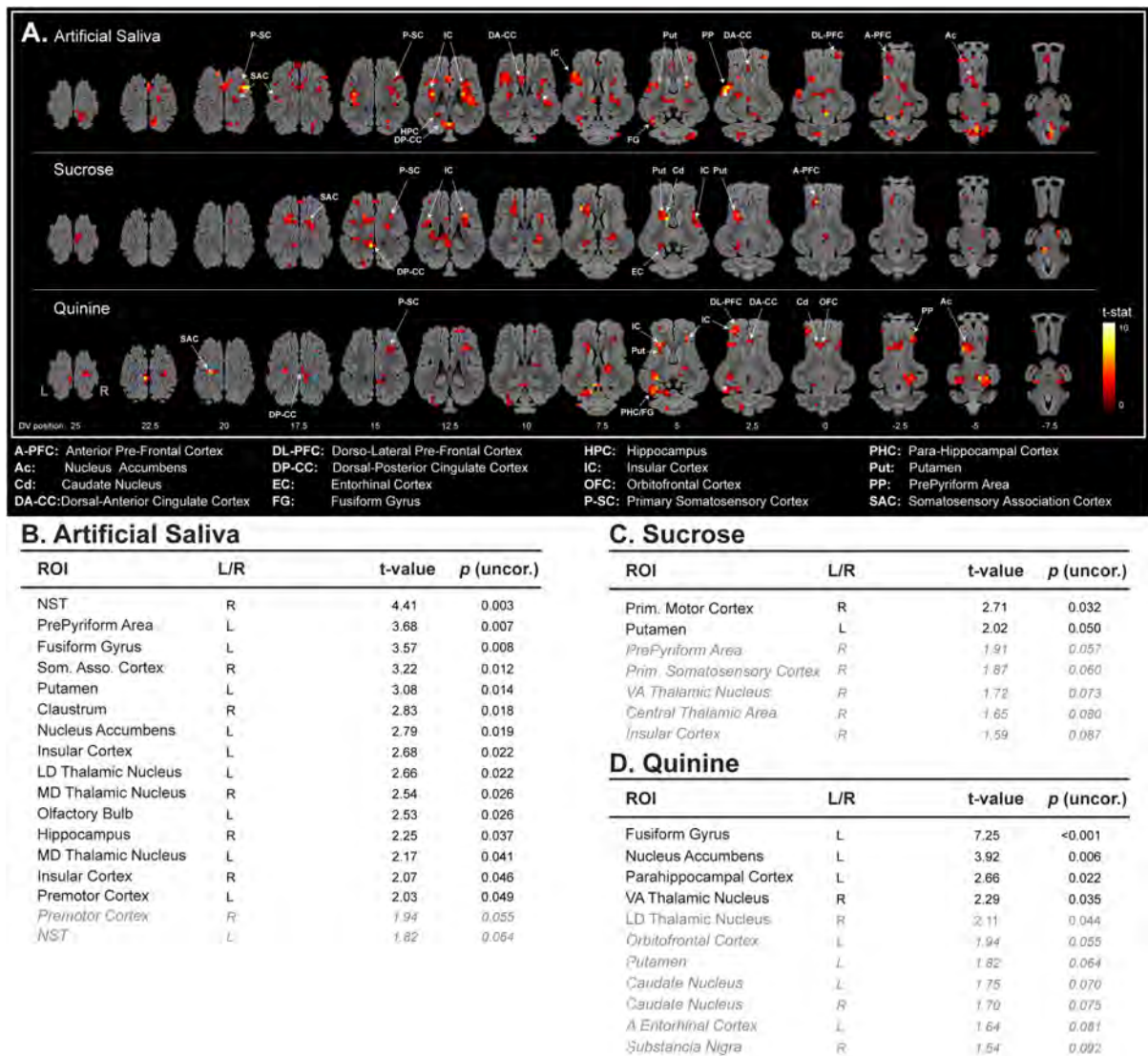


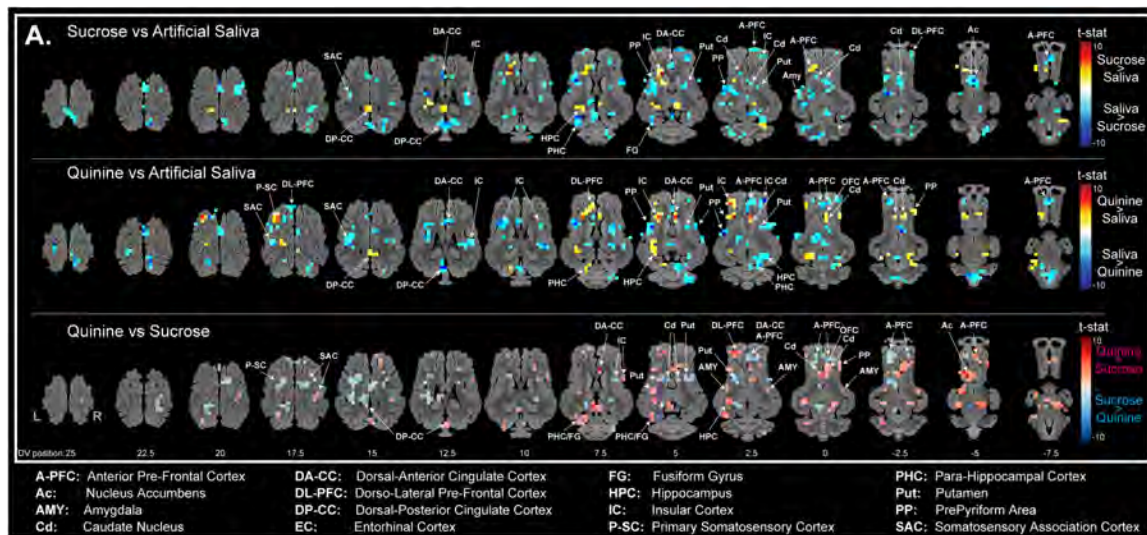
FIGURE 2 | (A) Horizontal maps of global brain BOLD responses to artificial saliva, sucrose, quinine, and between sucrose and quinine stimulations. *p*-Value threshold = 0.05; DV, dorsal-ventral position in mm related to the posterior commissure. ROI-based statistical analysis with ROIs from Saikali atlas (Saikali et al., 2010) for **(B)** artificial saliva, and **(C)** sucrose, **(D)** quinine. ROI abbreviations are detailed at the bottom of the panel **(A)**. Cerebellar brain ROIs were excluded, *p*-values <0.05 are presented in black normal font style, and *p*-value between 0.05 and 0.1 are presented in gray italic. Activation and decreased activation are separated by a line and are organized in *p*-value decreasing order.

cortex, the somatosensory association cortex, the dorsal posterior cingulate cortex, the insular cortex, and the putamen (**Figure 2A**). For instance, sucrose and quinine stimulation promote brain activation in the most anterior part of the insular cortex, which is in accordance with taste encoding (Rolls, 2016). However, sucrose and quinine stimulations promoted a reduced number of activated voxels in the brain (sucrose: $n = 174$ activated voxels and quinine: $n = 222$ activated voxels) compared with artificial saliva stimulation ($n = 399$ activated voxels), as illustrated in the insular cortex (**Figure 2A**). This is illustrated in the sucrose vs. artificial saliva and in the quinine vs. artificial saliva brain maps in which we detected fewer activated voxels with quinine stimulation than with artificial saliva stimulation

(**Figure 3A**). The ROI-based statistical analysis also showed more statistically activated brain regions with artificial saliva stimulation ($n = 15$ for $p < 0.05$, **Figure 2B**) than with sucrose ($n = 2$ for $p < 0.05$) or quinine stimulation ($n = 4$ for $p < 0.05$, **Figures 2C,D**).

Brain Responses to Sucrose Stimulation

Compared with artificial saliva stimulation, the sucrose stimulation also promoted activations, but in different subparts, in the anterior prefrontal cortex, the dorsal posterior cingulate cortex, a larger activation in the putamen, and an additional activation in the entorhinal cortex and the caudate nucleus (**Figure 2A**).



B. Sucrose vs Artificial Saliva

	ROI	L/R	t-value	p (uncor.)
Sucrose > Saliva	Central Thalamic Area	R	2.52	0.026
	Pulvinar Nuclei	L	1.81	0.065
Saliva > Sucrose	LD Thalamic Nucleus	L	-6.35	<0.001
	MD Thalamic Nucleus	R	-3.69	0.007
	Fusiform Gyrus	L	-3.32	0.011
	Hippocampus	R	-3.26	0.011
	Olfactory Bulb	L	-3.21	0.012
	Prepyriform Area	L	-2.83	0.018
	Nucleus Accumbens	L	-2.55	0.026
	Premotor Cortex	R	-2.00	0.051
	A Entorhinal Cortex	R	-1.98	0.052
	DA Cingulate Cortex	L	-1.95	0.054
	Parahippocampal Cortex	R	-1.89	0.059
	NST	R	-1.83	0.082
	Nucleus Accumbens	R	-1.52	0.094
	Substantia Nigra	R	-1.49	0.098
Globus Pallidus	R	-1.48	0.100	
Prim. Motor Cortex	R	-1.46	0.100	

C. Quinine vs Artificial Saliva

	ROI	L/R	t-value	p (uncor.)
Quinine > Saliva	LD Thalamic Nucleus	L	1.98	0.051
	A Entorhinal Cortex	L	1.73	0.072
	Geniculate Nuclei	L	1.67	0.078
	Fusiform Gyrus	L	1.52	0.095
Saliva > Quinine	Prepyriform Area	L	-2.31	0.035
	LD Thalamic Nucleus	L	-2.24	0.038
	Hippocampus	R	-2.24	0.038
	Clastrum	R	-2.13	0.043
	Globus Pallidus	L	-1.78	0.068
	Olfactory Bulb	L	-1.77	0.069
	Parahippocampal Cortex	R	-1.64	0.081
	Clastrum	L	-1.59	0.086
	Insular Cortex	L	-1.58	0.088
	NST	L	-1.54	0.092
NST	R	-1.51	0.096	
Premotor Cortex	R	-1.50	0.097	

D. Quinine vs Sucrose

	ROI	L/R	t-value	p (uncor.)
Quinine > Sucrose	Fusiform Gyrus	L	6.16	<0.001
	Caudate Nucleus	R	3.63	0.008
	Geniculate Nuclei	R	3.03	0.015
	Nucleus Accumbens	L	2.64	0.023
	A Entorhinal Cortex	L	2.20	0.040
	DA Cingulate Cortex	L	1.99	0.051
	Orbitofrontal Cortex	L	1.83	0.064
	Substantia Nigra	R	1.77	0.068
	LD Thalamic Nucleus	R	1.73	0.072
	Olfactory Bulb	L	1.48	0.100
Sucrose > Quinine	Globus Pallidus	L	-3.76	0.007
	Clastrum	R	-2.15	0.042

FIGURE 3 | (A) Horizontal maps of global brain responses between quinine and artificial saliva, as well as sucrose and artificial saliva stimulations. *p*-Value threshold = 0.05; DV, dorsal-ventral position in mm related to the posterior commissure. ROI-based statistical analysis with ROIs from Saikali atlas (Saikali et al., 2010) for **(B)** sucrose vs. artificial saliva, **(C)** quinine vs. artificial saliva, and **(D)** quinine vs. sucrose (uncorrected *p*-values). ROI abbreviations are detailed at the bottom of the panel **(A)**. Cerebellar brain ROIs were excluded, *p*-values <0.05 are presented in black normal font style, and *p*-value between 0.05 and 0.1 are presented in gray italic.

The ROI-based statistical analysis validated in part the global brain changes and showed an activation of the primary motor cortex and the putamen, a tendency toward activation of five other brain structures (Figure 2C).

The sucrose vs. artificial saliva brain map corroborated these observations (Figure 3A). Compared to artificial saliva, sucrose

promoted a decreased activation in the prepyriform area, the dorsal anterior cingulate cortex, the anterior and the ventral part of the anterior prefrontal cortex, the anterior part of the dorsal lateral prefrontal cortex, the fusiform gyrus, the ventral part of the caudate nucleus, the nucleus accumbens, the amygdala, and both higher activation and decreased activation in the insular

cortex and the putamen. The ROI-based statistical analysis of the sucrose vs. artificial saliva stimulation showed a statistical activation only in the central thalamic area and a statistical decreased activation in two thalamic areas, the fusiform gyrus, the hippocampus, the olfactory bulb, the prepyriform area, the nucleus accumbens, and a tendency toward lower activation in nine others brain structures such as, for instance, the entorhinal cortex, the dorsal anterior cingulate cortex, the para-hippocampal cortex, the nucleus accumbens, the substantia nigra, and the globus pallidus (Figure 3B).

Brain Responses to Quinine Stimulation

Compared with artificial saliva stimulation, the quinine stimulation promoted also activations, but in different subparts, in the prepyriform area, the dorsal posterior cingulate cortex, the dorsal lateral prefrontal cortex, a larger activation in the fusiform gyrus, the caudate nucleus, the nucleus accumbens, and an additional activation in the para-hippocampal cortex and the orbitofrontal cortex (Figure 2A).

The ROI-based statistical analysis supports in part the global brain changes and showed an activation of the fusiform gyrus, the nucleus accumbens, the para-hippocampal cortex, the ventral anterior thalamic nucleus, a tendency toward activation of seven other brain structures, i.e., the orbitofrontal cortex, the putamen, and the caudate nucleus (Figure 2D).

The quinine vs. artificial saliva brain maps corroborated these observations (Figure 3A). Compared to artificial saliva, quinine promoted a larger activation in the caudate nucleus, a decreased activation in the ventral part of the anterior prefrontal cortex, the putamen, and both activation and decreased activation in the insular cortex, the prepyriform area, the dorsal lateral prefrontal cortex, the dorsal posterior cingulate cortex, and the hippocampus. The ROI-based statistical analysis of the quinine vs. artificial saliva stimulation only showed a tendency toward activation in four brain structures, i.e., the lateral dorsal thalamic nucleus, the anterior entorhinal cortex, the geniculate nuclei, and the fusiform gyrus; a statistical decreased activation in the prepyriform area, the lateral dorsal thalamic nucleus, the hippocampus, the claustrum, and a tendency toward decreased activation in eight others brain structures, i.e., the globus pallidus, the olfactory bulb, the para-hippocampal cortex, the insular cortex, and the nucleus of the solitary tract (Figure 3C).

Comparison of Brain Responses to Sucrose vs. Quinine Stimulations

The quinine vs. sucrose brain map allowed discriminating the impact of each stimulation. Quinine promoted a higher activation in the dorsal lateral prefrontal cortex, the ventral part of the anterior prefrontal cortex, the prepyriform area, the para-hippocampal cortex, the fusiform gyrus, the hippocampus, the caudate nucleus, the posterior region of the putamen, the nucleus accumbens, and in the amygdala (Figure 3A, in red). Sucrose promoted a higher activation in the primary somatosensory cortex, the dorsal anterior cingulate cortex, the dorsal part of anterior prefrontal cortex, and in the putamen (Figure 3A, in

blue). Note that each stimulation showed higher activation in different subparts of the insular cortex, the anterior prefrontal cortex (larger activation for sucrose stimulation), the putamen (larger activation for sucrose stimulation), and the caudate nucleus (larger activation for quinine stimulation).

The ROI-based statistical analysis of the quinine vs. sucrose stimulation validated in part the global brain changes and showed a higher activation from quinine in the fusiform gyrus, the caudate nucleus, the geniculate nuclei, the nucleus accumbens, and the anterior entorhinal cortex, a tendency toward higher activation of five other brain structures including the dorsal anterior cingulate cortex, the orbitofrontal cortex, the substantia nigra, the lateral dorsal thalamic nucleus, and the olfactory bulb, whereas we could only detect a higher statistical activation with sucrose stimulation in the globus pallidus and the claustrum (Figure 3D).

DISCUSSION

For the first time, this proof-of-concept study describes in the minipig model the brain responses to contrasted gustatory stimulations with fMRI. After validation of our fMRI paradigm with visual stimulation, we were able to detect the brain responses elicited by artificial saliva, sucrose, and quinine oral gustatory stimulations. The statistical analysis also allowed investigating the specificity of quinine stimulation, as an aversive stimulation paradigm, vs. sucrose stimulation, as a pleasant stimulation paradigm.

As a prerequisite, we aimed to validate our experimental setup, image processing, and statistical analysis approaches with a visual stimulation paradigm, as investigated in a previous study (Gizewski et al., 2007). We were able to detect statistically significant brain responses in the contralateral visual cortex but also a trend toward activation in the ipsilateral visual cortex, which is in accordance with previous studies related to visual stimulation in pigs (Fang et al., 2006) and in minipigs (Gizewski et al., 2007). In this proof-of-concept study, the brain responses of five animals were sufficient to detect the brain activation elicited by visual stimulation (uncorrected statistic at a p -value level of 0.05). Note that correction for multiple comparisons with satisfactory p -values threshold in fMRI paradigms has been achieved in pigs only with deep brain stimulation (Min et al., 2012; Knight et al., 2013; Paek et al., 2015; Gibson et al., 2016; Settell et al., 2017), which is a highly invasive treatment deeply modifying brain activity. The ROI-based statistic without correction for multiple ROI comparison is a non-standard approach regarding to usual human fMRI-based statistical analyses, but was used to provide first evidence in this exploratory study. Considering the limited number of animals and the fact that they were anesthetized during the imaging procedure, detection of a specific BOLD signal in accordance with our hypotheses was already a satisfactory achievement. Of course, further studies should consider increasing the number of animals or stimulations per animal to reach a better statistical power and improving the anesthesia and/or stimulation paradigms to reduce inter-individual variability.

Even though artificial saliva is usually considered as a neutral stimulus (Hellekant et al., 1997; Clouard et al., 2014b), our results showed that it elicited a broad spectrum of brain responses in olfactorygustatory brain areas, limbic and corticostriatal areas, meaning that it is not a trivial stimulation. Our approach and the global brain maps of sucrose vs. artificial saliva stimulation obtained here are comparable to those described in pigs by Clouard et al. (2014b), who investigated the impact of oral sucrose 5% stimulation on brain blood flow. Interestingly, we could also detect decreased activation in the caudate nucleus, the nucleus accumbens and the amygdala, which was reported in obese humans with sucrose stimulation compared with non-obese humans (Green et al., 2011). The global brain map of quinine vs. artificial saliva stimulation can be compared to the study of Zald et al. (2002), in which bitter stimulation promoted an increased cerebral blood flow in the dorsal cingulate cortex, the orbitofrontal cortex and the nucleus accumbens. Overall, our data are in line with a recent meta-analysis searching common pattern of activity related to basic taste stimulation, i.e., activation in the insular cortex, the thalamic region, the hippocampus, the putamen, and the cingulate cortex (Yeung et al., 2017).

Based on behavioral data available in animals (McCutcheon et al., 2012; Clouard et al., 2014a; Roura et al., 2016; Roura and Fu, 2017) and humans (Veldhuizen et al., 2006; Rudenga et al., 2010; Dalenberg et al., 2015; Field et al., 2015), the quinine vs. sucrose stimulation paradigm is now used as a common paradigm to decipher the central integration of aversive vs. pleasant stimulations using neuroimaging approaches (Zald et al., 2002; Rudenga et al., 2010; Szalay et al., 2012; van den Bosch et al., 2014; Dalenberg et al., 2015; Field et al., 2015).

The amygdala, which has direct connections from lingual nerves (King et al., 2014), is involved in pleasant and unpleasant stimuli (O'Doherty et al., 2001) and has been reported to be more activated with quinine compared to water stimulation (Zald et al., 2002). However, increased activation in amygdala with sucrose compared to quinine stimulation was also reported in humans between sucrose likers and quinine dis-likers (van den Bosch et al., 2014). In a previous study in the human, the orbitofrontal cortex, which is involved in hedonic taste valence, was found modulated by the pleasantness of the gustatory stimulation (Small et al., 2003). The anterior cingulate cortex, which is involved in taste pleasantness (Grabenhorst and Rolls, 2008), was more activated with sucrose stimulation than with quinine stimulation, which suggests a higher activation in this brain region with pleasant vs. unpleasant stimulation (Small et al., 2003; Green et al., 2015). Overall, sucrose stimulation promoted less activation in the brain than quinine stimulation, as already described in the human (Zald et al., 2002; Szalay et al., 2012). As major actors of the reward system, the nucleus accumbens and the caudate nucleus (Liu et al., 2011) were found activated with sucrose stimulation (Smith, 2004; Haase et al., 2009; Jacobson et al., 2010), but other authors showed that sucrose might promote inhibition in the nucleus accumbens in rats, whereas quinine promoted activation (Roitman et al., 2005). In our study, quinine was more effective than sucrose to induce activation in both these brain structures, but a concentration of 5% sucrose (i.e., 0.15 M) might have not been

sufficient to promote a prominent response in the reward-related brain regions compared to the concentration used in the aforementioned studies (i.e., 0.64 M). Even though the limited number of animals and the use of uncorrected statistics are important limitations in our study, we managed to describe brain responses differences between quinine and sucrose stimulations in brain structures already highlighted in human studies as aforementioned. It is necessary to keep in mind that discrepancies with previous studies might be related to physiological differences in the olfaction of pigs compared to humans (Roura et al., 2016; Roura and Fu, 2017), to differences related to the tastants' concentration (Yeung et al., 2016, 2018) and/or novelty (Clouard et al., 2012a; Kishi et al., 2017), or to the hunger/satiety status during imaging (Haase et al., 2009). Finally, it is important to remind that our animals were anesthetized, which might modulate or attenuate brain responsiveness (Hendrich et al., 2001; Willis et al., 2001).

CONCLUSION

To date, among all fMRI studies performed in pigs or minipigs, this pilot study provides for the first time some preliminary evidences on brain responses to gustatory stimulation with pleasant and aversive compounds in the minipig model. Even though our study used a limited number of animals with uncorrected statistics, our overall approach can be of high interest for preclinical studies investigating the sensory integration from gustatory stimulation such as in young vs. elderly investigations (Green et al., 2011) or in pathologies such as metabolic syndrome (Green et al., 2015) or obesity (Szalay et al., 2012).

AUTHOR CONTRIBUTIONS

NC, MF, ER, and DV-L contributed in the experimental design. NC, EB, RJ, SQ, PM, and HS-J contributed in the technical development. NC, PM, and RJ performed the experiments. NC and PM analyzed the data. NC, HS-J, MF, ER, and DV-L wrote the manuscript.

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More Than Meets the Eye: The Impact of Materialism on Information Selection During Luxury Choices

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Visual attention is an important condition for consumer decision-making. However, not much is known on individuals' determinants of this visual attention. Using eye tracking, this study investigated how psychological values (i.e., materialism) modulate visual attention to specific sources of information (i.e., product, brand and additional information) in the context of luxury consumption. Participants were asked to perform a forced-choice experiment, where products were randomly assigned with luxury and non-luxury brands (Experiment 1) and product information (Experiment 2). Experiment 1 revealed that materialism was related to relatively higher attention to luxury as opposed to non-luxury and higher choice proportion of products displayed with a luxury brand. Experiment 2 showed that when providing additional product information (e.g., regarding the material) in addition to the brand, all participants chose luxury products more often. Interestingly, choices seemed to be driven by enhanced attention to brand for participants with high levels of materialism when choosing luxury products. In contrast, choices were driven by text for participants with low levels of materialism for non-luxury products. This suggests that individuals with high levels of materialism may prefer luxury products for different reasons than individuals with low levels of materialism: while the first focus on the symbolic dimension conveyed by the brand (Experiment 1), the latter pay attention to the actual product characteristics (Experiment 2). Taken together, our results suggest that materialism as a psychological value has an impact on visual attention and information selection during decision-making in the context of luxury consumption.

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INTRODUCTION

When you scroll down on your computer on a retailer website, what information do you attend to when trying to make your decision? Visual attention is defined as the degree to which people visually focus on a stimulus within their range of exposure (Solomon et al., 2010), and is an important precondition for product choice. Attentional mechanisms allow people to select a subset of information, while suppressing the non-selected information for further processing (Wedel and Pieters, 2008). This selection of information is a crucial step in purchase decisions (Milosavljevic and Cerf, 2008), suggesting that it may be helpful to measure visual attention and information acquisition using techniques such as eye-tracker to better understand the process leading to these kinds of decisions.

Several studies have been performed in the context of purchase decisions to investigate the role of visual attention processes. As Orquin and Mueller Loose (2013) illustrates in his review, visual attention is strongly related to eye movements. Recording eye-movement data allows to study the process of information acquisition, which is performed by eye fixations and saccades (Shi et al., 2012). Eye-tracking data thus does not only record the time spent looking at a product, but also the position and duration of each eye fixation (Chandon et al., 2009). Interestingly, previous research revealed a discrepancy between self-reported and eye-tracking measures (Graham and Jeffery, 2011), suggesting that people are usually not aware of their eye fixations (Chandon et al., 2009). Studies using eye-tracking measures have pointed out that during choice, a pre-decisional gaze bias occurs toward the preferred option (Chae and Lee, 2013). This bias, referred to as the gaze cascade (Shimojo et al., 2003), consists in a shift of attention toward the preferred choice alternative (Krajbich and Rangel, 2011; Willemsen et al., 2011). The preferred option is thus observed during a greater amount of time (Glaholt and Reingold, 2011; Glöckner and Herbold, 2011). The attentional Diffusion-Drift Model (aDDM, Krajbich and Rangel, 2011) suggests that gaze fixation is the mechanisms by which decision makers retrieve information about each option. According to this model, spending time looking at an option means that we accumulate evidence in favor of the fixated alternative (Krajbich and Rangel, 2011).

Information selection is achieved through two processes: bottom-up and top-down processes (Wedel and Pieters, 2008). Bottom-up processes correspond to a rapid and automatic way to capture attention (Milosavljevic et al., 2012). They refer to factors such as visual saliency (Glaholt and Reingold, 2011; Atalay et al., 2012; Milosavljevic et al., 2012; Janiszewski et al., 2013). In contrast, top-down processes refer to a voluntary attentional capture which requires personal and active search (Wedel and Pieters, 2008). This voluntary focus may be driven by the task, by people's previous knowledge, social identity (Xiao and Van Bavel, 2012), interests or goals (Milosavljevic and Cerf, 2008; Glöckner and Herbold, 2011). As an example of the impact of people's previous knowledge, brand usage (or familiarity with a brand) has been shown to diminish search costs for the consumer, leading them to greater effectiveness in terms of decision making (Chandon et al., 2009). Further evidence suggest that top-down processes drive attention toward the pieces of information that are relevant or critical for the ongoing decision (Ares et al., 2013; Orquin and Mueller Loose, 2013). For instance, consumers who are evaluating which product they intend to buy spend more time on text information (Rayner et al., 2001; Cisek et al., 2014), whereas consumers assessing product advertisements spend more time on pictorial information (Rayner et al., 2008). Xiao et al. (2016) suggest that social identity may tune visual attention. In their model, visual inputs are embedded with social values. As a consequence, people's social identity strongly influences their perception. Interestingly, these top-down influences may act on early attentional stages of visual perception (Brosch and Van Bavel, 2012; Xiao et al., 2016). More generally, people's motives, experiences and concerns may have an impact on their visual attention to a stimulus (Pool et al., 2016). As an example, research

has revealed that participants' pro-environmental orientation lead them to have a greater propensity to attend to climate change images (Sollberger et al., 2017). Another study suggested that attention paid to nutrition labels was influenced by participants' goals (Graham and Jeffery, 2011): focusing on health goals enhanced participants' attention and intensity of processing (Visschers et al., 2010), leading to an increase of choices of healthy products (van Herpen and Trijp, 2011). Thus, literature suggest that visual attention may be influenced by top-down processes such as personal characteristics, their goals and social identity (see e.g., Pool et al., 2016).

In this study, we were interested in how materialism as a psychological value may impact visual attention to specific sources of information in the context of luxury consumption. Materialism is defined as an extensive concern for material objects and worldly possessions (Belk, 1985), and leads people to have high commitment toward the acquisition and consumption of possessions (Rindfleisch et al., 1997; Dittmar, 2005). As a value, materialism has an impact on people's goals, and is considered to be a tendency to favor extrinsic aspirations (i.e., wealth, popularity, attractiveness, and conformity) over intrinsic aspirations (i.e., self-acceptance, affiliation, community feeling, safety, spirituality, hedonism, and health). Materialistic individuals, i.e., people with high levels of materialism, are more likely to look for prestigious products reflecting a high social status (Fournier and Richins, 1991; Wang and Wallendorf, 2006) than non-materialists. More specifically, materialism is a critical dimension of luxury consumers' values (e.g., Fournier and Richins, 1991) as it enhances interest for luxury brands (Gil et al., 2012), preference for luxury goods (Wong and Ahuvia, 1998; Prendergast and Wong, 2003) and brand label consideration (Audrin et al., 2017a,b).

Brand labels are extrinsic cues (Bredahl, 2004). Extrinsic cues are any piece of information about the product that is not directly part of the product itself (Zeithaml, 1988), such as its price or the label displayed on it. Intrinsic cues refer to the physical composition of the product. Taking the example of a handbag, intrinsic cues would include the color, the texture or the material of the product (Zeithaml, 1988). Literature has shown the importance of extrinsic cues in consumer decision-making. For example, consumers' expectancies about a brand impact experienced pleasantness when consuming the product (e.g., Allison and Uhl, 1964; McClure et al., 2004). When participants were drinking Coke and Pepsi without knowing what they are drinking, experienced pleasantness was similar for both drinks. However, when drinks were labeled with a brand (Coke or Pepsi), participants reported increased preferences toward Coke (McClure et al., 2004). Thus, consumers heavily rely on extrinsic cues to build their preferences (Kuusela et al., 1998; Kardes et al., 2001; Veale and Quester, 2009; Baer et al., 2017).

Here, we report two eye-tracker studies designed to assess how materialism impacts visual attention to extrinsic (i.e., pictorial) information (Experiment 1) as well as both extrinsic (i.e., pictorial) and intrinsic (i.e., text) information (Experiment 2) that was provided for a set of products in a forced-choice task. In the first experiment, we randomly presented products with either luxurious or non-luxurious brand labels

(i.e., pictorial information) to participants with high and low levels of materialism. Regarding Experiment 1, our hypotheses were that (1) all participants would choose more often products at which they look longer, (2) people scoring higher on materialism would look longer at products presented with a luxurious brand label and (3) people scoring higher on materialism would choose more often products presented with a luxurious brand. In Experiment 2 participants with high and low level of materialism were randomly presented with products in either a luxurious or a non-luxurious condition. The conditions were determined by both pictorial (i.e., brand label) and text (i.e., country of origin and material) information. Regarding Experiment 2, we hypothesized that (1) all participants would more often choose products at which they looked longer. Based on previous evidence suggesting that when providing with information about brand and quality, both participants with high and low level of materialism evaluate more positively luxury (Audrin et al., 2017a), we hypothesize that (2) all participants would show higher visual attention and choose the luxurious condition more often than the non-luxurious condition. Finally, as previous evidence reveals that materialism is related to high importance to brand labels and that people scoring low on materialism look for more information, we hypothesize that (3) pictorial information would be more determinant in the choices of participants scoring high on materialism, whereas textual information would be more determinant in the choices of participants scoring low on materialism.

PRE-TEST STUDY

We first attempted to identify luxurious and non-luxurious brands adapted to our sample. One hundred and sixty-five female students in the first year of a psychology degree at the University of Geneva were asked to name as many luxury brands as they could. Then, they were presented with 106 brands of ready-to-wear products and asked whether they knew them. These brands were selected from advertisements seen in the newspapers or on television. In addition, we selected brands mentioned in the GenY Prestige Brand Ranking (L2 Think Tank and Stren, 2010), which ranks the top luxurious brands for women. For each brand, a label was presented and participants were asked to click on “1” if they knew the brand and “0” if they did not. Based on these tests, we computed scores for each brand to select the most well-known luxurious and non-luxurious brands. Chanel[®], Gucci[®], Dior[®], and Louis Vuitton[®] appeared to be the most well-known luxurious brands and H&M[®], Zebra[®], GAP[®], and Forever21[®], the most well-known non-luxurious brands. Knowledge of the brands was moreover tested on participants of the eye-tracker experiments: on average, participants knew 93.67% of the brands ($SD = 8.25$).

EXPERIMENT 1

Methods

Participants and Procedures

To test the effect of luxurious vs. non-luxurious brands and its moderation by materialism on choice, 70 female psychology

students at the University of Geneva were recruited for Experiment 1 (mean age = 22 ± 3 years). Our stimuli were products designed to be worn by women, we consequently only invited females to participate. Participants were first asked to complete an online full version of the Aspiration Index (Grouzet et al., 2005) in order to measure their materialism. After several weeks, all participants who completed the Aspiration Index were invited to the lab to participate to the eyetracker experiment in exchange for course credits. When coming to the laboratory, participants were invited to sit in front of a computer in a cubicle. We presented a set of 46 images of ready-to-wear products from the following categories to the participants: belts, handbags, purses and scarves. The selected products came from brands most-likely unknown to our sample (i.e., brands unknown as assessed in the pre-test study above), which ensured that the products or their brand could not be recognized by our participants. Each product was presented with 1 out of 8 brands. Half of the products were randomly presented with one out of four luxurious brand, while the other half was presented with one out of four non-luxurious brands. The luxurious and non-luxurious brands were randomized between participants so that each product was seen with each of the brands across the whole sample (see **Figure 1**). The study was performed according to the rules and regulations of the University of Geneva and the declaration of Helsinki. All participants gave their written informed consent to take part in the study, which was officially approved by the ethics committee of the University of Geneva.

Data Acquisition

Materialism

To assess participants' level of materialism, we used the full version of the Aspiration Index (Grouzet et al., 2005), which refers to Kasser (1999)'s conceptualization of materialism as a balance between extrinsic and intrinsic aspirations. In the Aspiration Index (Grouzet et al., 2005), people assessed importance of 57 aspirations on a scale ranging from 1 (“not important at all”) to 9 (“extremely important”). These goals refer to 11 aspirations, which can be separated into two main dimensions, i.e., intrinsic and extrinsic dimensions. Extrinsic



FIGURE 1 | Schematic description of the task in Experiment 1 and 2.

dimensions refer to the importance given to one's own image (e.g., "I hope for the future that my image will be one other's find appealing"), popularity (e.g., "I will be admired by many people"), financial success (e.g., "I will have expensive possessions"), and conformism (e.g., "I will live up to the expectations of my society"). Intrinsic dimensions are the importance given to his own health (e.g., "I will feel energetic and full of life"), affiliation (e.g., "There will always be someone around to take care of me"), spirituality (e.g., "I will find personal answers to universal spiritual questions such as: Is there a supreme spiritual being? Is there life after death? What is the meaning of life?"), community (e.g., "I will assist people who need it, asking nothing in return"), hedonism (e.g., "I will have a lot of excitement in my life"), safety (e.g., "I will have few threats to my personal safety") and self-acceptance (e.g., "I will feel free"). Materialism scores were computed as the relative importance of extrinsic (mean of the extrinsic dimensions' scores) vs. intrinsic aspirations (mean of the intrinsic dimensions' scores) (Kasser, 1999; Grouzet et al., 2005). The more people considered extrinsic aspirations important compared to intrinsic aspirations, the more they were materialists. Our sample had an average of -1.589 score on materialism ($SD = 1.272$).

Eye-Tracking

Participants seated 60 cm away from a 43 cm-wide screen and were asked to keep their head in the same position during the experiment. Movement of the eyes were recorded at a 60-Hz frequency using infrared cameras of the Tobii eye-tracker located at the bottom of the screen. The eye-tracker was calibrated before each session. This phase consisted in the presentation of moving dots that participants were asked to follow without anticipating the dots' movement.

After this calibration, participants were presented with two ready-to-wear products side by side. For each trial, one product was presented with a luxurious brand and the other with a non-luxurious brand. The side of the luxurious/non-luxurious condition was randomized. Participants were asked to select the product they would prefer to get if they could obtain it. There was no time constraint. Each participant had to make 46 choices, where each of the 46 products was presented twice (each time with the same brand label, every time against another product). After each choice, a fixation cross was displayed in the center of the screen to wait for the next pair of products to be presented. The order of presentation was randomized between participants.

Data Analyses

Data analyses were performed with R (R Development Core Team, 2008), lmerTest (Kuznetsova et al., 2017), and lme4 packages (Bates et al., 2015). (Generalized) Linear Mixed Model ((G)LMM) analyses were performed on eyetracker and behavioral data. We used participants and stimuli as random effects (i.e., random intercepts) to control for variance due to participants and items, respectively (Judd et al., 2012). For the fixed effects, we assigned the coding $-1/+1$ as advised by Judd et al. (2012), which allowed us to interpret these effects as main effects. We visually inspected residual plots to detect deviations from homoscedasticity or normality. Descriptive analyses (mean

and s.e.) are reported below. Concerning the eye-tracker data, analyses were performed on the total fixation time. Due to its non-normal distribution, fixation time was log-transformed, but for the ease of interpretation, means were back-transformed for **Table 1**. Our model contained Condition (luxurious vs. non-luxurious condition), Materialism and Information (product vs. brand) as fixed effects. Concerning the behavioral data, we performed analyses on participants' choices (**Table 2**). For each choice, we predicted the probability that the product on the left would be chosen. Selecting the product on the left or on the right as a dependent variable is equivalent, as the position (right or left) of the luxurious and non-luxurious brands was randomized. To analyse this dichotomous variable, we performed a multilevel logit model. The fixed effects were Condition (luxurious vs. non-luxurious condition), Materialism (materialist vs. non-materialist) and the Time (time spent before making the choice in milliseconds). Descriptive statistics for the choice variable are reported in **Table 2**.

Results

Total Fixation Time

Results for the total fixation time showed a main effect of Information [$b = 0.787$, $IC_{95\%} = [0.763; 0.812]$, $t_{(8,125)} = 62.992$, $p < 0.001$; see **Table 3**], revealing that participants spent more time looking at the product picture than at the brand. The Condition \times Materialism interaction effect was significant [$b = -0.013$, $IC_{95\%} = [-0.026; -0.001]$; $t_{(8,089)} = -2.047$, $p = 0.041$], supporting our hypothesis that materialism had an impact on the time spent on luxury vs. non-luxurious condition. Interestingly, results revealed that the more participants are materialistic, the less they look at non-luxury. There was no further significant effect.

Choice

Results showed a main effect of Condition ($b = 0.232$, $IC_{95\%} = [0.112; 0.354]$, $z = 3.771$, $p < 0.001$; **Table 4**), where products associated with luxurious information were more often chosen than products associated with non-luxurious information. The

TABLE 1 | Descriptive statistics for the Total Fixation Time variable (mean and s.e.).

Materialism	Condition	Information	Time (ms)	s.e.
-1 s.d.	Luxury	Image	958.337	84.227
-1 s.d.	Luxury	Brand	176.542	11.632
-1 s.d.	Non-luxury	Image	957.088	80.370
-1 s.d.	Non-luxury	Brand	182.390	11.626
mean	Luxury	Image	944.758	56.846
mean	Luxury	Brand	179.456	7.850
mean	Non-luxury	Image	929.435	54.242
mean	Non-luxury	Brand	184.375	7.847
+ 1 s.d.	Luxury	Image	932.756	79.051
+ 1 s.d.	Luxury	Brand	182.032	10.917
+ 1 s.d.	Non-luxury	Image	904.994	75.431
+ 1 s.d.	Non-luxury	Brand	186.128	10.912

TABLE 2 | Descriptive analyses for the Choice variable (mean and s.e.).

Materialism	Condition	Proportion of choice	s.e.
-1 s.d.	Luxury	50.8%	0.023
-1 s.d.	Non-luxury	49.12%	0.023
mean	Luxury	51.58%	0.016
mean	Non-luxury	48.41%	0.017
+1 s.d.	Luxury	52.29%	0.023
+1 s.d.	Non-luxury	47.70%	0.023

TABLE 3 | Results for the Total Fixation Time variable, *** $p < 0.001$; * $p < 0.05$.

Fixed Effects	b	SE	p-value
Intercept	0.579	0.002	0.001***
Condition	0.017	0.012	0.137
Information	0.787	0.013	0.001***
Materialism	0.018	0.038	0.641
Condition × Information	0.005	0.012	0.629
Condition × Materialism	-0.013	0.006	0.041*
Information × Materialism	-0.001	0.003	0.261
Condition × Information × Materialism	-0.001	0.008	0.801

Random effects	σ^2	SE
Participants		
Intercept	0.153	0.391
Stimuli		
Intercept	0.003	0.609

marginal effect of Time ($b = 0.051$, $IC_{95\%} = [-0.000; 0.103]$, $z = 1.941$, $p = 0.052$) supported the hypothesis that the more people looked at the product, the more they chose it. Finally, the Condition × Materialism interaction was significant ($b = 0.086$, $IC_{95\%} = [0.023; 0.148]$, $z = 2.708$, $p = 0.006$), supporting the hypothesis that the impact of materialism depended on the product: the more participants were materialistic, the more they would choose luxury products over non-luxury products ($b = 0.12$, $z = 2.562$, $p < 0.001$).

Discussion

The results presented above revealed that the longer participants looked at a product, the higher the probability that this product was chosen. While the result is marginally significant in our study, it is congruent with previous evidence (Glaholt and Reingold, 2011; Atalay et al., 2012; Milosavljevic et al., 2012) suggesting that higher attention to a product is driven by enhanced interest (Fink et al., 1996), thus leading to enhanced preference. Our results reveal for the first time that this is also true in the context in luxury consumption.

Our results further suggest that despite their longer attention to the product itself compared to the extrinsic cues, participants still integrated extrinsic cues (i.e., brand label) into their choices. The more participants were materialistic, the less they looked at products displayed with a non-luxurious brand. These results

TABLE 4 | Results for the Choice variable, *** $p < 0.001$; * $p < 0.05$.

Fixed Effects	b	SE	p-value
Intercept	-0.318	0.128	0.013*
Materialism	-0.037	0.036	0.304
Condition	0.232	0.062	0.001***
Time	0.051	0.026	0.052.
Condition × Materialism	0.086	0.032	0.006***

Random Effects	σ^2	SE
Participants		
Intercept	0.041	0.202
Stimuli		
Intercept	0.398	0.631

revealed that the mere association of a brand with a product can lead participants to look at it differently, and to choose it differently, depending on their materialism. This is in line with previous evidence revealing the importance of external cues in consumer choices (e.g., McClure et al., 2004), but we revealed here that this can also be observed in the context of luxury consumption.

Finally, our results reveal that psychological values such as materialism modulate the impact of extrinsic cues (e.g., Sörqvist et al., 2015). While the effect was not strong in our results regarding the pattern of visual attention, results on the choice variable revealed that the brand had a different impact on participants' choice, depending on their level of materialism. These results show further evidence that the brand was a strong vector of the luxury dimension of a product for materialistic people (Audrin et al., 2017a,b). More generally, this result echoes previous results revealing the strong link between materialism and luxury (Gil et al., 2012).

In the next experiment, we studied how providing textual information about the product in addition to the brand label modulates participants' visual attention and subsequent choices. We hypothesize that, in contrast to Experiment 1, where only brand information was provided, providing supplementary information about the product quality will lead both participants with high and low levels of materialism to prefer luxury.

EXPERIMENT 2

Products are embedded with multiple cues (Miyazaki et al., 2005) such as quality information and brands information. When facing multiple congruent cues, people usually take them into account in an additive way (Anderson, 1981; Miyazaki et al., 2005). However, research suggest that individual characteristics may impact the way multiple cues are integrated in the process of decision-making. For instance, Ahluwalia et al. (2000) revealed that depending on their brand commitment, consumers gave different diagnostic weights for positive and negative information.

In this experiment, we wanted to assess how materialism as a value impacts information integration during choice. To this

end, we provided text information in addition to the brand such as the country of origin and the material of the products. Our hypothesis was that when provided with supplementary intrinsic information, both participants with high and low materialism will prefer luxury. We suggest however that this may be driven by different reasons: materialistic participants can be expected to predominantly consider the symbolic aspect embedded in luxury, as previous research has shown that people high in materialism focused on the brand in the context of luxury consumption (Gil et al., 2012; Audrin et al., 2017a). Thus, we hypothesize that, as in Experiment 1, people with high level of materialism would primarily focus on the brand. In contrast, we hypothesize that consumers with low levels of materialism would primarily look for more objective information about the product as provided by the textual information, as literature suggests that they like luxury preferably for the quality it guarantees (Audrin et al., 2017a). Thus, we suggest that people low in materialism will mostly look at intrinsic textual information about the product. Taken together, we hypothesized that (1) all participants would show higher visual attention and choose luxurious condition more often than non-luxurious condition (Model 1), (2) all participants would choose more often products at which they looked longer (Model 2) and finally (3) pictorial information would be more determinant in the choices of people with high level of materialism, whereas textual information would be more determinant in people's with low level of materialism choices (Model 2).

Methods

Participants and Procedure

Among 195 female students in psychology at the University of Geneva, 60 were recruited based on their materialism scores on the Aspiration Index (Grouzet et al., 2005). Four of them were removed from the final sample because the eye-tracker was not able to detect their eyes, resulting in a sample of 56 participants (*mean age*: 22 ± 4 years). Our final sample consisted of 24 people with high levels of materialism (*mean* = 0.29 ± 0.42) and 32 people with low levels of materialism individuals (*mean* = -2.55 ± 0.52).

When coming to the laboratory, participants were invited to sit in front of a computer in a cubicle. The same set of 46 images of ready-to-wear products were presented as in Experiment 1. Half of the products were randomly assigned to in the luxury condition, while the other half was presented in the non-luxury condition. The luxury and non-luxury conditions were randomized between participants so that each product was seen with each of the brands across the whole sample. The eye-tracker was calibrated before each session. The task was similar to the one described in Experiment 1. In order to test the interaction between Materialism, Condition and Information, we manipulated the text information provided with each product. Each product was presented with a brand, as well as with information about the product's price, country of origin, and material. This information was gathered from the websites of the brands in question, and adjusted according to the presented product. Half of the products were presented in the luxurious condition (i.e., country of origin, price, material), while the other

half was presented in the non-luxurious condition. The number of words was the same in the text associated with luxurious and non-luxurious brands (3 words for the country where the product was made and 2 words for the composition; see **Figure 3** for a prototypical example). The study was performed according to the rules and regulations of the University of Geneva and the declaration of Helsinki. All participants gave their written informed consent to take part in the study.

Data Acquisition

Materialism

One hundred and ninety-five participants were presented with the Aspiration Index, and we computed their materialism scores as the relative importance of extrinsic aspirations vs. the importance of intrinsic aspirations. Based on these materialism scores for all participants, we selected the upper (high level of materialism) and lower (low level of materialism) quartile of the initial sample to participate to the experiment.

Eye-Tracking Data

When coming to the laboratory, participants were seated 60 cm away from a 43 cm-wide screen and were asked to keep their head in the same position during the experiment. As for Experiment 1, movement of the eyes were recorded at a 60-Hz frequency using infrared cameras of the Tobii eye-tracker, located at the bottom of the screen.

Data Analyses

To answer our first hypothesis (i.e., that all participants will show higher visual attention and choose luxurious condition more often than non-luxurious condition), we performed a linear mixed model on the total fixation time (Model 1). We entered participants and stimuli as random effects. As fixed effects, we introduced Information (i.e., picture vs. text information), Condition (luxurious vs. non-luxurious condition), and Materialism (high vs. low materialism) and their interaction. Descriptive statistics are reported in **Table 5**.

In order to assess our second (i.e., all participants will choose more often products at which they looked longer) and third hypotheses (pictorial information will be more determinant in participants high on materialism, whereas textual information will be more determinant in the choices of participants with low levels of materialism, respectively), a generalized linear mixed model was performed on the choices made by participants (Model 2). For each choice, we predicted the probability that the product on the left would be chosen. The fixed effects were Condition (luxurious vs. non-luxurious condition) and Materialism (high vs. low on materialism). We further introduced two variables referring to the time spent on the information variable. The first variable accounted for the relative time spent on the picture (i.e., product and brand) on the left as opposed to the total time spent on the pictorial information before making the choice (Picture Time). The second variable accounted for the relative time spent on the text information of the product on the left as opposed to the total time spent on the text information before making the choice (Text Time). Participants and stimuli were introduced as random

TABLE 5 | Descriptive statistics (mean and s.e.) for the Total fixation time variable.

Materialism	Information	Condition	Time (ms)	s.e.
High	Text	Luxury	632.693	50.735
High	Picture	Luxury	1136.966	52.348
High	Text	Non-luxury	524.014	53.117
High	Picture	Non-luxury	1095.82	46.878
Low	Text	Luxury	691.535	41.558
Low	Picture	Luxury	1189.684	38.569
Low	Text	Non-luxury	623.427	47.714
Low	Picture	Non-luxury	1190.373	47.756

TABLE 6 | Descriptive analyses (mean and standard error (s.e.)) for the Choice variable.

Materialism	Condition	Proportion of choice	s.e.
High	Luxury	55.8%	0.034
High	Non-luxury	44.1%	0.034
Low	Luxury	55.5%	0.030
Low	Non-luxury	44.4%	0.030

effects (random intercepts). Descriptive statistics are reported on **Table 6**.

Results

Total Fixation Time

Results of Model 1 showed a main effect of Information [$b = -0.146$, $IC_{95\%} = [-0.155; -0.138]$, $t_{(7,048)} = -34.366$, $p < 0.001$], revealing that participants spent more time looking at the pictorial elements than at the text information. Results further revealed a main effect of Condition [$b = 0.016$, $IC_{95\%} = [0.007; 0.023]$, $t_{(6,994)} = 3.913$, $p < 0.001$; **Table 7**], supporting our hypothesis that all participants looked more at luxurious products than at non-luxurious products. The Condition \times Information interaction was significant [$b = -9.25 \times 10^{-3}$, $IC_{95\%} = [-0.017; -0.001]$; $t_{(6,994)} = 2.305$, $p = 0.023$], showing that the difference between the time spent looking at luxurious and non-luxurious products was more important when participants focused on text information ($t = -3.688$, $p < 0.001$) than when they looked at the picture ($t = -1.495$, $p > 0.05$). The Information \times Materialism interaction was marginally significant [$b = -8.069 \times 10^{-3}$, $IC_{95\%} = [-0.016; 0.003]$, $t_{(6,997)} = 2.305$, $p = 0.058$, see **Figure 2**], suggesting that the impact of materialism on time spent looking at picture vs. text was different. Specifically, this suggests that for both groups, the time spent looking at the picture was longer than the time spent looking at the text, but that this difference was slightly more important for participants with high levels of materialism. Results revealed no further significant effect.

Choice

Results from Model 2 (i.e., with Condition, Materialism and Time) showed a main effect of Condition ($b = 0.258$, $IC_{95\%}$

TABLE 7 | Results for the Total fixation time variable, *** $p < 0.001$; * $p < 0.05$.

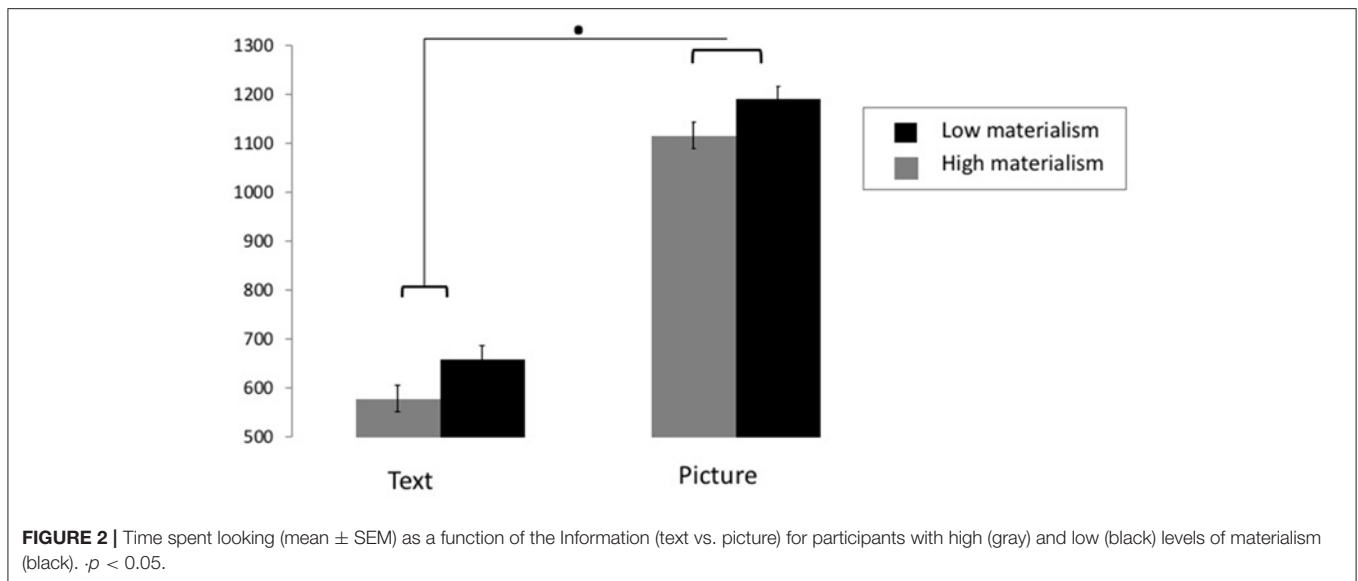
Fixed effects	b	SE	p-value
Intercept	2.810	0.024	0.001***
Condition	0.016	0.004	0.001***
Information	-0.147	0.002	0.001***
Materialism	-0.001	0.023	0.795
Condition \times Information	-0.009	0.004	0.023*
Condition \times Materialism	0.005	0.004	0.253
Information \times Materialism	-0.008	0.004	0.059.
Condition \times Information \times Materialism	0.002	0.004	0.485
Random effects	σ^2	SE	
Participants			
Intercept	0.03	0.17	
Stimuli			
Intercept	0.001	0.041	

$= [0.169; 0.349]$, $z = 5.640$, $p < 0.001$; **Table 8**), where luxury products were chosen more often than non-luxurious products. The main effects of Picture Time ($b = 3.547$, $IC_{95\%} = [2.936; 4.153]$, $z = 11.429$, $p < 0.001$) and of Text Time ($b = 1.079$, $IC_{95\%} = [0.780; 1.388]$, $z = 6.957$, $p < 0.001$; see **Table 8**) revealed that, as hypothesized, the more participants looked at the text and pictorial information, the more they chose it. The interaction between the Picture Time and Text Time was significant ($b = -1.904$, $IC_{95\%} = [-3.656; -0.059]$, $z = -2.033$, $p = 0.042$), indicating that the impact of the time spent looking at the picture on the choice weakened when the time spent looking at the text increased. Moreover, the interaction effect between Condition, Materialism and Picture Time was marginally significant ($b = 0.582$, $IC_{95\%} = [-0.024; 1, 469]$, $z = 1.915$, $p = 0.055$; **Figure 3**). This interaction revealed that when participants with low levels of materialism looked longer at non-luxurious picture, they chose them more often ($z_{\text{low-materialism}} = 5.325$, $p < 0.001$). On the other hand, participants with high levels of materialism who looked longer at the picture of luxurious condition chose them more often ($z_{\text{highmaterialism}} = 5.794$, $p < 0.001$).

Finally, the interaction effect between Condition, Materialism and Text Time was significant ($b = -0.386$; $IC_{95\%} = [-0.660; -0.09]$, $z = -2.702$, $p = 0.006$; **Figure 4**). This interaction revealed that when participants who scored high on materialism looked longer at text information of non-luxurious products, they would choose these products more often ($z = 1.882$, $p = 0.059$). In contrast, when participants scoring low on materialism looked longer at the text information of luxurious products, they would choose these products more often ($z = 5.233$, $p < 0.001$).

Discussion

Supporting our hypothesis, results from Experiment 2 showed that when providing multiple congruent sources of information about a product, all participants chose luxury products more often. Our results highlight that individual characteristics have



an impact on how these multiple sources of information are taken into consideration when making a choice (Ahluwalia et al., 2000). Eye-tracking data revealed different patterns of visual attention for the groups: the more participants with high levels of materialism paid attention to the *picture* of the product (i.e., brand and picture), the more often they chose luxury products. On the contrary, the more participants with low levels of materialism looked at the *text* information, the more often they chose luxury products. These results suggest that individual values and goals lead to different patterns of visual attention and information integration during decision-making and product choice. Participants scoring high on materialism considered specifically the symbolic aspect embedded in luxury (i.e., they mostly focus on the brand): the more they looked at pictorial information, the more they chose luxury products. Participants scoring low on materialism looked for textual information about the product: the longer they looked at text information, the more they chose luxury products. This suggests that the preference for luxury may be related to specific dimension of luxury (Audrin et al., 2017a) depending on peoples' values. People with high levels of materialism favor symbolic dimensions by focusing on pictorial brand-related element (Audrin et al., 2017a,b). By contrast, participants with low levels of materialism pay relatively more attention to factual product information (Audrin et al., 2017a).

GENERAL DISCUSSION

In this work, we investigated how materialism modulates visual attention to specific sources of information in the context of luxury consumption. Using eye-tracking, we tested how visual attention allocated to pictorial brand information (Experiments 1, 2) and textual quality information (Experiment 2) was related to product choices for participants with high and low levels of materialism.

Experiment 1 tested the importance of pictorial brand-related information. Results revealed that higher levels of materialism were related to higher visual attention to the luxurious condition (i.e., product and brand). Increased visual attention furthermore enhanced the probability of choosing luxury products. Experiment 2 tested the importance of both pictorial and textual information, revealing that when adding supplementary textual information about the products, all participants paid increased attention and chose the luxurious condition more often. Differences in materialism may have lead participants to choose luxurious products for different reasons: the longer participants with higher levels of materialism looked at pictorial information, the more often they chose luxury products. In contrast, the longer participants with low levels of materialism looked at textual information, the more they chose luxury products.

Our results provide congruent evidence for the gaze cascade effect suggested by Shimojo et al. (2003). As suggested by (Shimojo et al., 2003), eye movements and eye gaze reveal a shift of attention toward one alternative (Krajbich and Rangel, 2011), and are involved in preference formation. This preference formation may further indicate interest toward the observed alternative (Shimojo et al., 2003). Our results are congruent with this literature, as the more participants looked at a product or its feature, the more frequently they chose it. Moreover, our results reveal that materialism, as a psychological value, may have an impact on visual attention. This is congruent with previous evidence revealing the importance of top-down processes, notably the importance of people's motives, experiences and concerns, on visual attention (Pool et al., 2016).

Results from a retail context (Chandon et al., 2009) suggest that gaze may be an indicator of *attention*. On the other hand, Shimojo et al. (2003) suggest that this may be an indicator of *interest*. Finally, Fink et al. (1996) suggest that *interest* may lead to higher *attention* toward an option. Our results seem to provide congruent results with Fink et al.'s work: while previous

TABLE 8 | Results for the Choice variable (Model 2), *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Fixed effects	b	SE	p-value
Intercept	-0.197	0.104	0.057
Materialism	-0.09	0.056	0.099.
Condition	0.258	0.046	0.001***
Picture time	3.547	0.31	0.001***
Text time	1.079	0.155	0.001***
Condition × Materialism	-0.047	0.047	0.317
Materialism × Picture Time	0.081	0.305	0.791
Condition × Picture Time	-0.100	0.304	0.743
Materialism × Text Time	-0.107	0.149	0.476
Condition × Text Time	-0.027	0.143	0.853
Picture Time × Text Time	-1.904	0.937	0.042*
Materialism × Condition × Picture Time	0.582	0.304	0.055.
Materialism × Condition × Text Time	-0.386	0.143	0.006**
Condition × Picture Time × Text Time	1.239	0.938	0.186
Materialism × Picture Time × Text Time	0.282	0.934	0.763
Condition × Materialism × Text Time × Picture Time	0.144	0.935	0.878
Random effects	σ^2	SE	
Participants	0.05	0.241	
Intercept			
Stimuli	0.14	0.377	
Intercept			

research has suggested that materialism was related to higher *interest* toward luxury (Gil et al., 2012), our result reveal that this interest was related to higher *attention* to luxury. Future research should however specifically test the relation between materialism, interest and attention toward luxury.

As pointed out in the literature, when making a decision, individuals use attribute information as a way to estimate their probable satisfaction with the choices they are facing. However, products are often embedded with multiple attributes, which makes the integration of all pieces of information too difficult and too costly in terms of cognitive processes. People often focus on one single source of information, in order to make their decision easier (Johnson et al., 2012). This strategy allows consumers to reduce the cognitive effort, and to specifically focus on the attribute they perceive as the most important (Johnson et al., 2012). Our results suggest that this strategy was also used in our experiments, and further highlights that psychological values modulated the selection of the most important attribute.

Our findings support the suggestion that materialism, measured as a psychological value, might lead to a propensity to attend to specific aspects of luxury. As the previous literature reveals, materialistic people focus on the conspicuous aspects provided by luxury possessions (Gil et al., 2012; Audrin et al., 2017a), further suggesting that they give importance to the brand

as it can be displayed overtly. Conversely, people with low levels of materialism may consume luxurious products because of their quality (Audrin et al., 2017a).

Taken together, our results provide new insights to the field of research on consumer decision-making in the context of luxury consumption. To the best of our knowledge, our study is the first to manipulate displayed brands and text information of ready-to-wear products in an eye-tracking setting. Our results provide evidence for the importance of the brand in consumers' preferences establishment. Notably, our results show how strong the impact of brand information may be to (materialistic) consumers, as the brands were randomly presented with products, but still lead to a preference for the respective products. This result, consistent with previous research (e.g., Audrin et al., 2017a,b), points out that values are important factors to be taken into account when studying consumer decision-making. While the behavioral (choice variable) results revealed strong evidence in favor of our hypotheses, evidence was weaker regarding the eye-tracking data, which gave only moderate support to our hypotheses. This kind of discrepancy between self-reported and eye-tracking measures has been previously shown in the literature (Graham and Jeffery, 2011). However, it suggests that these results should be taken cautiously, and that the experiments reported here should be replicated in order to show more compelling evidence regarding the impact of personal values on visual attention in the context of luxury consumption. The measure of fixation duration should also be taken cautiously. Indeed, Krasich et al. (2018) revealed that mind wandering (i.e., the tendency to zone out, when the attention shifts away from the on-going task toward unrelated thoughts such as grocery shopping or upcoming vacations) was related to longer fixation durations, thus suggesting that longer fixation time is not a necessarily a proof of enhanced attention or interest.

Two additional limitations of our study are related to our sample. First, our participants were exclusively students, who may not be very familiar with luxury consumption, as luxury products are high-priced and thus not affordable to students. It would be interesting to evaluate the extent to which our results may apply to actual luxury consumers. Second, we focused our research on female participants. This was related to the products chosen, which were exclusively designed for women. An interesting follow-up would be to evaluate whether the results observed in our study are also true for men. Indeed, as Stokburger-Sauer and Teichmann (2013) mentioned, female participants have more positive attitudes toward luxury brands. Thus, assessing how men are sensitive to brands vs. textual information may provide further explanations on what people look for when consuming luxury.

CONCLUSION

Our results point out how psychological values (i.e., materialism) modulate visual attention to specific sources of information (i.e., pictorial brand vs. text quality information). Eye-tracking data

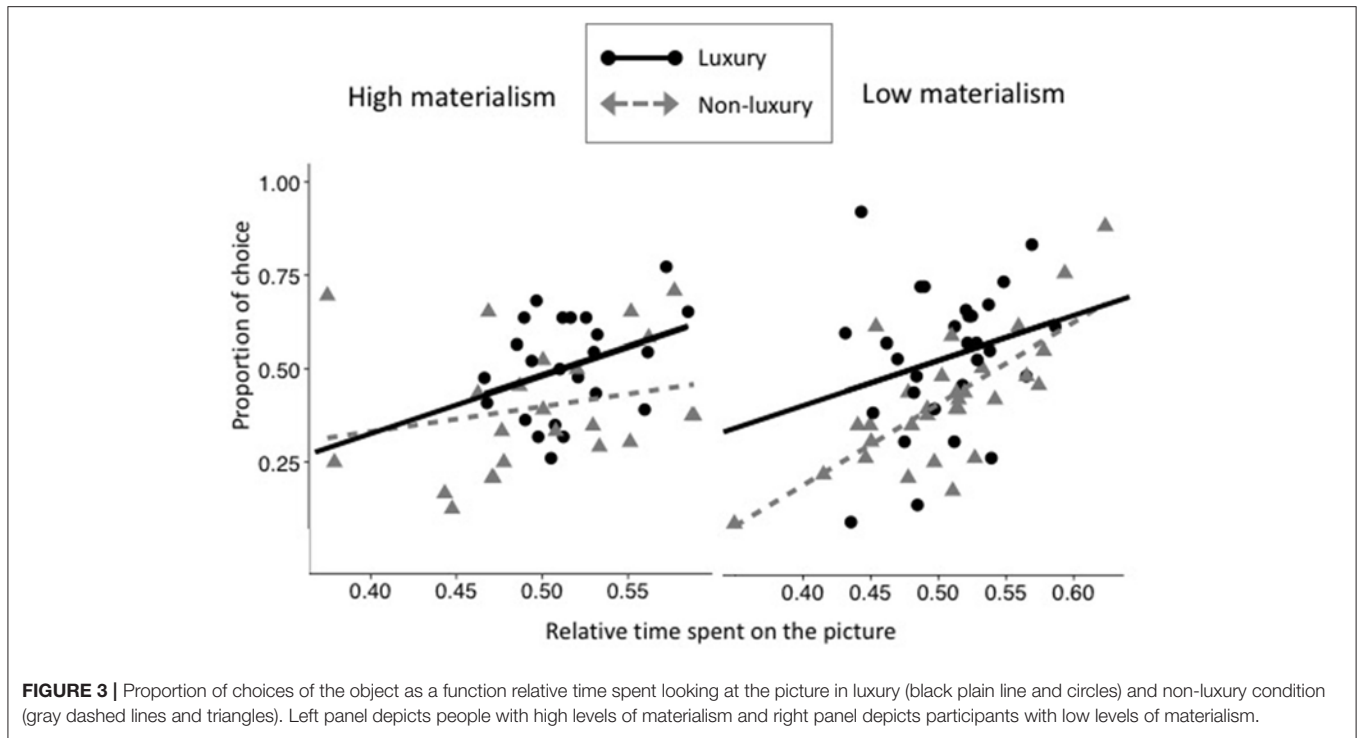


FIGURE 3 | Proportion of choices of the object as a function relative time spent looking at the picture in luxury (black plain line and circles) and non-luxury condition (gray dashed lines and triangles). Left panel depicts people with high levels of materialism and right panel depicts participants with low levels of materialism.

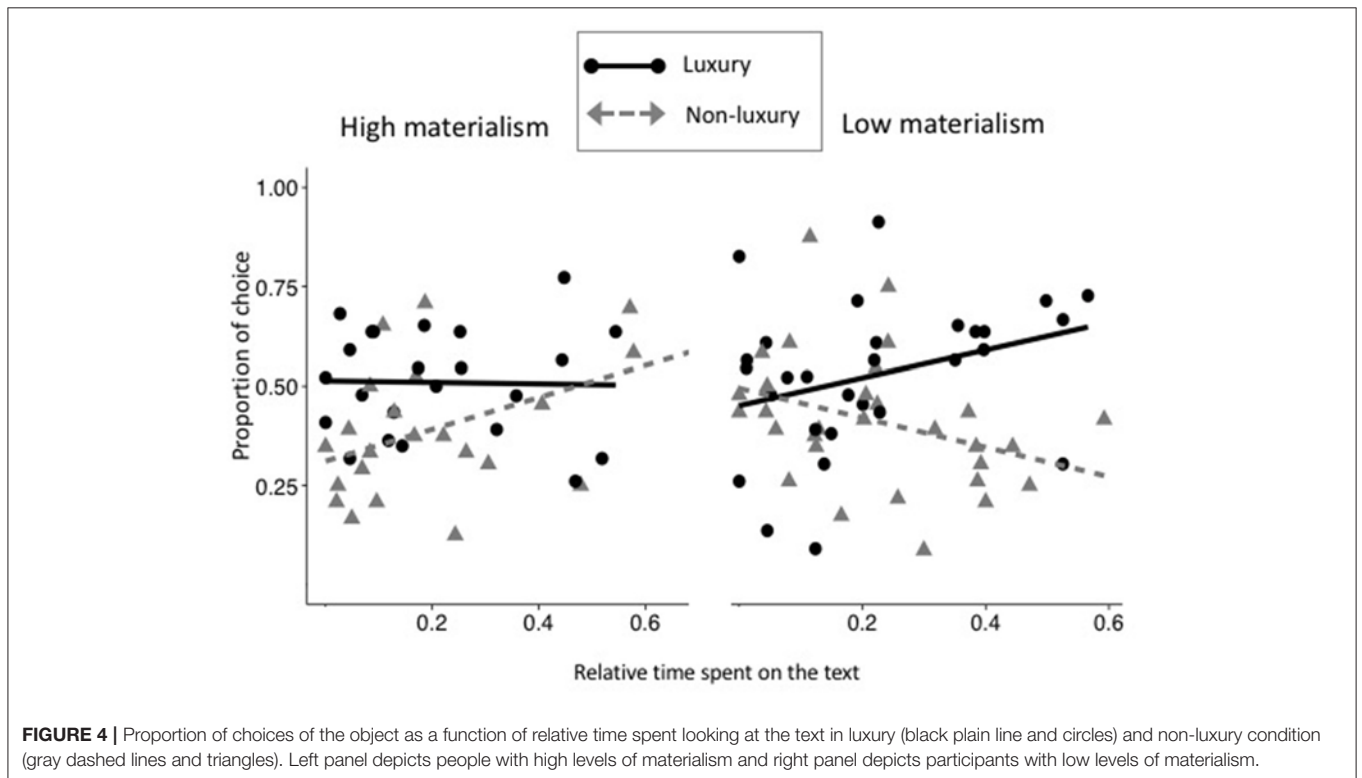


FIGURE 4 | Proportion of choices of the object as a function of relative time spent looking at the text in luxury (black plain line and circles) and non-luxury condition (gray dashed lines and triangles). Left panel depicts people with high levels of materialism and right panel depicts participants with low levels of materialism.

revealed that levels of materialism modulate the importance allocated to specific sources of information in the process of luxury decision-making: when only brand information was

available, materialism was related to enhanced preference for luxury. However, when supplementary information about product quality was available, all participants tended to

choose luxury more often. Interestingly, while people with high levels of materialism focused on the product picture and the brand, participants with low levels of materialism focused more on the text information about product quality when choosing luxury products. To summarize, our results suggest that materialism as a psychological value may impact how visual attention is directed toward specific sources of information in the context of luxury consumption.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

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AUTHOR CONTRIBUTIONS

CA, TB, DS, and JC contributed conception of the study. CA designed the study, organized the database, performed the statistical analysis, wrote the first draft of the manuscript. TB, DS, and JC contributed to manuscript revision, read and approved the submitted version.

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Current Status on the Functional Characterization of Chemosensory Receptors of *Cydia pomonella* (Lepidoptera: Tortricidae)

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Cydia pomonella (Lepidoptera: Tortricidae) is a major pest of apple, pear and walnuts. For its control, alternative strategies targeting the olfactory system, like mating disruption, have been combined with insecticide applications. The efficacy of these strategies headed the direction of efforts for the functional characterization of codling moth chemosensory receptors to implement further control methods based on chemical sensing. With the advent of transcriptomic analysis, partial and full-length coding sequences of chemosensory receptors have been identified in antennal transcriptomes of *C. pomonella*. Extension of partial coding sequences to full-length by polymerase chain reaction (PCR)-based techniques and heterologous expression in empty neurons of *Drosophila melanogaster* and in Human Embryonic Kidney cells allowed functional studies to investigate receptor activation and ligand binding modalities (deorphanization). Among different classes of antennal receptors, several odorant receptors of *C. pomonella* (CpomORs) have been characterized as binding kairomones (CpomOR3), pheromones (CpomOR6a) and compounds emitted by non-host plants (CpomOR19). Physiological and pharmacological studies of these receptors demonstrated their ionotropic properties, by forming functional channels with the co-receptor subunit of CpomOrco. Further investigations reported a novel insect transient receptor potential (TRPA5) expressed in antennae and other body parts of *C. pomonella* as a complex pattern of ribonucleic acid (RNA) splice-forms, with a possible involvement in sensing chemical stimuli and temperature. Investigation on chemosensory mechanisms in the codling moth has practical outcomes for the development of control strategies and it inspired novel trends to control this pest by integrating alternative methods to interfere with insect chemosensory communication.

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INTRODUCTION

The codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) is a major pest insect of commercial crops such as apple, pear and walnuts of Palearctic and Nearctic regions (Witzgall et al., 2008).

Integrated with insecticides, alternative methods are commonly used to control this insect (Star  et al., 2008; Odendaal et al., 2015; Arnault et al., 2016; Iraqui and Hmimina, 2016). Among these methods, mating disruption, which targets the olfactory system of *C. pomonella*

males through the use of female sex pheromones, demonstrated efficient results to limit crop infestation (Hathaway et al., 1974; Ridgway et al., 1990; Light et al., 2001; Light, 2016). Furthermore, odors emitted by host-plants (kairomones), are combined with pheromones to enhance male attraction for the codling moth (Knight and Light, 2001; Light et al., 2001; Witzgall et al., 2001, 2005; Yang et al., 2004).

In insects, odors such as pheromones and kairomones are detected by olfactory sensory neurons (OSNs) that innervate specialized sensilla on their antennae (Buck and Axel, 1991; Chess et al., 1992; Vosshall et al., 2000; Carlson, 2001; Kurtovic et al., 2007). On the dendritic membrane of OSNs, odorants and pheromones mostly bind a class of seven-transmembrane proteins known as odorant receptors (ORs; Clyne et al., 1999). Deciphering mechanisms of receptor/ligand interactions and understanding pharmacological, kinetic properties and activation modalities of OR proteins, unveil promising aspects to improve strategies for the control of pest insects (Jones et al., 2011; Pask et al., 2011, 2013; Röllecke et al., 2013; Bobkov et al., 2014). Identification of ligands for specific ORs (deorphanization) among odors emitted from females and plant-hosts of the codling moth facilitates our understanding of the neurobiological and behavioral aspects at the base of the chemical ecology of *C. pomonella*. This contributes to possible application of novel ligands for semiochemical-based control strategies.

This mini-review reports the state of the art of current findings on the functional characterization of codling moth ORs as well as findings of a novel transient receptor potential (TRP) channel expressed in the olfactory system of *C. pomonella*. This contribution introduces ongoing studies on the molecular aspects of chemical sensing of the codling moth and their possible application to current control strategies targeting the olfactory system of the insect.

IDENTIFICATION OF CHEMOSENSORY RECEPTORS OF *Cydia pomonella*

By means of a polymerase chain reaction (PCR)-based technique, the 3' end of gene transcripts encoding putative members of *C. pomonella* ORs (*CpomORs*) have been initially identified from total ribonucleic acid (RNA) samples extracted from antennae (Garczynski et al., 2012). In this study, a similar method described by Buck and Axel (1991) was used to design degenerate forward primers based on polypeptide sequence alignments of the C-terminus of 12 members of the pheromone receptor subfamily of *Bombyx mori* (Lepidoptera: Bombycidae) and *Heliothis virescens* (Lepidoptera: Noctuidae). Forward primers were used to amplify partial 3'-ends starting from retro-transcribed 3'-cDNA templates generated by SMARTTM kits (Clontech, Mountain View, CA, USA). Among amplified 3'-ends, the first set of *CpomORs* were identified. This method represented the first effort in the isolation of *CpomORs* from antennal RNA samples, leading optimization of further RACE-PCR approaches to amplify the full-length coding sequences of other chemosensory genes of this insect, aimed to address their phylogenetic and functional characterization.

With the advent of transcriptomic analysis, a wider investigation was conducted by the use of 454-next generation sequencings (NGS) of antennal RNA-samples (Bengtsson et al., 2012). For the first time, a wide asset of assembled fragments of gene coding sequences was identified, revealing 14 candidate ionotropic receptors (IRs), one candidate gustatory receptor (GR) and 43 candidate ORs. Among these, five ORs were members of the putative pheromone receptors (PRs) subfamily: a monophyletic clade in Lepidopteran insect OR phylogeny, with receptors that predominantly respond to odors emitted by females (Jacquin-Joly and Merlin, 2004; Ihara et al., 2013; Leal, 2013). Among the five candidate PRs reported by Bengtsson et al. (2012), two PRs represented some of the same ORs identified in the previous work of Garczynski et al. (2012). With the aim to complement these studies, using Illumina-based RNA-sequencing, assembly of a transcriptome from male, female and larval olfactory tissues of the codling moth, a more complete list of chemosensory receptors of *C. pomonella* was updated to 21 IRs, 20 GRs and 58 putative ORs, among which, 11 represented members of the PR-clade (Walker et al., 2016; **Table 1**). Identification of IRs and GRs in antennal transcriptomes of the codling moth was in accordance with the reported findings of their functional importance in insect chemosensation (Clyne et al., 2000; Robertson et al., 2003; Benton et al., 2009; Montell, 2009; Ai et al., 2010; Silbering et al., 2011; Rytz et al., 2013; Missbach et al., 2014; Sanchez-Alcaniz et al., 2018). Despite their importance, most of the efforts to functionally characterize chemosensory receptors of the codling moth targeted ORs, with particular focus on members of the PR-subfamily.

FUNCTIONAL CHARACTERIZATION OF *CpomOR3*

CpomOR3 represents the first OR of the codling moth that was isolated, heterologously expressed and functionally characterized (Bengtsson et al., 2014). Expression of this receptor was conducted in *Drosophila melanogaster* ab3A (Dobritsa et al., 2003; Gonzalez et al., 2016) and aT1 (Kurtovic et al., 2007; Montagné et al., 2012) empty neurons, to screen a panel of ligands among pheromones, synergists and antagonists known for their activation of the olfactory system of *C. pomonella*. Activation of *CpomOR3* was demonstrated for the plant volatile ethyl-E,Z-2,4-decadienoate, commonly known as pear ester (Jennings et al., 1964; Berger and Drawert, 1984; Light et al., 2001; Yang et al., 2004; Knight et al., 2005; Willner et al., 2013). Phylogenetic analysis demonstrated *CpomOR3* to be a PR-candidate. Activation of a putative PR to the synergist pear ester was in accordance with neurological effects identified when this compound was tested with the primary sex pheromone component of the codling moth (codlemone) on AL-glomeruli of the insect (Trona et al., 2010, 2013). *CpomOR3* response to pear ester gave further support to the role of this compound as a kairomone, already known to enhance male attraction in orchards when combined with female pheromones (Light et al., 2001; Yang et al., 2004). These findings suggest a possible role of

TABLE 1 | Updated list of *Cydia pomonella* odorant receptors (CpomORs) in comparison among results from Garczynski et al. (2012); Bengtsson et al. (2012) and Walker et al. (2016), based on their techniques (brackets).

Walker et al. (2016) (<i>illumina</i>)	Bengtsson et al. (2012) (454)	Garczynski et al. (2012) (<i>RACE-PCR</i>)	Status	Clade
CpomOrco	CpomOR2	-	Complete ^a	Co-receptor
CpomOR1	CpomOR4	CpomOR11	Complete ^{a,b}	PR ^M
CpomOR2a	CpomOR5	CpomOR1a	Incomplete ^b	PR
CpomOR2b		CpomOR1a		
CpomOR2c		CpomOR11a		
CpomOR3	CpomOR3	-	Deorphanized^{a,b;1,2}	PR
CpomOR4	CpomOR6	CpomOR4	Complete	PR
CpomOR5	-	-	Complete ^b	PR ^M
CpomOR6a	CpomOR1	-	Deorphanized^{a,b;2}	PR ^M
CpomOR6b	-	-	Complete ^b	
CpomOR7	-	-	Complete	PR ^M
CpomOR8	-	-	Complete	PR
CpomOR9	-	-	Incomplete	PR
CpomOR10	CpomOR28	-	Complete ^b	OR
CpomOR11	CpomOR11	-	Incomplete	OR ^L
CpomOR12	-	-	Incomplete	OR
CpomOR13	CpomOR8	-	Complete	OR
CpomOR14	CpomOR14	-	Complete	OR
CpomOR15	CpomOR20	-	Complete	OR
CpomOR16	-	-	Complete	OR
CpomOR18	CpomOR10	-	Complete	OR ^L
CpomOR19	CpomOR19	-	Deorphanized^{b;3}	OR
CpomOR20	CpomOR18	-	Complete	OR
CpomOR21	-	-	Incomplete	PR ^F
CpomOR22	CpomOR15	-	Complete ^b	PR ^F
CpomOR25	CpomOR21	-	Complete	OR
CpomOR26	-	-	Complete	OR
CpomOR27	CpomOR27	-	Complete	OR
	CpomOR29			
CpomOR28	CpomOR26	-	Complete ^b	OR
CpomOR29	-	-	Complete	OR
CpomOR30	CpomOR30	-	Complete	OR ^F
CpomOR31	-	-	Complete	OR ^M
CpomOR32	-	-	Complete	OR
CpomOR35	CpomOR35	-	Complete	OR
CpomOR37	CpomOR36	-	Complete	OR
	CpomOR39			
CpomOR38	-	-	Incomplete	OR
CpomOR39	CpomOR38	-	Complete ^b	OR
CpomOR40	CpomOR33	-	Complete	OR
CpomOR41	-	-	Complete	OR ^F
CpomOR42	-	-	Complete	OR
CpomOR44	-	-	Complete	OR
CpomOR46	CpomOR16	-	Complete	OR
CpomOR47	-	-	Complete	OR
CpomOR49	-	-	Complete	OR
CpomOR53	CpomOR9	-	Complete	OR
CpomOR54	CpomOR7	-	Complete	OR
	CpomOR41			
CpomOR56	CpomOR37	-	Complete	OR
CpomOR57	CpomOR31	-	Complete	OR
CpomOR58	CpomOR34	-	Complete	OR
CpomOR59	CpomOR12	-	Complete	OR
CpomOR60	-	-	Complete	OR
CpomOR61	CpomOR17	-	Complete	OR
CpomOR62	-	-	Complete	OR
CpomOR63	CpomOR23	-	Complete	OR
CpomOR64	CpomOR24	-	Complete	OR ^L
CpomOR65	CpomOR22	-	Complete	OR
CpomOR66	CpomOR32	-	Incomplete	OR
CpomOR67	-	-	Complete	OR
CpomOR68	-	-	Complete	OR

(Continued)

TABLE 1 | (Continued)

Walker et al. (2016) (<i>Illumina</i>)	Bengtsson et al. (2012) (454)	Garczynski et al. (2012) (<i>RACE-PCR</i>)	Status	Clade
CpomOR71	-	-	Complete ^b	OR ^L
CpomOR72	CpomOR40	-	Complete	OR
Not found	CpomOR13	-	-	-
Not coding	CpomOR43	-	-	-
Not coding	CpomOR44	-	-	-

^MReceptors with male-bias expression; ^Freceptors with female-bias expression; ^Lreceptors with larval-bias expression (Walker et al., 2016). ^aReceptors heterologously expressed in Human Embryonic Kidney (HEK293T); ^breceptors heterologously expressed in *Drosophila* empty neurons (Figure 1). Deorphanized receptors are indicated with numbers based on published data: ¹Bengtsson et al. (2014); ²Cattaneo et al. (2017b); ³Gonzalez et al. (2015). Accession to the updated dataset of CpomORs is available in Walker et al. (2016).

kairomones in the mate-choice behavior and in the reproductive isolation of tortricids (Trona et al., 2013; Bengtsson et al., 2014).

To better elucidate mechanisms involving pear ester sensing for CpomOR3, functional characterization experiments based on *Drosophila* empty neurons have been implemented through the heterologous expression of this receptor in Human Embryonic Kidney (HEK293T) cells (Cattaneo et al., 2017b). The use of an *in vitro* method represented an alternative to the common approaches for the functional characterization of insect ORs based on *Drosophila* empty neurons. The choice of this method was supported by successful attempts on the expression of PR-candidates from moths belonging to Bombycidae (Grosse-Wilde et al., 2006), Noctuidae (Grosse-Wilde et al., 2007), Saturniidae (Forstner et al., 2009) and Tortricidae (Steinwender et al., 2015). Comparison of heterologous expression between *Drosophila* OSNs and HEK293T is shown in Figure 1.

In search of other possible ligands for CpomOR3, screening of a compound library on HEK293T cells validated activation of the receptor to both pear ester and the analogous methyl-(E, Z)-2,4-decadienoate. Sensing of an analogous methyl-ester for the codling moth was reported for the first time by demonstrating larval attraction from emissions of ripe Bartlett pear (Knight and Light, 2001), although origins of methyl ester as a plant-emitted odorant are still debated. Indeed, aside from emission by Bartlett pear, methyl ester was found in the head, thoraxes and fecal pellets of the bark beetle *Pityogenes chalcographus* (Coleoptera: Curculionidae; Birgersson et al., 1990). In addition, methyl ester was also found in emissions from stink bugs of the genus *Euschistus* (Heteroptera: Pentatomidae; Aldrich et al., 1991; Tognon et al., 2016).

A remaining question is if interaction of the analogous methyl ester with the same receptor of ethyl-(E,Z)-2,4-decadienoate may result in a similar effect in the antennal lobe as an evidence of its synergism with codlemone (Trona et al., 2010, 2013).

FUNCTIONAL CHARACTERIZATION OF CpomOR6a

Heterologous expression of codling moth receptors in HEK293T cells also deorphanized the PR candidate CpomOR6a as responsive to (E, E)-8-12-dodecadien-1-yl acetate (Codlemone acetate; Cattaneo et al., 2017b). Combining heterologous expression in *Drosophila* aT1, (E,Z)- and (Z,Z)-geometric isomers of codlemone acetate were also identified as partial

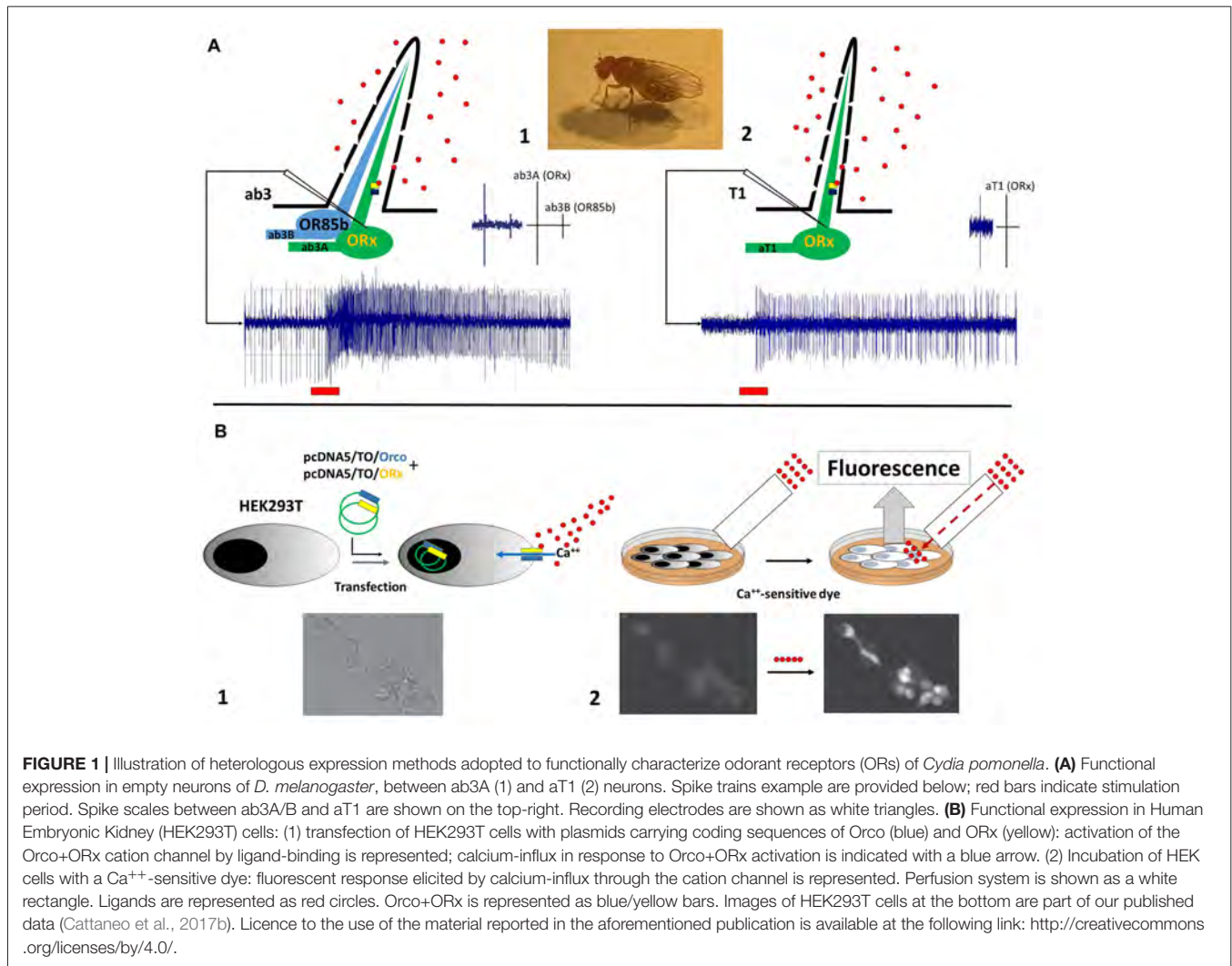
ligands of the receptor. Together with these ligands, CpomOR6a sensed (E)-10-dodecadien-1-yl acetate and, although with less specificity, (Z,E)-8-12-dodecadien-1-yl acetate.

Codlemone acetates are main pheromone components emitted by female moths closely related to *C. pomonella* (Frerot et al., 1979; Roelofs and Brown, 1982; Davis et al., 1984; Witzgall et al., 1996; Chambers et al., 2011). Although receptors of codlemone acetates of these species have not been isolated and deorphanized yet, overall sequence similarities and relatively high expression in *C. nigricana* and *Hedya nubiferana*, suggested the gene locus OR6 to express a conserved receptor between *C. pomonella* and these tortricid species (Gonzalez et al., 2017). While speculative, a possible explanation of the existence of the codlemone acetate receptor in *C. pomonella* may be as a remnant of the former ancestor of the insect. However, conserving a receptor dedicated to detect other species may be important for reproductive isolation of the codling moth. Otherwise, since moths emitting codlemone acetates share the same host range with *C. pomonella*, detection of codlemone acetates may facilitate host finding for the codling moth. The evolution of a receptor specialized for the detection of a main pheromone compound, like codlemone, may likely represent a step towards allopatric speciation of *C. pomonella*.

Among candidate PRs of the codling moth (Table 1), the most likely sensor for codlemone is CpomOR1 given its abundant expression in OSNs of male moths (Bengtsson et al., 2014; Walker et al., 2016). Although heterologous expression methods in HEK cells and *Drosophila* empty neurons were unable to demonstrate CpomOR1 responsiveness to codlemone (Cattaneo et al., 2017b), future deorphanization attempts will unveil if this prediction holds true. Another remaining question is whether the transcript variant CpomOR6b has the same response spectrum as CpomOR6a and what might be its relevance, especially considering the lack of knowledge on alternative splicing in lepidopteran PRs (Garczynski and Leal, 2015).

FUNCTIONAL CHARACTERIZATION OF CpomOR19

Although CpomOR19 is not a PR-candidate in *C. pomonella*, testing heterologous expression of this receptor in *Drosophila* ab3As is part of documented deorphanizations of chemosensory receptors of the codling moth (Gonzalez et al., 2015).



CpomOR19 is responsive to 1-indanone; several analogs of this compound (2-methyl-1-indanone, 2-ethyl-1-indanone and 3-methyl-1-indanone) elicit responses of different sensitivities by this receptor. These compounds are renowned for their “non-host” origins (Klein et al., 1990; Anderson et al., 1993; Nagle et al., 2000; Okpekon et al., 2009; Rukachaisirikul et al., 2013). CpomOR19 binding to indanes represented the first deorphanization of a receptor of the codling moth to compounds emitted by non-hosts. Interestingly, different sensitivities between 1-indanone and its analogs for the CpomOR19 binding is consistent with observations reported between pear and methyl esters on CpomOR3, where one carbon of the alkyl group may determine different binding affinity, perhaps due to differences in the polarity of the compounds (Cattaneo et al., 2017b).

By use of the same method, activation of the ortholog SlitOR19 of the African cotton-leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) demonstrated conservation in binding 1-indanone and analogous. When compared, ab3A expressing CpomOR19 and SlitOR19 showed increased response to indanes,

when substituted with alkyl groups at position two and three of the five-membered ring. On the contrary, indanes provided with methyl substituents on the benzene ring largely did not activate these receptors. Furthermore, indanes provided with alcohols, hydrocarbons and amine groups also did not activate any of the two receptors, which suggested a conserved function for CpomOR19 and SlitOR19 orthologs, despite the phylogenetical and ecological distance of their respective moths. A recent report on *Spodoptera* ORs provides a blueprint for prediction of SlitOR ligands based on the interaction of phylogeny and chemical structure (de Fouchier et al., 2017). Given evidences of conserved function between CpomOR19 and SlitOR19, prediction of SlitOR ligands may benefit future studies on deorphanization of CpomORs.

PHYSIOLOGICAL PROPERTIES OF CpomORs

Expression of CpomOR genes in HEK cells was undertaken by co-transfecting the CpomOrco co-receptor subunit of the

codling moth (**Figure 1B**). Functional studies of CpomOrco demonstrated heteromeric complexes of the co-receptor with OR subunits being more sensitive than homomeric co-receptor complexes, as previously demonstrated for Orco-based channels of other insects (Jones et al., 2011; Pask et al., 2011; Kumar et al., 2013; Turner et al., 2014). By the use of the main ligand VUAA (Jones et al., 2011), calcium response was characterized by faster activation/deactivation kinetics for CpomORco+OR than CpomORco alone. Testing inhibitors like amiloride derivatives (ADs; Pask et al., 2013; Röllecke et al., 2013) demonstrated similar effects for both homomeric and heteromeric complexes. When HEK cells were tested by whole-cell and outside-out patch-clamp recordings, activation of CpomOrco+OR complexes resembled modalities of ligand-gated cation channels: responses to multiple stimulations were characterized by constant amplitudes and stable kinetic parameters, which is indicative of the ionotropic nature of insect OR receptors (Sato et al., 2008).

Despite that the molecular mechanisms at the base of signal transduction of insect olfactory systems still remain unknown (Krieger and Breer, 1999; Jacquín-Joly and Merlin, 2004; Sakurai et al., 2014), results on the functional characterization of *C. pomonella* ORs are consistent with the idea that all insect or, perhaps, even all arthropod chemosensory receptor channels (among ORs and IRs) can be characterized by somewhat common pharmacology (Bobkov and Ache, 2007; Abuin et al., 2011; Bobkov et al., 2014). Although, this might be called into question given some evidence pointing towards metabotropic signaling modalities for insect ORs (Sargsyan et al., 2011; Getahun et al., 2013; Ignatious Raja et al., 2014).

TRANSIENT RECEPTOR POTENTIAL CHANNELS OF *Cydia pomonella*

A second analysis of sequencing data from Bengtsson et al. (2012), unveiled further transcripts related with ligand-gated cation channels belonging to the class of TRP. In several organisms, TRP-channels enable sensing of multiple stimuli from the environment (Liedtke and Heller, 2007). Among chemical stimuli, several compounds commonly found in food plants and spices are reported to activate TRPs (Caterina et al., 1997; Jordt et al., 2004; Xu et al., 2006; Bautista et al., 2007). Interestingly, TRP-active compounds are reported for their ability to repel insects (Leung and Foster, 1996; Barnard, 1999) and, in particular, to activate the olfactory system of tortricid and noctuid moths (Cattaneo et al., 2014; Wei et al., 2015).

In *C. pomonella*, five TRPs have been found in the antennae belonging to the TRPC (TRP, TRPC) and the TRPA subfamily (Pyrexia, water witch, TRPA5; Cattaneo et al., 2016). Up to now, *CpomTRPA5* is the only TRP of the codling moth that has been extended to the full length. Interestingly, five variants of the spliced-coding sequence have been found, demonstrating different expression patterns among body parts of the codling moth. Analysis of the *CpomTRPA5* mRNA sequence demonstrated the transcript undergoing to mRNA editing by insertion of 15 additional nucleotides within the third exon of the full-length sequence, which is a mechanism

occurring for K⁺ channels of multiple organisms, including insects (Holmgren and Rosenthal, 2015). Evolutionary studies suggested the relatedness of *TRPA5* gene to the thermal sensor *Pyrexia* (Peng et al., 2015), which has also been described as a thermal-gated K⁺-channel of insect (Lee et al., 2005).

Identification of TRPs in *C. pomonella* represented the first documented finding within this species for this particular class of chemoreceptors. Identification of *CpomTRPA5* and its spliceforms is among the first documented existences of this particular subunit for arthropod TRPAs (Peng et al., 2015). Relatedness of *CpomTRPA5* with *Pyrexia* suggested a possible role of the CpomTRPA5 receptor as a thermal sensor, which is consistent with behavioral evidences for the codling moth of odor-guided responses in relation with temperature (Witzgall et al., 1999).

By means of methods adopted to test activation of mammalian TRPAs expressed in HEK cells (Bassoli et al., 2009, 2013; Cattaneo et al., 2017a), functional characterization studies of CpomTRPA5 may be conducted to better elucidate possible roles of this receptor in chemical and physical sensing modalities of the codling moth.

FUTURE PERSPECTIVES

The technologies adapted to the setup of transcriptomic and heterologous expression studies for the functional characterization of chemosensory receptors of *C. pomonella*, may offer new opportunities to address longstanding questions in the field of insect ecology, with a practical outcome for the implementation of its control strategies.

Two out of the three codling moth ORs that have been deorphanized, belong to the clade of putative Pheromone Receptors. Although attempted, the receptor for the main pheromone codlemone has not been functionally characterized. To validate a possible role of CpomOR1 as a main candidate sensor (Bengtsson et al., 2012; Walker et al., 2016; Cattaneo et al., 2017b), future experiments will verify if co-expression of CpomOR1 with CpomOR6a in *Drosophila* aT1 neurons is sensitive to codlemone. This approach is supported by evidences of response to codlemone acetates from OSNs of *C. pomonella* responding to codlemone (Bäckman et al., 2000), which may suggest a possible role of the CpomOR6a subunit to sense this pheromone. In addition, studies on several insects demonstrated co-expression of different OR subunits in the same OSN (Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldman et al., 2005; Ray et al., 2007; Koutroumpa et al., 2014; Karner et al., 2015; Lebreton et al., 2017), and stoichiometry of OR heteromers is still debated (Larsson et al., 2004; Benton et al., 2006; Wicher, 2018).

In support of the control of the codling moth with mating disruption, novel trends are leading the direction of studies to integrate targeting of sensing modalities of codling moth females. Indeed, methods based on mating disruption demonstrated inefficacy to the control of the codling moth at high population in the orchards, as well as on the top of tree branches, where the pheromone cloud is limited (Witzgall et al., 1999).

Identification of CpomORs with a female-biased expression (Bengtsson et al., 2012; Walker et al., 2016) motivates the use of heterologous methods to address their functional characterization (Swedish Research Council Formas, Project Reg. No. 2016-01281 “Control of Apple Pest Insects with Fruit and Yeast Odorants”). This approach may identify novel ligands active on female olfactory systems. Among these ligands, odors emitted by fruits and their associated microbes may be tested, given the importance of yeasts for attractiveness of egg-laying females (Witzgall et al., 2012).

Recent studies based on CRISPR/Cas9 editing of the codling moth, demonstrated efficacy of this method to address knockdown of functional OR proteins, which resulted in affection of fecundity and fertility, with edited females producing nonviable eggs (Garczynski et al., 2017). Future targets may combine heterologous expression methods, with the use of CRISPR/Cas9 to generate OR-edited insects, as a complementary approach to address the functional characterization of codling moth receptors.

Future trends integrating research on the olfactory system of *C. pomonella* may target larval chemical sensing as complementary to the current approaches addressing the functional characterization of adult ORs (Formas Mobility Starting Grant Reg. No. 2018-00891 “Control of Fruit Pests by Targeting Larval Chemical Sensing,” submitted). Indeed, chemosensory mechanisms at the base of larval behavior are long renowned for the codling moth (Knight and Light, 2001; Jumean et al., 2005) and expression of CpomORs with a larval-bias has been reported (Walker et al., 2016).

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- Broader discoveries on the molecular bases of the olfactory mechanisms of *C. pomonella* will enhance current control strategies interfering with the insect’s chemosensory communication. Development of novel methods targeting olfaction may help limit the use of insecticides, with beneficial effects on the quality of life for apple growers, consumers, as well as public living around the orchard areas, reducing further the conflict between agricultural and urban worlds.

AUTHOR CONTRIBUTIONS

AMC wrote the manuscript.

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Using Temporal Expectation to Assess Auditory Streaming in Mice

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Auditory streaming is the process by which environmental sound is segregated into discrete perceptual objects. The auditory system has a remarkable capability in this regard as revealed in psychophysical experiments in humans and other primates. However, little is known about the underlying neuronal mechanisms, in part because of the lack of suitable behavioural paradigms in non-primate species. The mouse is an increasingly popular model for studying the neural mechanisms of perception and action because of the range of molecular tools enabling precise manipulation of neural circuitry. Here we present a novel behavioural task that can be used to assess perceptual aspects of auditory streaming in head-fixed mice. Animals were trained to detect a target sound in a one of two simultaneously presented, isochronous pure tone sequences. Temporal expectation was manipulated by presenting the target sound in a particular stream either early (~2 s) or late (~4 s) with respect to trial onset in blocks of 25–30 trials. Animals reached high performance on this task ($d' > 1$ overall), and notably their false alarms were very instructive of their behavioural state. Indeed, false alarm timing was markedly delayed for late blocks compared to early ones, indicating that the animals associated a different context to an otherwise identical stimulus. More finely, we observed that the false alarms were timed to the onset of the sounds present in the target stream. This suggests that the animals could selectively follow the target stream despite the presence of a distractor stream. Extracellular electrophysiological recordings during the task revealed that sound processing is flexibly modulated in a manner consistent with the optimisation of behavioural outcome. Together, these results indicate that the perceptual streaming can be inferred via the timing of false alarms in mice, and provide a new paradigm with which to investigate neuronal mechanisms of selective attention.

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1. INTRODUCTION

In an acoustic scene comprising multiple auditory sources, humans and animals can readily identify and track a relevant sound source amongst the background noise, and switch from tracking that source to follow another (Fritz et al., 2007; Shamma et al., 2011). Such ease however belies the complexity of the underlying processes: the auditory system must parse sounds from various sources into discrete perceptual objects (a process known as “auditory stream segregation”; Bregman, 1994), and may further enhance the representation of relevant inputs to best support behavioural needs (Crick, 1984; Fritz et al., 2007; Shamma et al., 2011). Switching attention from

one source to another has been shown to modulate sensory signal representation at various levels of the auditory pathway, notably in the auditory cortex (AC) (Fritz et al., 2003, 2007; Mesgarani and Chang, 2012; Lakatos et al., 2013; Rodgers and DeWeese, 2014). However, little is known about the underlying mechanisms. This may be due to the complex and numerous neural pathways involved in modulating the activity in AC during attentional processes (Froemke et al., 2012; Pinto et al., 2013; Nelson and Mooney, 2016; Winkowski et al., 2017). To understand the contribution of each pathway, it is necessary to develop experimental paradigms enabling their specific manipulation.

The mouse is an attractive experimental model because of the wealth of genetic and molecular approaches that are available to manipulate anatomically defined neural circuits (Havekes and Abel, 2009; Garner and Mayford, 2012; Harris et al., 2014; Park and Carmel, 2016). Moreover, mice rely on auditory signals for a wide range of behaviourally relevant tasks (Pereira et al., 2012; Konopka and Roberts, 2016; Itatani and Klump, 2017), and are capable of flexible behaviour (Bissonette and Powell, 2012; Jaramillo and Zador, 2014; Hamilton and Brigman, 2015). However, no behavioural paradigm exists yet in mice to study the effect of selective attention on auditory streaming. Current paradigms of auditory selective attention in mice (Ahrens et al., 2014; Rodgers and DeWeese, 2014; Wimmer et al., 2015) mostly rely on briefly presented stimuli, which do not allow for auditory stream formation (Bregman, 1994; Moore and Gockel, 2012). In contrast, behavioural tasks are far more extensively developed in primates (Lakatos et al., 2013; Calderone et al., 2014), owing to their greater capacity to learn complex rules (Bissonette and Powell, 2012). Translating behavioural tasks from primates to mice would enable deeper insight of the neural mechanisms underlying auditory processing and perception.

Here, we present a novel behavioural task inspired from primate models (Lakatos et al., 2013), that can be used to assess the effect of selective attention on auditory streaming in head-fixed mice. By introducing biases in the acoustic stimuli and studying the timing of mice behavioural decisions, we were able to assess whether the animals switched from listening to one sound source to another within an auditory mixture in single behavioural sessions. Our paradigm is advantageous as it enables the quantification of attentional state upon electrophysiological signals acquired acutely. By recording neural activity in the AC of mice during ongoing behaviour, we reveal that sound processing is flexibly modulated in a manner consistent with the optimisation of behavioural outcome. Future use of this behavioural paradigm combined with molecular tools to manipulate neural circuits would offer great insight on the underlying basis of auditory selective attention and streaming.

2. RESULTS

2.1. Design and Validation of a New Auditory Task Involving Selective Attention and Auditory Streaming

We developed a Go/No-Go auditory selective attention paradigm in mice, modelled after a related study in primates (Lakatos et al.,

2013). Two auditory streams were simultaneously presented to the subject (**Figure 1A**); one stream was composed of high frequency tones each separated by short time intervals (High stream), and the other stream was composed of low frequency tones separated by longer time intervals (Low stream). Mice were rewarded for correctly detecting a target frequency-modulated sound embedded within one of the two streams (**Figure 1B**). We guided the subject toward attending to one specific stream by fixing the target features for blocks of consecutive trials. During the first trials of a block, only the target of a given stream was presented (target only condition; S0), then the single stream associated with that target was also presented (single stream condition; S1), and finally both streams were simultaneously presented (dual stream condition; S2) (**Figure 1C**). The target alone (S0) and single stream (S1) conditions were intended as cues indicating upcoming target features in the dual stream (S2) condition. We ensured that the animals could switch attentional state during single behavioural sessions by using blocks of ~40 trials, and by presenting the different blocks in strictly alternating fashion.

As we used a rather small number of trial in each block compared to previous studies in rodents (Jaramillo and Zador, 2010, 2014), it was critical to ensure that our subjects could identify the change in target feature probability. Because rodents are known to be particularly sensitive to temporal biases (Buhusi et al., 2009; Jaramillo and Zador, 2010; Tosun et al., 2016), we differentiated the target timing for each of the two streams. Specifically, high frequency targets were presented early (~2 s from trial onset; Early trial block), and low frequency targets were presented late (~6 s from trial onset; Late trial block). Any difference in behavioural response timing observed across blocks would indicate that mice were sensitive to the block design despite the small number of trial used per block. It is important to highlight that in the dual stream (S2) condition, the stimuli presented before the targets were identical in both block types, as displayed in **Figure 1A**. Mice achieved high performance in this target detection task (**Figure 1D** and **Supplementary Figures 1,2**; $d' > 1$ for both blocks, S2 condition). Furthermore, they were sensitive to the trial block design, as revealed by the change in their false alarm (FA) response pattern between Early and Late blocks (**Figure 2**). Notably, FA in the Late block were significantly delayed compared to those in the Early block (**Figure 2B**; FA response time: Early block = $1,358 \pm 57$ ms, Late block = $3,720 \pm 633$ ms, median \pm median absolute deviation; $p < 0.001$, WSR test, $N = 25$ sessions in 4 mice, only FA in S1 and S2 were used).

Having shown that mice timed their behavioural responses according to known target temporal biases (i.e., Early or Late), we explored whether mice specifically attended to the cued auditory stream. To answer this question, we analysed FA response patterns more finely. Notably, FA responses appeared aligned to tone onsets in the single stream (S1) but not in the target-only (S0) condition (**Figure 3A**). We formally quantified the locking of FA to tone onset by measuring the vector strength (VS) of the FA reaction time (RT) distribution in both conditions (**Figure 3B**). For all mice, the VS was lower in the target alone (S0) condition compared with the single stream (S1) condition (**Figures 3C,D**; $VS = 0.46 \pm 0.06$ in

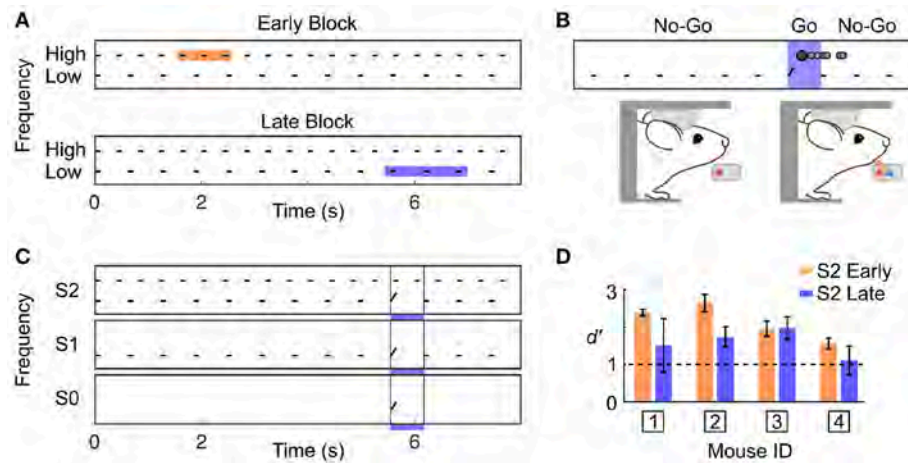


FIGURE 1 | Complex discrimination task involving auditory selective attention. **(A)** The stimulus consisted of two trains of 100 ms pure tone; one with High frequency tones separated by 300 ms, and one with Low frequency tones separated by 517 ms. A frequency-modulated target could occur at three positions with equal probability in a trial (shown by the colored squares). High frequency targets were presented Early, and Low frequency targets were presented Late. **(B)** Water-restricted mice were head-fixed onto an apparatus enabling the detection of licking events. The first lick detected in a trial was counted as the response (represented as the darker filled dot on top of the stimulus trace). The mouse was required to not lick before the occurrence of a target. If the mouse responded in the target window, a drop of water was administered (Hit). **(C)** Target timing and frequency content was biased using a trial block design. A block was composed of three trial types: target only (S0), single stream (S1), and dual stream (S2). Once the S2 condition was reached, mice had to perform 20–25 correct trials for the stimulus to switch to the other block type. **(D)** Averaged d' over the last 3 training sessions prior to electrophysiological recording for each mouse in the S2 Early and Late condition. d' -values above 1 indicate good performance.

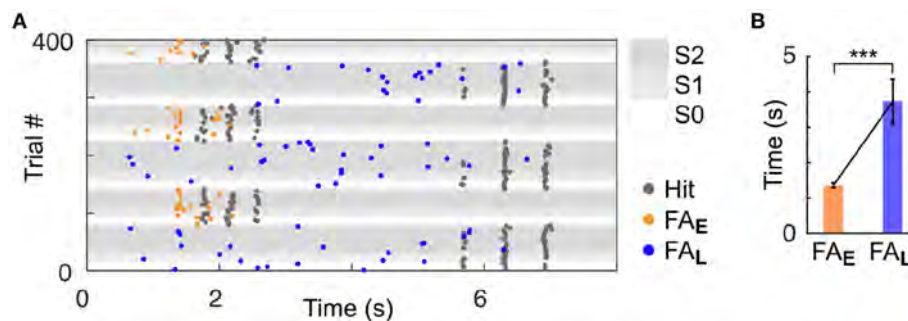


FIGURE 2 | Mice were sensitive to temporal bias as revealed by their false alarm timing. **(A)** Example session of a mouse (ID 3). Each dot represents a response (Hit or FA). FA are color-coded based on the block type (Early or Late). **(B)** Significant increase in the median of FA time (measured from trial onset) in the Late vs. Early condition. Error bars represent median absolute deviation. $***P < 0.001$.

S1 condition, $VS = 0.07 \pm 0.01$ in S0 condition; $p < 0.01$, WSR test). Moreover, significant VS was only found in the single stream (S1) condition (**Figure 3C**, starred distributions), indicating that mice did not use the trial onset to time their response with such specificity. This result confirmed that FA were not randomly executed, but instead timed to stream tone onsets.

Based on this finding, we hypothesized that mice sensitive to the target spectral bias (i.e., High or Low) may selectively attempt to respond to tones of the cued stream rather than in the non-cued stream, a strategy that increases the chance of responding correctly. To test this hypothesis, we reported the FA in the dual stream (S2) condition to the nearest High and Low tone onset, in cases where either the High stream

(Early block) or Low stream (Late block) was cued (**Figure 4**). The difference between RT distributions indicated whether the subject adapted its behaviour according to the spectral probability of the target. We observed considerable heterogeneity in behavioural response strategies of the individual mice: one animal executed FAs mainly in response to tones in the High stream, two animals executed FAs mainly to the Low stream, and one animal (# 2; **Figure 4**) timed its FAs to the cued stream. These results indicate that mice can be trained on this selective attention paradigm, and that different animals employ different behavioural strategies under the same task conditions. Assessment of FA timing patterns to high and low streams can be used to establish which behavioural strategy is being followed.

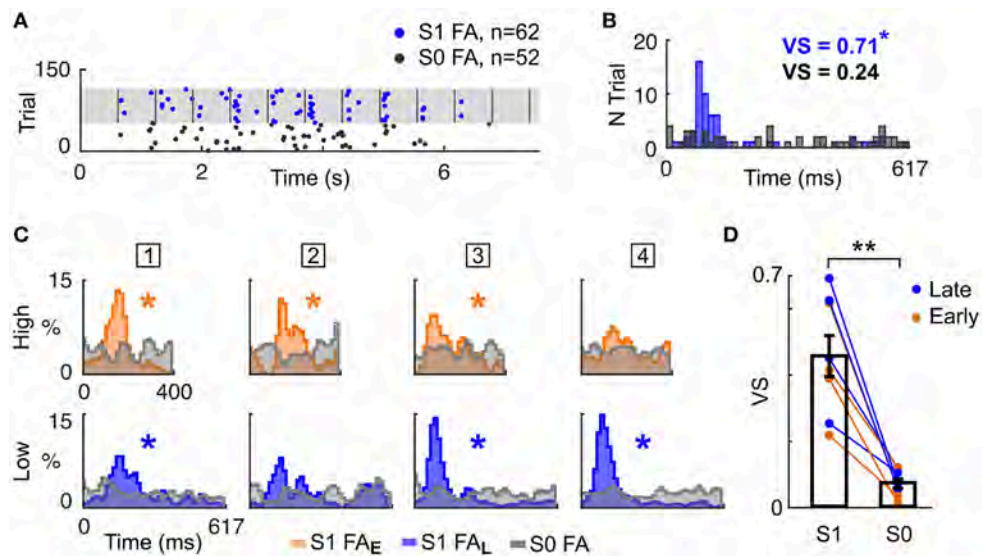


FIGURE 3 | Mice timed their false alarm responses to tone onsets. **(A)** Example FA during the Late block in a single session. Each dot represents a FA response, in the S0 (grey) or S1 (blue) condition. Vertical grey lines indicate sound onsets in S1 condition. **(B)** FA reaction time (RT) distributions for S0 and S1 conditions (same colors as in **A**). The Vector Strength (VS) indicates the peak strength of the distribution, and is reported in the inset ($*p < 0.05$). **(C)** Normalised RT distribution for FA in S1 conditions for each mice (one mouse per column, the mouse ID is presented in the square box) computed using all sessions. RT are measured either from High (top) or Low (bottom) tone onsets. The distributions are color-coded based on the block type (Early or Late). The RT distribution of a mouse generating FA timed to sound onsets presents a peak, as assessed by the VS ($*p < 0.05$). RT distributions are smoothed (1 bin std Gaussian filter) for display. **(D)** The VS was significantly higher in the S1 than S0 condition. Each line indicates the VS change between S1 and S0 condition for a mouse generating FA in a specific block type (Early or Late). $**P < 0.005$.

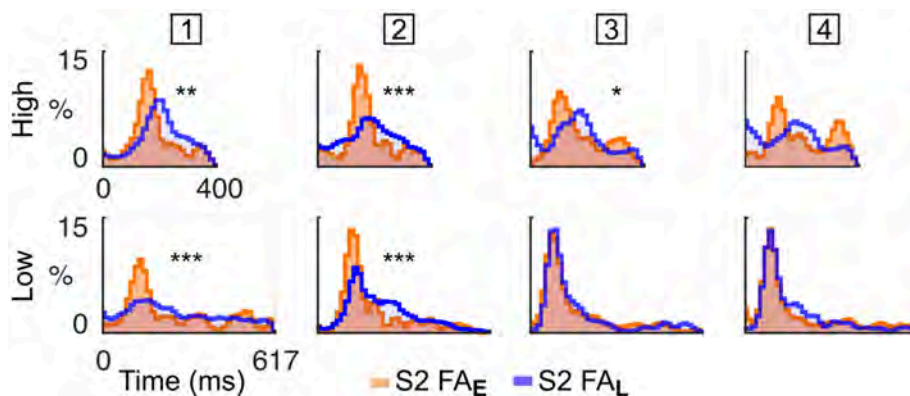


FIGURE 4 | Mice were sensitive to spectral bias as revealed by their false alarm timing. Normalised RT distribution for FA in S2 condition for each mice (one mouse per column, the mouse ID is presented in the square box) computed using all sessions. RT are measured either from High (top) or Low (bottom) tone onsets. The distributions are color-coded based on the block type (Early or Late). RT distributions are smoothed (1 bin std Gaussian filter) for display. The p -value from a KS test comparing the RT distributions in Early and Late conditions is displayed as an indication of response bias ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$; Bonferroni correction factor 8). The first mouse times its FA mainly to the High tones. The second mouse times its FA to the cued tones. The third and fourth mice time their FA mainly to the Low tones.

2.2. Selective Attention Modulated Neural Responses in Auditory Cortex Irrespectively of Frequency Tuning

Having shown that mice can be trained to selectively attend to the cued stream, we sought to determine whether neural activity in the AC varied according to the identity of the cued stream. We recorded single cells in the AC of behaving mice using tetrodes.

Each cell was assigned to a frequency-preference group (High responder or Low responder) based on its response to tones in the single stream (S1) condition (**Figures 5A,B**). All cells classified as High responder or Low responder were significantly activated by pure tones of a single frequency, either to the High frequency ($n = 11$ cells) or to the Low frequency ($n = 6$ cells) tones only.

We first focused the analysis on the activity recorded during the Early time zone (EZ; 0–1.6s from trial onset) occurring before

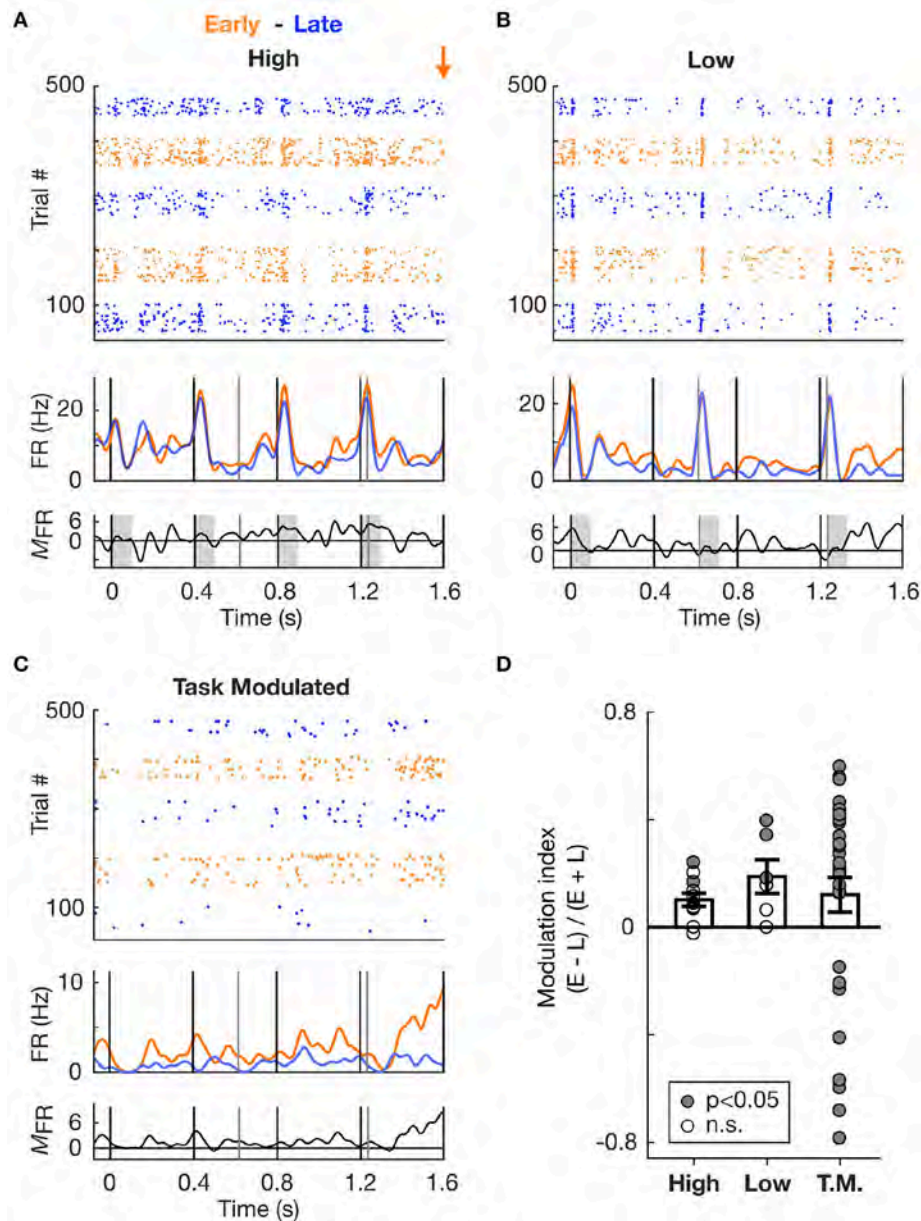


FIGURE 5 | Increase in spiking activity prior to target occurrence irrespective of frequency tuning. **(A)** Increase in spiking activity in Early vs. Late trials for an example cell tuned to the High frequency sound. Top and second panels: Raster plots and corresponding mean PSTHs of spiking activity during Hit trials in S2 condition, color-coded based on block type (Early or Late). The time axis is truncated so as to encompass only the Early zone (EZ; 0–1.6 s from trial onset). The orange arrow marks the onset time of the first target in the Early block. The vertical lines indicate tone onset (black: High frequency, grey: Low frequency). Third panel: Firing rate modulation (measured as the difference) between the Early and Late PSTHs. Values above zero indicate an increase in firing rate in the Early vs. Late condition. **(B)** Same as in **(A)**, but for a cell tuned to the Low frequency. **(C)** Same as in **(A)**, but for a cell classed as Task Modulated (T.M.). **(D)** Modulation of the firing rate between Early and Late conditions, displayed for each cell type. A value above 0 indicates an increase in the Early vs. Late trials. Each dot represents a cell, and the dot is filled with grey color if the firing rate of the cell significantly differed in Early vs. Late condition.

the first Early target onset in the dual stream (S2) condition. Since the stimuli in the dual stream (S2) Early and Late blocks were identical during the EZ, any difference in stimulus processing between Early and Late blocks could be attributed to top-down influences reflecting expectations. **Figure 5D** presents the modulation of the summed activity in the Early compared to Late

block for the different cell types, with values above 0 indicating an increase in firing rate in the Early block. Both High- and Low-responder cell populations displayed an increase in firing rate, as assessed by the average modulation index above 0. This increase was significant at the single cell ($n = 5$ cells, $p < 0.05$, WSR test) and population ($p < 0.01$, WSR test) levels.

To further confirm whether this increase in activity was not dependent on the cell's tuning, we measured the firing rate modulation of cells which were not significantly tuned to either Low or High tones, but which exhibited a high discrimination performance between Early and Late trials (AUROC > 0.6, using the sum of the PSTH to assess firing rate change in the EZ). These cells were labelled as Task Modulated (Figure 5C), and while they were not significantly responsive to High and Low tones, they might still be responsive to other sounds or to other aspects of the pure tones such as the offset (Sollini et al., 2018). As displayed in Figure 5D, the average modulation index for the Task Modulated cell population was also above 0, indicative of an increase in firing rate in Early compared to Late trials. Most of these Task Modulated cells had a significant modulation ($p < 0.05$, WRS test), which can be accounted for by the selection criterion based on high AUROC values. Therefore neural activity in the EZ of Early block trials was elevated relative to Late block trials, irrespective of the frequency tuning of individual cells, suggesting that the temporal proximity of an action (e.g., a lick) and/or reward is associated with a global increase in excitability in the AC.

To confirm whether the increase in firing rate was localised to the time prior to likely target occurrence, we measured the firing rate modulation of cells during Late trials at two different time intervals, one near trial onset and one near Late targets. These intervals were defined as early (0–1.6 s from trial onset) and late (4–5.6 s from trial onset), as is illustrated in Figure 6A. Importantly, both time zones encompassed a similar amount of Low and High tones (although organised in a different pattern relative to each other), and had similar duration. This allowed for a direct comparison of the mean firing rate during these two periods. Since High and Low cells were mostly responsive to single tones, the difference in tone presentation pattern was considered negligible when comparing the firing rates between the intervals. The Figure 6A presents an example cell (classed as Task Modulated) displaying an increase in firing rate over the course of Late trials. This effect was not an artefact, as analysis of the autocorrelogram and spike waveforms presented in Figure 6B confirmed. The minimal contamination in the refractory period argues against electrical noise generating the increase in activity visible in Figure 6A. This observation was highly consistent across cells, as most cells displayed an increase in firing rate during the period close to the Late target (Figure 6C). This effect was significant both at the single cell ($p < 0.05$, WSR test) and population ($p < 0.01$, WSR) levels. However, the Task Modulated cells were selected based on their ability to discriminate late and early zones (AUROC > 0.6), and might thus constitute a different population than the one presented in Figure 5D.

2.3. Neural Activity in Auditory Cortex Was Modulated by Upcoming Behavioural Decision

The previous finding indicated that the increase in firing rate observed preceding a likely target was not involved in the specific enhancement of sensory processing. Instead, this modulation

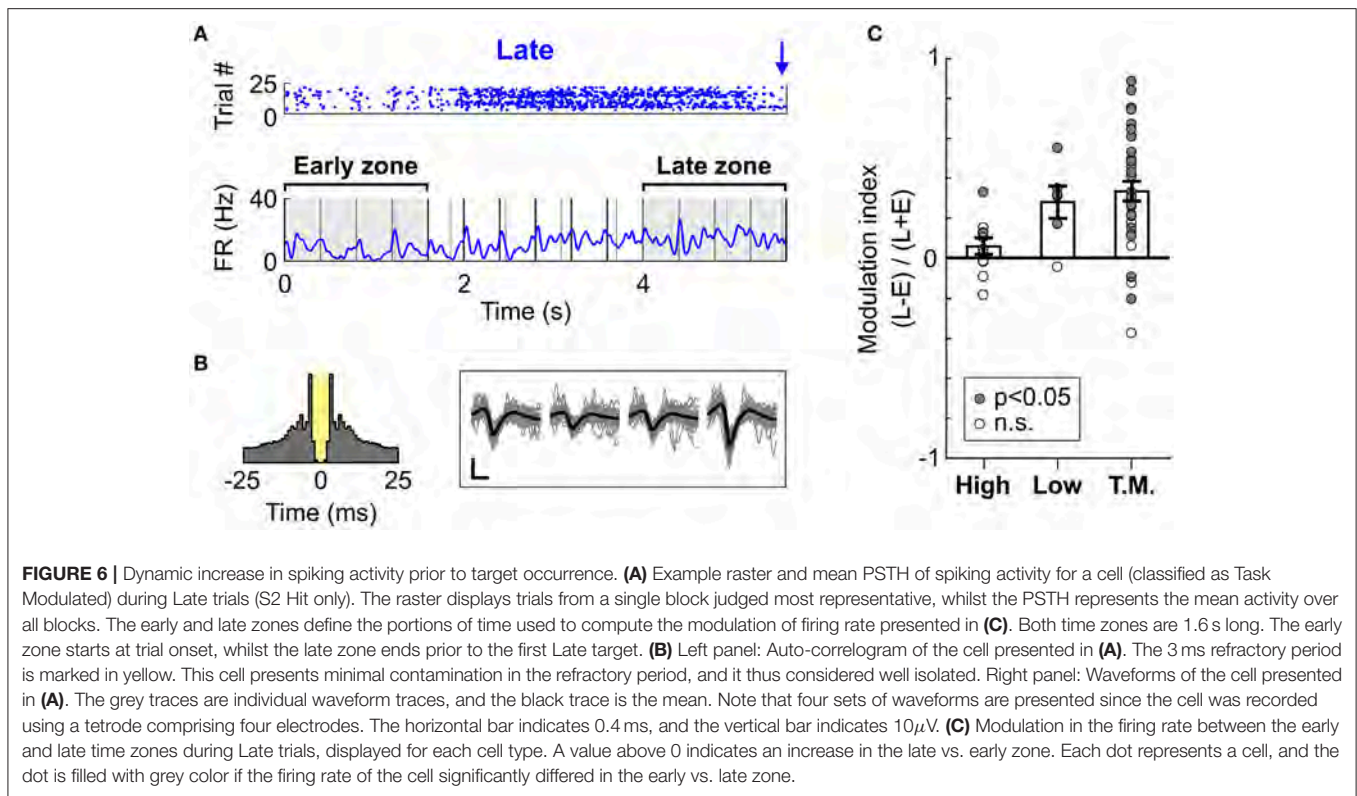
might reflect response preparation or reward anticipation, and might thus occur prior to any response, be it a Hit or FA. To test this hypothesis, we assessed whether cells with a high discrimination performance between Early and Late Hit trials could accurately classify the FA response types (Figure 7). Throughout this section, the activity during the Early and Late Hit trials is labelled as training data, whilst the activity during the Early and Late FA trials is labelled as test data. Early FA were defined as the FA responses made in the 1.2–2.8 s time window post trial onset in the Early S2 block, and Late FA were defined as the FA responses made in the 4–7 s time window post trial onset in the Late S2 block. For all trials, the activity considered was the average firing rate in the 0–1.2 s time window post trial onset. Firstly, a cell was classified as informative if its discrimination performance (i.e., the AUROC) was above 0.6 when comparing the activity in Early and Late S2 Hit trials (training data; Figure 7A). Then, a logistic regression classifier was trained on the training data (removal of 5% of trials for cross-validation). The accuracy of this classification typically matched the AUROC value. Then, the activity during each FA trial (test data) was presented to the classifier (see Figure 7B) and classification accuracy calculated. Since the number of Early and Late FA was generally unequal, it was important to build a baseline distribution for the classification accuracy. For example, a cell classifying any activity as originating from the class Early could obtain 90% classification accuracy if 9 out of 10 trials were truly Early. To generate a baseline classification accuracy distribution, the order of the true labels was shuffled, and these newly generated labels were compared against the predicted labels (process repeated 500 times). Out of the cells with high classification accuracy for Hit trials ($n = 30$), 9 cells were found to classify FA trial types above chance ($p < 0.05$; $n = 1/3$ for High cells; $n = 1/3$ for Low cells; $n = 7/24$ for Other cells). On average, classification accuracy was $66.5 \pm 2.2\%$ for those cells, whilst the mean of the baseline distribution was $51.7 \pm 1.4\%$. These results confirmed that the spiking activity of a subpopulation of cortical neurons mirrored the behavioural response specificity.

3. DISCUSSION

In a world where the senses are continuously stimulated, optimisation of information processing is believed crucial for perception and resulting behavioural actions (Crick, 1984; Fritz et al., 2007; Harris and Mrsic-Flogel, 2013). To understand how such optimisation occurs is a major quest in sensory neuroscience. The mouse is an advantageous model in which to study the neural processes underlying sensory refinement, as it is possible to precisely record and manipulate defined neural circuit components.

3.1. Auditory Attention and Streaming Could Be Inferred From the Timing of False Alarms

Here, we developed a novel behavioural task in mice to assess perceptual aspects of auditory streaming. Animals were trained to detect a target sound in one of two simultaneously presented,



isochronous pure tone sequences. Whilst mice reached high performance in this task, incorrect responses (false alarms; FA) were highly informative on the individual's strategy. Using FA responses together with classical measures of performance such as d' enabled us to critically assess each animal's behavioural state. Notably, we show that a subset of animals was able to adapt their behaviour according to task specificity.

Mice are known to adjust the timing of their behavioural decisions according to learned probabilities so as to maximise reward outcome (Buhusi et al., 2009; Tosun et al., 2016). We therefore propose that the observation of incorrect response timing during any behavioural task may offer great insight on the subject's behavioural strategy and attentional state.

3.2. Activity in Auditory Cortex Depends on Behavioural Context

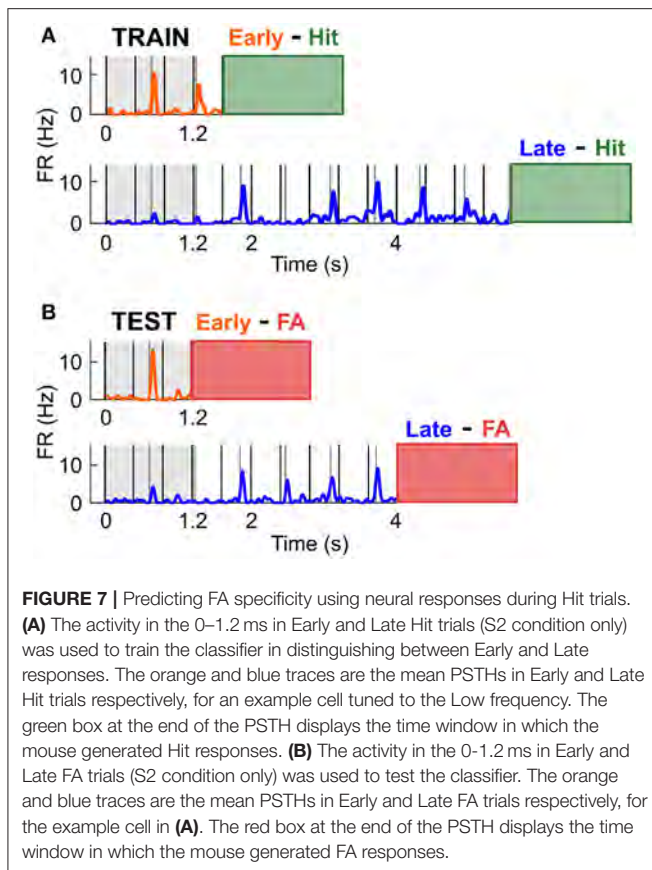
By recording in auditory cortex during ongoing behaviour, we related modulation of sensory encoding to changes in behavioural states. Specifically, activity in the AC was modulated by target feature expectation, in a manner that reflected the timing of the upcoming target, but not the spectral content of that target. Most cells displayed an increase in spiking activity prior to the first target during early trials, independently of their tuning profile. Moreover, a classifier trained based on the activity of a subset of AC cells during Hit trials was sufficient to decode response timing during FA trials. This implies that mice use similar behavioural strategies during Hit and FA, and that these strategies impact on neural processing at the level of the AC.

Given these findings, it appears that the prime difference in activity between Early and Late trials was not related to sensory processing *per se*, but rather reflects other aspects of the behavioural context. This was surprising, as the activity in auditory cortex is traditionally thought of as dedicated to the processing of auditory signals. These modulations might instead reflect the encoding of other task aspects, such as movement preparation, reward expectation, or task rule (Hu, 2003; Shuler and Bear, 2006; Rodgers and DeWeese, 2014; Bagur et al., 2017). Neural activity at the level of AC has recently been proposed to encode more than simply stimulus features (Rodgers and DeWeese, 2014; Bagur et al., 2017; Francis et al., 2018), however the underlying mechanisms are yet unknown. Future experiments will enable to delineate the contribution of specific anatomical pathways, including both sub-cortical (Thorn et al., 2010; Froemke et al., 2012; Pinto et al., 2013; Wimmer et al., 2015; Nelson and Mooney, 2016) and cortical structures (Gremel and Costa, 2013; Rodgers and DeWeese, 2014; Winkowski et al., 2017), in the modulation of AC activity during varying attentional states.

4. MATERIALS AND METHODS

4.1. Animals

Mice were housed under a 12/12 h light/dark cycle with food and water available *ad libitum*, except during behavioural training days. Electrophysiological recordings and behavioural training were performed during the dark phase of the cycle. All experiments were conducted under the UK Animals (Scientific



Procedures) Act 1986. Four adult female mice (C57BL/6, Charles River UK Ltd & Harlan UK Ltd) were used in this study. Mice were aged 16–18 weeks and weighed 25–35 g on the day of electrophysiological recording.

4.2. Head-Implantation

Mice were anaesthetised with isoflurane (1–2 % v/v) and placed in a stereotaxic frame (Angle 2, Leica Microsystems, Germany). A custom-made plastic head-implant was fixed to the exposed cranium using tissue glue (Histoacryl, Braun Corporation, USA) and dental cement (Associate Dental Products Ltd). A grounding pin was inserted above the cerebellum, and secured using dental cement. The location of the future craniotomy was measured using a pipette referenced to Bregma (−2.7 mm AP, ±2.8 mm ML), and marked by a cross made on the skull using a surgical blade. The exposed skull was then covered with Kwikcast (World Precision Instruments), and the animal recovered. Analgesia was provided by injecting carprofen (5 mg/kg) sub-cutaneously (SC) 20 min prior to recovery.

4.3. In vivo Electrophysiology

Upon the day of electrophysiological recording, the animal was anaesthetised using isoflurane and surgically prepared. The Kwikcast was removed, exposing the skull over both hemispheres. Right- and left-hemisphere craniotomies (1 × 1 mm) were made over the cross-marked locations. The dura was

removed, and the brain was lubricated with PBS. Agar (1 % in PBS) was applied over the PBS as a moisturising sealant. Once hardened, the agar was covered with a layer of Kwikcast. The mouse was administered with analgesics subcutaneously, and left to recover in a heating chamber until locomotor and grooming activity were fully recovered.

Once the animal was recovered from the craniotomy, it was fixed in the apparatus using zinc screws attached to the head-implant. The back of the animal was gently restrained using a half-tube composed of soft fabric, clamped down by a grounded metal plate. Once a craniotomy was made, up to two subsequent recordings were made in that hemisphere. Recordings were made in the other hemisphere successively. Mice underwent left or right craniotomies first in balanced proportion across the cohort.

All recordings were made using silicon microelectrode comprising multiple tetrodes (A32, 4x2Tet, NeuroNexus, USA), advanced in the brain using a micromanipulator (IVM, Scientifica, UK) tilted by a 35 degree angle from the vertical line. The electrode penetration depth was typically 2.610 mm. Data were acquired via Digital Lynx 16SX system (Neuralynx, USA) and stored on a PC.

4.4. Auditory Stimulus Presentation

Auditory stimuli were pre-generated and calibrated (5–100 kHz flat spectrum ± 1.5 dB SPL) using Matlab (Mathworks, USA) and presented free-field (ES1; Tucker Davis Technologies, USA) via an RZ6 Processor (using RPvdsEX software; Tucker Davis Technologies, USA). The start and end of all stimuli were ramped with a 3 ms cosine ramp. All sounds presented were 100 ms long. A High (14 kHz) and Low (5 kHz) carrier frequency oddball streams were generated, by presenting pure tones isochronously and by introducing a target instead of a pure tone. Per trial, only one target could be presented. A target was a rising frequency modulated sweep (4.4 octave/s) starting at the stream carrier frequency (i.e., at either 5 or 14 kHz). The stimulus onset asynchrony (SOA) of the low and high streams was fixed at 617 ms and 400 ms respectively. At the beginning of a trial, the two streams started concurrently.

4.5. Behavioural Setup

During behavioural training, mice were fixed onto a rigid metal platform, also used during electrophysiological recording. An Arduino UNO (www.arduino.cc) served as a controller, interfacing with a PC via the serial port. Outputs from the Arduino were sent to the PC and saved in text format using custom-written scripts in Python. Data from the text file were further analysed and plotted using custom-written Python scripts so as to display the mouse performance online during training sessions. During electrophysiological recording, arduino digital commands were sent to the digital port of the Digital Lynx so as to align neural traces with digital triggers during post-processing.

A lick port was used to monitor the animal's behaviour. The lick port was composed of an infra-red LED and sensor electrical circuit, a water delivery system and a vacuum system used for water removal. Briefly, a drop of water was delivered upon the opening of a solenoid valve clamping the water delivery tube. The control signal was a digital square pulse sent by an

Arduino. Similarly, a vacuum removal system was triggered upon the activation of a solenoid valve, connected to a negative air pressure. The lick port was mounted onto micromanipulators so as to position it finely compared to the mouse jaw. The speaker was positioned in front of the animal.

4.6. Behavioural Paradigm

Licking for a water reward was used to indicate a response. A single lick was considered as a Go-response. Mice were trained on a dual-stream oddball paradigm. An oddball stream was composed of a sequence of pure tones of a given frequency (High or Low), containing a target (frequency modulated) sound. Mice were cued to expect a target in a particular stream using a trial block design. Per block, the target alone was first presented (S0 condition) until the mouse reached 5 Hit trials. Subsequently, the oddball stream associated with that target was presented (S1 condition) until the mouse performed 10 Hit trials. Finally, the two streams were presented concurrently (S2 condition) until the mouse performed 20–25 Hit trials. High frequency target occurred Early (~ 2 s from trial onset), whilst Low frequency target occurred Late (~ 6 s from trial onset).

At the end of each trial, the vacuum pump was turned on for 500 ms to remove the excess water present in the lick port. A random delay (100–150 ms) was applied before presenting any stimulus following vacuum offset. The reward window started 100 ms after target onset, and ended when the next sound of the corresponding stream occurred, i.e., the length of the reward window was the stream SOA minus 100 ms.

4.7. Measure of Behavioural Performance

All sounds presented before the target window were considered as one No-Go cue, and all sounds presented after as another No-Go cue. One trial could thus give 4 outcomes depending on the mouse response timing: (1) if the mouse licked before the target window, a false alarm (FA) was counted; (2) if the mouse licked in the target window, a correct rejection (CR) + Hit were counted; (3) if the mouse licked after the target window, a CR + Miss + FA were counted; (4) if the mouse did not lick during the trial, a CR + Miss + CR were counted.

Performance was measured using the sensitivity index d' , defined as $z(P_{Hit}) - z(P_{FA})$, where z is the z -transform, P_{Hit} and P_{FA} the probability of Hit and FA respectively. P_{Hit} was defined as N_{Hit}/N_{Go} , i.e., the number of Hit trials divided by the number of Go cues presented (note that $N_{Go} = N_{Miss} + N_{Hit}$). Similarly, P_{FA} was defined as N_{FA}/N_{No-Go} , i.e., the number of FA trials divided by the number of No-Go cues presented (note that $N_{No-Go} = N_{CR} + N_{FA}$). In the case of $P_{Hit} = 0$ or $P_{FA} = 0$, the value was replaced with $1/N_{Go}$ or $1/N_{No-Go}$ respectively. In the case of $P_{Hit} = 1$ or $P_{FA} = 1$, the value was replaced with $(N_{Go} - 1)/N_{Go}$ or $(N_{No-Go} - 1)/N_{No-Go}$ respectively.

4.8. Training Protocols

4.8.1. Water Regulation

Following a minimum of 5 days post-surgery recovery, the mouse was placed under water restriction. On the first day of water restriction, no water was given. On the second day, 1 mL of water

was given. Subsequently, the mouse began its training protocol on the behavioural setup, where it received most of its daily water dose. The minimal water dose given per day was fixed to 1 mL. If the mouse did not perform enough trials in a single session so as to reach the daily dose, the complementary water volume was given at the end of the training session once the mouse had been removed from the head-fixing apparatus. Mice were typically trained 6–7 days per week.

Once under water restriction, the weight of the mouse was measured daily, prior to the delivery of water. In the case of the animal's weight decreasing below 80% of an aged-match, water unrestricted litter-mate, the animal was given water ad libitum for a day. In order to give a precise dose to an animal in isolation, the mouse was placed on a scale, and its weight recorded. The dose of water was then administered either directly to the mouse's mouth using a pipette, or by squirting the water out onto the scale's plastic flooring. The difference in body weight measurement pre- and post- water delivery ensured that the animal had drunk its daily dose.

4.8.2. Habituation

The first 3 training sessions consisted of habituating the mouse to the head fixation apparatus and learning the association between lick port and water delivery. During these habituation sessions, the reward delivery valve was replaced by a syringe full of water that could be manipulated by the experimenter so as to deliver water into the lick port on demand.

During each session, the mouse was fixed into the setup, and a pipette full of water was first advanced to the animal's mouth so as to prompt licking behaviour. Once the mouse had started to express exploratory licking behaviour, the lick port was filled with water and advanced so as to be reached by the animal's tongue. The lick port was filled with water until the animal displayed signs of satiation, i.e., stopped licking and attempted to push away the lick port with its forelimbs. The mouse was then removed from the apparatus, and supplementary water was given if necessary.

4.8.3. Target-Reward Association

Following the habituation sessions, the mouse underwent typically 7 sessions to associate the water delivery with the target sounds. During the first 4 sessions, an automatic reward (water drop of 5 μ L) was given 100 ms after sound onset. During the 3 consecutive sessions, the mouse had to trigger a reward by licking in the reward time window. The two (High and Low) target sounds were presented per session, using a 20–30 trials block design.

4.8.4. Refrain Licking Prior to Target Onset

During 5 sessions following the target-reward association sessions, the mouse was required not to lick before the occurrence of a sound (note that unlike in the FM discrimination task, all sounds presented at this stage are targets in the case of oddball tasks). The length of time the mouse was required to withhold licking for was randomly selected from a uniform distribution whose boundaries were incrementally increased over

the sessions ([200–500], [400–700], [600–1,000], [800–1,200], [1,000–1,400] ms).

4.8.5. Introduction of Single Stream

For the following 8 sessions, pure tones were introduced so as to form a single-stream oddball paradigm. Within a training session, the intensity of the pure tone was augmented from 0 dB up to the intensity level used for the target sound using 10 dB increment steps. The amount of hit trials required to augment the intensity was diminished across sessions (50-30-20-15-10-7-5-3). The mouse was required not to lick before the occurrence of the target, using the longest interval used in the previous training phase. The SOA varied depending on the frequency of the stream pure tone.

4.8.6. Introduction of Distractor Stream

For the following 4 sessions, pure tones were introduced so as to form another single-stream. Within a training session, the intensity of the extra tones were augmented after each 10 hit trials from 40 dB up to the level used for the target sound using 10 dB increment steps. The amount of hit trials required to present extra tones was diminished across sessions (20-15-10-7).

4.8.7. Reinforcing False Alarms

For the following 7 sessions, response to a No-Go sound (FA) was negatively reinforced by presenting a short noise burst upon the response, stopping any upcoming sound presentation, and subsequently applying a silent time out (random delay of 4–6 s). The vacuum valve was turned on after the time out had passed. If a response was made after having missed target, the sequence was terminated after presenting four regular tones past the target and the trial ended regularly (i.e., without time out).

4.8.8. Introduction of a Temporal Bias

For the following 5 sessions, the time window in which a target could occur was modified across session. For T1, only the window of the Low target varied so as to occur gradually later in the trial. Per session, the target window was fixed according to a trial block design (Early or Late block). On the final day of training, the Late window was ~6 s, and the Early window was ~2 s. Reinforcement of FA was always applied during this training stage.

4.9. Statistics

Blinding and randomization of neurophysiological data were not performed. Unless stated otherwise, results are presented as mean \pm standard error of the mean. KS refers to the Kruskal Wallis test. WRS refers to the Wilcoxon rank sum test, and WSR to the Wilcoxon signed rank test.

Statistics on circular data were performed using the Kuiper and the Rayleigh tests (Wilkie, 1983). The vector strength (VS) was considered significant if $N \cdot VS^2 > k$ at the α level, where N was the number of phases used to compute the VS, and $k = [2.9957 \ 4.6052 \ 5.2983 \ 6.9078]$ for corresponding α values [0.05 0.01 0.005 0.001] (Wilkie, 1983).

Throughout this text, the strength of p -value is indicated by stars: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ unless stated otherwise.

Significant spiking response to a tone was measured using the activity in the 100 ms window pre-tone (spontaneous) and post (evoked) tone onset. A 10 ms bin PSTH was generated for each trial, and the evoked response at each bin was compared (KS test) against the spontaneous response. A cell was classified as significantly evoked if either 3 bins passed the threshold of $p < 0.01$ or if one bin passed the threshold of $p < 0.001$ (Bonferroni correction factor 10).

The discrimination performance (DP) for trial type (such as Early/Late) classification was computed using logistic regression and receiver operating characteristic (ROC) analysis. Smoothed-PSTH on single trials were generated for the two conditions to compare (5 ms bin, convolved with 20 ms std Gaussian function for smoothing). A logistic regression model was fit at each bin of the single trial responses. The ROC curve was computed using the probability estimates from the logistic regression model. The DP was defined as the area under the ROC curve (AUROC).

5. DATA STATEMENT

Data can be made available by the authors upon request.

ETHICS STATEMENT

This study was carried out under a UK Home Office Project Licence (PPL 70/8837) in accordance with the Animals (Scientific Procedures) Act 1986 following the recommendations of Imperial College Animal Welfare and Ethical Review Board (AWERB).

AUTHOR CONTRIBUTIONS

GC designed the study, conducted experiments and analysis, and wrote the manuscript. PC designed the study, contributed to analysis, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00205/full#supplementary-material>

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How Many Clocks, How Many Times? On the Sensory Basis and Computational Challenges of Circadian Systems

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A vital task for every organism is not only to decide *what* to do but also *when* to do it. For this reason, “circadian clocks” have evolved in virtually all forms of life. Conceptually, circadian clocks can be divided into two functional domains; an autonomous oscillator creates a ~24 h self-sustained rhythm and sensory machinery interprets external information to alter the phase of the autonomous oscillation. It is through this simple design that variations in external stimuli (for example, daylight) can alter our sense of time. However, the clock’s simplicity ends with its basic concept. In metazoan animals, multiple external and internal stimuli, from light to temperature and even metabolism have been shown to affect clock time. This raises the fundamental question of cue integration: how are the many, and potentially conflicting, sources of information combined to sense a single time of day? Moreover, individual stimuli, are often detected through various sensory pathways. Some sensory cells, such as insect chordotonal neurons, provide the clock with both temperature and mechanical information. Adding confusion to complexity, there seems to be not only one central clock in the animal’s brain but numerous additional clocks in the body’s periphery. It is currently not clear how (or if) these “peripheral clocks” are synchronized to their central counterparts or if both clocks “tick” independently from one another. In this review article, we would like to leave the comfort zones of conceptual simplicity and assume a more holistic perspective of circadian clock function. Focusing on recent results from *Drosophila melanogaster* we will discuss some of the sensory, and computational, challenges organisms face when keeping track of time.

Keywords: circadian clock, biological oscillator, multisensory integration, bayesian modeling, *Drosophila melanogaster*, sensory conflict

INTRODUCTION

A good decision is not absolute. It varies depending on context. Foraging for food can be a good or bad decision depending on the presence of a looming predator. The key is to optimize behavior for the current context. But what if the context is in perpetual flux? Owing to the spin of our planet on its longitudinal axis, the vast majority of life on earth exists in 24-h cycles of environmental change.

The sun rises and sets. Dawn comes and goes. Temperatures change, and both predator and prey alike sleep and wake. Thus, good decisions depend as much on *when* to do, as they do on *what* to do.

This critical importance of time for decision-making necessitates the existence of internal clocks. These “circadian” oscillators give temporal structure to behavior. The circadian system of *Drosophila melanogaster* presents a remarkable tool to investigate how external environmental changes can impact internal timekeeping that best prepares an organism for time-appropriate tasks. The circadian clock has a set period (i.e., one full cycles takes ~24 h) and its time, or phase, can be adjusted by incoming sensory information. This flexibility is paramount to a system that is orchestrating numerous behavioral and physiological processes while external stimuli are constantly changing.

In this review article we set out to highlight the complexities of the *Drosophila melanogaster* circadian clock, in which “decisions” must be made with regard to external environmental cues. These fundamental timing decisions are made by individual neurons in the context of multimodal sensory information. By discussing the complexities that exist on the molecular, cellular, network and behavioral level, we propose computational approaches that may be fruitful to gain further insight into the nuances of circadian systems.

THE MOLECULAR CLOCK

To understand how the wider circadian system keeps time, we must first begin with the oscillatory building blocks that comprise it. Time is computed at the level of individual cells and integral to this cellular timekeeping is a molecular clock that is driven by the autonomous oscillations of so called “clock genes”. The autonomous oscillators are driven by a series of molecular transcriptional/translational feedback loops (TTFL) of which components autoregulate their own expression (Figure 1), reviewed in Hardin (2011).

The molecular clock starts with the transcription factors, CLOCK (CLK) and CYCLE (CYC), activating transcription of the primary TTFL genes, *per* and *tim*, through binding to E-box regions in their gene promoters (Hao et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). Transcription occurs between ~ZT4 to ~ZT18 (where ZT0 is lights on and ZT12 is lights off) and translation of these mRNAs generate the protein products PERIOD (PER) and TIMELESS (TIM). The PER protein is inherently unstable, and is rapidly targeted for proteasomal degradation by the kinase DOUBLETIME (DBT; Kloss et al., 1998, 2001; Price et al., 1998). However, the PER-DBT dimer can be stabilized through dimerization with TIM allowing the PER-TIM-DBT complex to accumulate in the cytosol, around 6–8 h after *per* and *tim* transcription activation or ~ZT12 (Curtin et al., 1995; Gekakis et al., 1995; Price et al., 1995; Zeng et al., 1996).

Phosphorylation of PER and TIM by the kinases, CK2 and SGG (Martinek et al., 2001; Lin et al., 2002; Akten et al., 2003), initiates nuclear accumulation of PER and TIM (Nawathean and Rosbash, 2004; Top et al., 2016). Inside

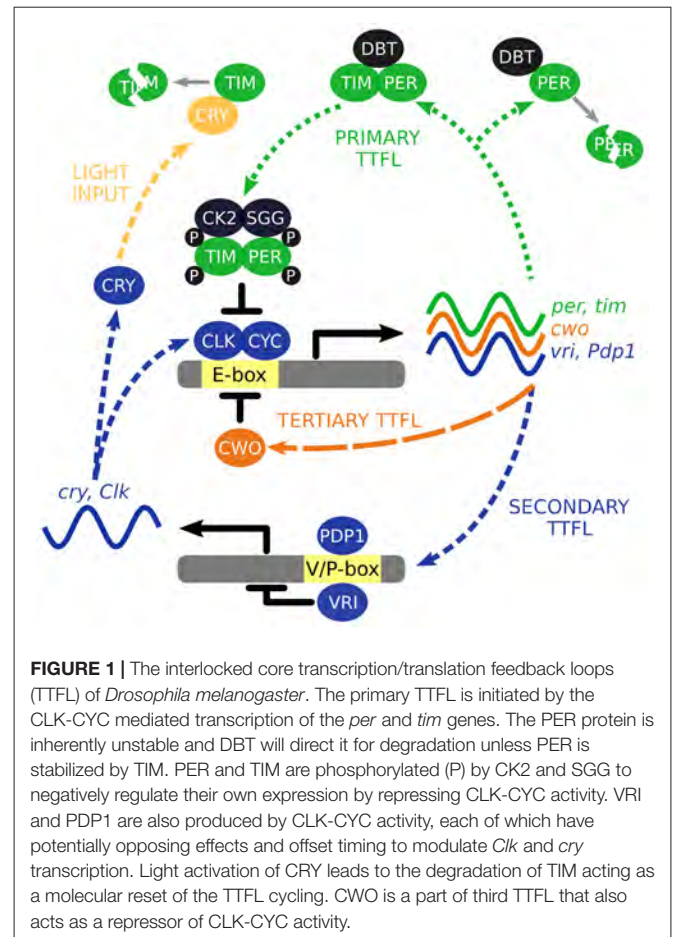


FIGURE 1 | The interlocked core transcription/translation feedback loops (TTFL) of *Drosophila melanogaster*. The primary TTFL is initiated by the CLK-CYC mediated transcription of the *per* and *tim* genes. The PER protein is inherently unstable and DBT will direct it for degradation unless PER is stabilized by TIM. PER and TIM are phosphorylated (P) by CK2 and SGG to negatively regulate their own expression by repressing CLK-CYC activity. VRI and PDP1 are also produced by CLK-CYC activity, each of which have potentially opposing effects and offset timing to modulate *Clk* and *cry* transcription. Light activation of CRY leads to the degradation of TIM acting as a molecular reset of the TTFL cycling. CWO is a part of third TTFL that also acts as a repressor of CLK-CYC activity.

the nucleus, the active form of PER inhibits CLK-CYC activation of *per* and *tim* transcription between ~ZT18 to ~ZT4 (Lee et al., 1998, 1999; Bae et al., 2000; Menet et al., 2010). Consequently, PER and TIM concentrations decline in the cytosol, reducing nuclear abundance of PER and TIM, and ultimately removing the inhibition of CLK-CYC activity. The molecular cycle then starts again, *circa* 24 h later.

The primary TTFL is bolstered by additional interlocked loops. In a second TTFL CLK-CYC also activates transcription of *vri* and *Pdp1ε/δ* between ~ZT4 and ZT16 (Blau and Young, 1999; Cyran et al., 2003). VRILLE (VRI) protein concentration peaks several hours before PDP1ε/δ (~ZT14 vs. ~ZT18) and both act at VRI/PDP1-boxes to modulate *Clk* and *cry* transcription (Cyran et al., 2003; Glossop et al., 2003). PER-TIM-DBT activity from the primary TTFL interlinks the two loops. The *cry* gene encodes a blue-light photoreceptor that upon photic activation promotes TIM degradation essentially acting as a molecular reset switch of the primary TTFL (Emery et al., 1998; Stanewsky et al., 1998). In the third TTFL, CLK-CYC promotes expression of *clockwork orange* that competitively binds to the same E-box regions to negatively regulate CLK-CYC mediated transcription (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007).

THE CENTRAL CLOCK—A NETWORK OF COUPLED OSCILLATORS

If the oscillating proteins of the TTFLs are the gears that direct timekeeping, the central pacemaker neurons are analogous to a synchronizer that sets a coherent time in context with the external environment. The architecture of this central pacemaker in flies consist of ~150 neurons comprising distinct subgroups identified in the brain through cytological staining of clock genes (**Figure 2**; Helfrich-Förster and Homberg, 1993; Helfrich-Förster, 1995). The subgroups are named after their morphology and anatomical location; each brain hemisphere has four small ventral lateral neurons (s-LN_v) that express the pigment dispersing factor (PDF), a fifth PDF-negative s-LN_v, four large PDF-positive ventral lateral neurons (l-LN_v), six dorsal lateral neurons (LN_d), 17 group one dorsal neurons (DN1), two group two dorsal neurons (DN2), ~40 group three dorsal neurons (DN3) and three lateral posterior neurons (LPN; Helfrich-Förster et al., 2007; Nitabach and Taghert, 2008; Schubert et al., 2018).

The dual oscillator model, originally hypothesized for nocturnal rodents (Pittendrigh and Daan, 1976), has historically been used to also describe the *Drosophila* crepuscular activity

patterns, proposing separate autonomous oscillators that control activity peaks observed at dusk and dawn. The PDF+ LN_v are required for the fly's morning activity peak (and free-running rhythms; Renn et al., 1999; Grima et al., 2004; Stoleru et al., 2004), while the LN_d and 5th PDF- LN_v are important for the evening peak (Grima et al., 2004), thus, these two groups are labeled the morning (M) and evening (E) oscillators respectively. In practice these designations are too strict as the roles and hierarchy of some cellular oscillators change under different environmental conditions (Rieger et al., 2006, 2009; Murad et al., 2007; Picot et al., 2007).

Current pacemaker models depict a variable—both neurochemically and electrically—coupled neuronal network that differentially responds to incoming multimodal stimuli (Yao and Shafer, 2014; Schlichting et al., 2016; Yao et al., 2016) reviewed in detail in Hermann-Luibl and Helfrich-Förster (2015); Top and Young (2018).

PERIPHERAL CLOCKS

In addition to the central pacemaker clocks, circadian oscillators also exist in several tissues outside the central nervous system and are likely to regulate organ or tissue specific functions. Peripheral clocks have largely been characterized through clock TTFL protein rhythms identified either by immunohistochemistry or by luciferase reporter expression being driven by clock gene promoters. Located in a number of tissues, peripheral clocks are heterogeneous in both their molecular machinery and their relationship to the central clock (Ito and Tomioka, 2016).

At the molecular level, CRY appears to act not only as a photoreceptor but also as a core component of the primary TTFL. In some peripheral clocks of *cry* mutants—in which CRY normally fulfils both roles—light entrainment is lost, however they also lose molecular oscillations of *per* and *tim* (Ivanchenko et al., 2001; Krishnan et al., 2001). In these peripheral clocks it appears CRY not only integrates photic information into the clock, but it also plays a role in driving oscillations of the clock, possibly acting as a repressor (Collins et al., 2006). Alternatively, CRY may act exclusively as a photoreceptor with persistent free running rhythms observed in certain tissues of *cry* mutants. This indicates that in these peripheral clocks CRY is not required to drive oscillations of the clock (Ito et al., 2008).

Phase relations between central clocks and peripheral clocks are also heterogeneous. For example, peripheral clocks in the Malpighian tubules and chemosensory sensilla can directly entrain to external stimuli and maintain entrained rhythms even without information from central clock neurons (Hege et al., 1997; Krishnan et al., 1999). A different phase relationship is observed between oenocytes and the central clock. PDF release from clock neurons into the hemeolymph can set the phase of the distally located oenocytes, which control rhythmic release of mating pheromones (Krupp et al., 2013). The different phase relationships identified between central and peripheral clocks add yet another layer of complexity to the timekeeping system.

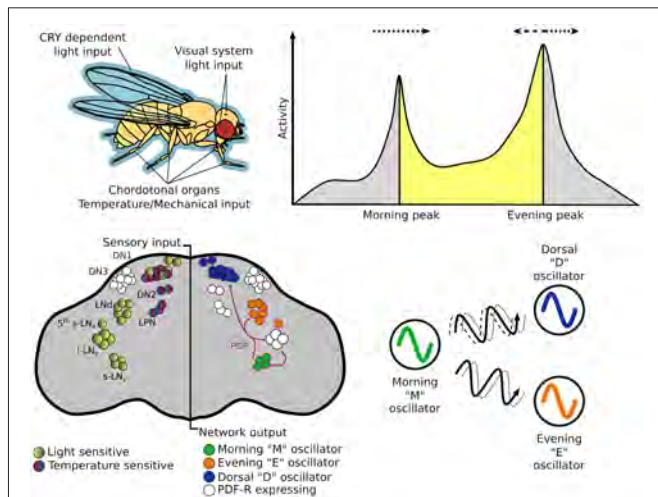


FIGURE 2 | The circadian system of *Drosophila melanogaster* from input stimulus to output behavior. Light information may enter the circadian system through the cuticle of the fly to directly act on CRY, which is expressed in many central and peripheral clock cells. Alternatively, it may enter via sensory cells of the visual systems—compound eye, H-B eyelets or the ocelli that make direct—or indirect—synaptic contact with central clock neurons. While flies have many thermo- and mechano-sensing organs, chordotonal organs (ChOs) have been directly implicated in communicating both temperature and mechanical stimuli to the circadian system. The neuronal populations of central pacemakers differ in their sensitivity to external stimuli as well as their neurochemical and electrical connectivity. Pacemaker neurons expressing CRY preferentially entrain to light cues while others not expressing CRY have demonstrated preference for temperature cues. Neurochemical differences can denote distinct functional groups—for example the M oscillator neurons express and release pigment dispersing factor (PDF) that can lead to phase changes in molecular oscillations of cells expressing PDF-R. This can result in phase advances or delays of downstream processes that in turn can advance or delay behavioral outputs.

LOCOMOTOR ACTIVITY RHYTHMS

Circadian rhythms exist at the cellular and network levels, yet it is the appropriate timing of behaviors that makes this system adaptive for the organism. Behavioral output of a circadian clock is typically measured as the organism's activity pattern; this is no different for *Drosophila*. Simple infra-red beam-breaking monitors record the activity of individual flies isolated in small glass tubes with enough food to last the duration of the experiment. These monitors are placed inside incubators in which the environmental conditions can be accurately controlled according to the test paradigm. In the simplest experiments, transitions between environmental conditions, or Zeitgebers ("time giver" in German), occur almost instantaneously (e.g., 12 h of light followed by 12 h of dark—LD). Here, *Drosophila* exhibits the previously mentioned bimodal activity pattern, with M and E activity peaks occurring at the Zeitgeber transition points. If the flies are then released into constant, free-running conditions, devoid of temporal information, rhythmic activity persists. However, the rhythms become almost exclusively unimodal possibly due to the merging of the previous M and E activity peaks (Wheeler et al., 1993; Helfrich-Förster, 2000). The timing of this free-running activity peak is a common read-out of "clock time" or circadian time. By adjusting the timing of cue onset/offset, the entrainability of the autonomous oscillator, which adjusts subsequent free-running rhythms to new schedules, can be tested. The speed of entrainment and relative amplitudes of autonomous oscillations depend on the strength and mode (e.g., light, temperature, vibration) of the specific Zeitgeber signal. Indeed, the precise activity profile observed depends on both the environmental conditions and the genetic background of the fly (Schlichting and Helfrich-Förster, 2015).

ENTRAINMENT

Photic information is communicated to clock neurons both directly via CRY, and indirectly via visual photoreceptors residing in the compound eyes, ocelli and Hofbauer-Buchner eyelets. CRY mediates cell-autonomous perception of light in a number of tissues that harbor circadian rhythms via its interaction with TIM in the primary TTFL (Plautz et al., 1997; Emery et al., 2000; Lin et al., 2001). This direct input pathway makes the *Drosophila* circadian system particularly sensitive to light, capable of entraining to low light intensities (0.03 lux; Bachleitner et al., 2007), and can exhibit significant phase shifts following brief light pulses (Levine et al., 1994; Egan et al., 1999; Vinayak et al., 2013). Visual pathways to the clock are not as well defined and do not reset the clock with the same efficiency as CRY dependent pathways (Emery et al., 2000; Helfrich-Förster et al., 2001). However, it appears that a subset of rhodopsin photoreceptors communicate visual photic information to the clock by a novel phototransduction pathway (Stanewsky et al., 1998; Ogueta et al., 2018).

Temperature cycles (TC) can also entrain the circadian clock, and although this can occur in a tissue autonomous fashion in peripheral clocks, signaling from peripheral sensors

play an important role in entraining the central clock (Glaser and Stanewsky, 2005; Sehadova et al., 2009). Thermoreceptors, expressed in chordotonal organs (ChOs) have been identified in the thermotransduction pathway, relaying information to the clock across different TC regimes (Wolfgang et al., 2013; Chen et al., 2015). Furthermore, genes important to the structural integrity of ChOs have also shown importance for entrainment to TC (Glaser and Stanewsky, 2005; Sehadova et al., 2009). Specifically, *nocte* (*no circadian temperature entrainment*) was identified in a screen for mutants that could entrain to LD but not to TC (Glaser and Stanewsky, 2005). Interestingly, the *nocte* mutant cannot entrain to combined LD and TC, hinting at a role in a sensory integration pathway that appears to eventuate in the DN1 central clock neurons (Chen et al., 2018).

ChOs also play a role in entrainment to mechanical inputs and this appears dependent on where the ChO is located. ChOs are stretch sensitive organs that populate almost every exoskeletal joint mediating proprioception in the legs as well as hearing in the antennae (Kavlie and Albert, 2013). Flies with no functional ChOs fail to entrain to 12-h vibration/12-h silence cycles, whereas flies lacking only antennal ChOs entrain better than their wild type controls (Simoni et al., 2014). This may be another feedback loop in the circadian system where output activity feeds information back to the clock.

NATURAL CONDITIONS

In order to perform more ecologically relevant experiments, environmental conditions can also be shifted in relation to each other in order to closely mimic natural conditions. This can be taken even further by placing the activity monitors outside the predictable conditions of the laboratory in the real-world environment. Under semi-natural "Summer" conditions an extra activity peak is observed that coincides with the temperature maxima termed the afternoon (A) peak (Vanin et al., 2012). Evidence suggests that A peak activity is induced by activation of the TRPA1 thermosensor in the AC neurons, rather than the circadian clock pacemakers (Tang et al., 2013; Green et al., 2015). The ecological relevance of the A peak is hypothesized to be an escape response from afternoon heat which is made evident by the occurrence of this peak being largely environmentally controlled. Closer observations of the fly activity support this hypothesis. Inactive flies at a preferable temperature (28°C) quickly retreat to the relative coolness of their food at an uncomfortable temperature (31°C) and are subsequently induced to erratic hyperactivity at noxious temperatures (35°C; Menegazzi et al., 2012). The phase of the A peak, however, still appears to be modulated by both the clock and environmental conditions (Menegazzi et al., 2012; Vanin et al., 2012). Interestingly, a functional clock appears to suppress the A peak at non-noxious temperatures. Clock mutant flies, deficient in either PER or CLK, exhibit A peak activity under milder TC in which wild-type flies exhibit bimodal activity patterns (Currie et al., 2009; Menegazzi et al., 2012; Vanin et al., 2012). This could be the clock mutant's lack of time perception being unaware of the daily afternoon peaks of temperature, whereas wild-type flies anticipate the regular afternoon increase

in temperature knowing it is nothing to be concerned about until a critical threshold is crossed at noxious temperatures. This is a more explicit decision-making process governed by the circadian clock—knowing what stimuli to respond to and what stimuli can safely be ignored.

CONFLICT CONDITIONS

The complexity of the number of potential interactions that could occur between the central clock pacemakers and the peripheral clocks is only now starting to be realized. How many clocks contribute to the animal's overall sense of time? The historical view that one master pacemaker clock actively sets the phase of all the downstream clocks is being challenged. Perhaps the central pacemaker sets the time of central processes and regional peripheral clocks set time locally? To test this, offset environmental conditions have been used to understand the molecular, and subsequent behavioral repercussions, of receiving potentially conflicting environmental cues. During antiphasic conflict of light and temperature (a 12-h maximal misalignment between the two cues), the activity patterns of the flies demonstrate preferential entrainment to light (Yoshii et al., 2010; Harper et al., 2016). An assessment of the relative strength of photic and thermic input under these conditions suggests light to be the victor. However, observation of fly activity under other misalignment schedules reveals that flies appear to follow temperature cues when the misalignment is short (2–4 h) and light cues when the misalignment is long (>7 h). Misalignment between this (5–7 h) produces a novel behavior of sustained activity over the course of misalignment (termed “plateau” or P behavior; Harper et al., 2016). P behavior was not observed in the clock-less *per* mutants or the light-input pathway impaired *cry* mutants. The *per* mutant generally displayed arrhythmic behavior with brief startle responses to environmental changes while, due to their lack of the CRY photoreceptor, *cry* mutants entrained preferentially to temperature cues. The lack of the P behavior in these two clock mutants during sensory conflict demonstrates that the behavior depends on a functional clock and is not the result of environmental masking. Furthermore, a severe dampening of PER oscillations in the central pacemakers under conflict conditions support the hypothesis that sensory conflict is causing the abnormal P behavior. This contrasts with results from a subsequent study in peripheral clocks. During similar conflict conditions, bioluminescent PER reporters expressed in peripheral clocks, revealed peripheral molecular rhythms remained entrained to the light cue (Harper et al., 2017). Again, CRY expression appears to be key for this light cue entrainment in these tissues as peripheral molecular rhythms in *cry* mutants follow the temperature cue under conflict conditions. The fact that molecular rhythms in these peripheral clocks do not collapse as observed in the central clock could indicate that these peripheral clocks do not contribute to locomotor rhythms or alternatively that asynchrony between central and peripheral clocks results in abnormal behavior. Further studies are required to unpack this relationship.

PROBABILISTIC CONSIDERATIONS AND FUTURE COMPUTATIONAL APPROACHES

The “circadian clock” is a complex, highly interconnected system, which attempts to determine—or predict—the state of the world from noisy or incomplete data. Probabilistic modeling provides a powerful conceptual framework for the theoretical, and experimental, analysis of such a system.

In a circadian setting, we can think of Zeitgebers as observable variables, from which the clock must compute the otherwise unobservable time of day. Bayesian integration provides an optimal algorithmic method by which to combine different sources of information. Here, the goal of a Bayesian observer (e.g., the “circadian clock”) is to compute the conditional density function that specifies the probability of the time of day from the given external cues (e.g., light and temperature). Further complexity is added by the temporal structure of circadian data, requiring that probability distributions be calculated over sequences of observations. Hidden markov models (HMMs) are one way of representing such temporal distributions and are used widely in tasks such as speech recognition (Rabiner, 1990), computational genomics (Eddy, 2004) and decoding neural spike data (Escola et al., 2011). More recently, HMMs have begun making their way into the circadian field, effectively modeling rhythms of both molecular (Bieler et al., 2014) and behavioral data (Harper, 2017). We expect such probabilistic frameworks to be crucial for understanding the relationships between circadian clock components across all levels of the system.

DISCUSSION

Complexity exists at all levels of the circadian system. Multiple TTFL maintain the ticking of the individual cellular clocks. This timing can then be communicated, neurochemically or electrically, to other cells that use the information to set the phase of their own internal clocks and orchestrate downstream processes. However, the flow of this information is not unidirectional. Multiple central oscillators exchange timing information set by incoming sensory information to compute time. This raises a further question of how the circadian system weights each oscillator's contribution (a problem well-suited to probabilistic modeling).

Existing methods used to quantify circadian function are imperfect. They are typically measured in free running conditions in order to avoid masking effects that occur as a stimulus response to environmental condition transitions. This necessarily removes the context in which circadian clocks operate and results in rapidly dampened rhythms—both molecular and behavioral. Ideally the phase of the circadian system would be measured during the entrainment period to alleviate the rapid dampening effects of free run, and also reducing the overall length of the experiment. Again, probabilistic models pose an interesting tool for extracting key circadian metrics—such as phase, period and rhythm strength—from noisy time series data in the presence of changing Zeitgebers.

Finally, a better quantitative understanding of the computations—and possible conflicts—occurring in the circadian system is also relevant in the context of the multitude of clock-related pathologies in humans (Roenneberg and Merrow, 2016). These pathologies may be the result of the circadian system simply making the wrong decisions, e.g., due to conflicting data (light at night, social jetlag, trans-meridian travels, etc.). A deeper understanding of where the errors in the decision making processes occur may be a decisive first step toward identifying and treating these pathologies.

AUTHOR CONTRIBUTIONS

JS, RH and JA together wrote and designed different aspects of the article.

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Gentle Handling Attenuates Innate Defensive Responses to Visual Threats

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Innate defensive responses to threats are essential for animal survival. The complexity and variability of innate defensive behaviors can be due to individual experiences, environmental factors, and internal states. However, it is not completely understood if the gentle handling involved in sensory processing affects innate defensive responses to visual threats. Here, we report attenuation of innate defensive responses after gentle handling accompanied by de-excitation of the intermediate layer (IL) and deep layer (DL) of the superior colliculus (SC) but not of the superficial layer (SL). Our theoretical analysis of the c-Fos network revealed an increased correlation in module 1, which maybe generally functionally associated with fear emotional, a decreased correlation in module 2, which maybe generally functionally associated with sensory processing. The IL of the SC appeared to have the highest correlation with the two modules. We verified the dynamic activities of the IL of SC in response to overhead looming stimulus using fiber photometry. Retrograde labeling of 18 regions of interest (ROIs) showed that the IL received significant inputs from the cortical areas, thalamus, hypothalamus, and brainstem. These data suggest the sensory processing involved in the modulatory roles of the SC in innate fear processing.

Keywords: gentle handling, innate fear, sensory input, superior colliculus, overhead looming stimulus

INTRODUCTION

Innate defensive responses to threats are essential for animal survival (LeDoux, 2012; Anderson and Adolphs, 2014; Janak and Tye, 2015; Tovote et al., 2015). Although defensive behaviors are stereotyped, their complexity and variability can be due to individual experiences (Morgan, 1896), environmental factors, and internal states (LeDoux, 2012; Anderson and Adolphs, 2014; Anderson, 2016). However, whether sensory input regulate the innate fear behavioral output is not well defined.

Overhead looming stimuli that mimic aerial predators have been shown to trigger stereotyped defensive responses across species (Yilmaz and Meister, 2013; Muijres et al., 2014; Temizer et al., 2015). Recently, looming-evoked defensive behavior and its underlying neural circuitry via the superior colliculus (SC) have received increasing attention (Yilmaz and Meister, 2013; Shang et al., 2015; Wei et al., 2015; De Franceschi et al., 2016; Huang et al., 2017; Vale et al., 2017; Evans et al., 2018; Li et al., 2018; Salay et al., 2018). SC is a laminated retinal

recipient structure, of which its superficial layer (SL) receives direct retina input and primarily responds to visual stimuli. The intermediate layer (IL) and deep layer (DL) of the SC contain neurons that respond to multimodal (somatosensory, auditory, visual) sensory inputs (Wurtz and Goldberg, 1971; Stein, 1984).

Laboratory housing, husbandry, and handling methods have been shown to affect the internal state of the animals (Grandin, 1997; Poole, 1997; Deacon, 2006; Hurst and West, 2010; Clarkson et al., 2018). Unpleasant somatosensations such as tail handling and rough handling reduce the response to reward and increase stress levels (Grandin, 1997; Clarkson et al., 2018). Conversely, a gentle touch plays an essential role in the pleasant somatosensory processing conserved across species (Lumpkin et al., 2010; Abaira and Ginty, 2013). In mammals, a gentle touch is the expression of preference between mates, parenting, and affiliation (Barnett, 2005; Ebisch et al., 2016). It has also been demonstrated that massage has positive effects on health in human, grooming reduces stress levels in primates, and tactile stimulation alleviates the effects of stress in fish (Field et al., 2005; Soares et al., 2011). Our previous study demonstrated that stress accelerates the defensive responses to looming in mice (Li et al., 2018). However, it remains unknown if the repeat pleasant somatosensation of gentle handling affects innate defensive behaviors to visual threats.

Here, we found that gentle handling attenuated innate defensive responses to overhead looming. In addition, the IL and DL (but not the SL) of the SC had significantly reduced activities in response to overhead looming after repeated gentle handling. Functional analysis of the c-Fos network revealed that the highest correlation was between the IL of the SC in two modules among the 16 ROIs. Moreover, we verified the dynamic activities of the IL of SC to overhead looming stimulus using fiber photometry. Retrograde labeling of 18 ROIs showed that the IL had significant inputs from the cortical areas, thalamus, hypothalamus, and brainstem, suggesting the potential modulatory roles of the SC in innate fear and sensory processing.

MATERIALS AND METHODS

Animals

All husbandry and experimental procedures in this study were approved by the Animal Care and Use Committee at the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences (Shenzhen, China). Male (6 weeks of age) C57BL/6 mice obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were kept in a quiet room (22–25°C) with a 12 h light-dark cycle, and were given food and water *ad libitum*.

Viral Injection

Mice were anesthetized with intraperitoneal (i.p.) injection of pentobarbital sodium (0.5% w/v, 80 mg/kg) and placed in a stereotaxic frame (RWD Life Science, Shenzhen, China). Microinjection needles were inserted unilaterally directly above the IL of the SC, using the following coordinates from Bregma: AP, −3.85 mm, ML, ±0.8 mm, and DV, −1.85 mm. The cholera

toxin subunit B (CTB) Alexa Fluor 594 Conjugate and adeno-associated virus (AAV) expressing EGFP driven by a neuron calcium/calmodulin-dependent kinase II (CaMKII) promoter (AAV9-CaMKII α -GCaMP6-EGFP; BrainVTA Co., Ltd., China) were injected using a 10 μ L microsyringe with a 33 gauge metal needle (Hamilton; Sigma, United States) connected to the UltraMicroPump 3 microsyringe pump injector (World Precision Instruments, United States) and its controller (Micro4; World Precision Instruments) at a rate of 100 nL/min. After injection, the needle was left in place for an additional 10 min to allow diffusion of the virus particles away from the injection site, and then it was slowly withdrawn.

Histology, Immunohistochemistry, and Microscopy

To quantify the expression of c-Fos positive neurons in the whole brain induced by overhead looming stimuli after consecutive 7 days of gentle handling or no-handled, the two group of mice were sacrificed 1.5 h after presentation of overhead looming stimuli on the 8th day. Mice were perfused by overdosing with chloral hydrate (10% w/v, 300 mg/kg, i.p.) and transcranially perfused with cold 1 M PBS followed by ice-cold 4% paraformaldehyde (PFA; Sigma) in 1 M PBS. Brains were removed and submerged in 4% PFA at 4°C overnight to postfix, and then transferred to 30% sucrose to equilibrate. The coronal brain sections (40 μ m) were obtained with a cryostat microtome (CM1950; Leica, Germany). Freely floating sections were washed with PBS, and blocked for 1 h at room temperature in blocking solution containing 0.3% Triton X-100 and 10% normal goat serum (NGS). Then the sections were incubated overnight with rabbit monoclonal anti-c-Fos (1:300, #2250; Cell Signaling Technology, United States) diluted in PBS with 3% NGS and 0.1% TritonX-100. The sections were incubated for 1 h at room temperature with Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200; Jackson Laboratory, United States). Finally, the sections were mounted, coverslipped with DAPI (1:50,000, #62248; Thermo Fisher Scientific, United States), and photographed using the Olympus VS120 virtual microscopy slide scanning system (Olympus; Japan). The images were acquired using identical gain and offset settings, and analyzed with ImageJ, Image Pro Plus, and Adobe Photoshop software. ROIs were traced with reference to the “The mouse brain in stereotaxic coordinates, George Paxinos and Keith B. J. Franklin,” and c-Fos immunoreactivity was quantified using Image Pro Plus that was checked by comparing to manual counts by a trained double-blind observer.

Behavior Assay

Gentle Handling

After obtained from the laboratory animal center, the mice have 1 week to habituation to the new animal facility and cage homes. Gentle handling was performed in the arena.

Briefly, mice were placed individually on a piece of medical absorbent cotton for 3 min handling once per day for 7 consecutive days. Handling sessions were performed by gently and thoroughly touching the mouse body through the cotton.

New cages with the previous bedding were used to house the mice that had been handled. Handling sessions were performed in the same room by the same experimenter. The mice in the non-handled group received the same approach without the handling treatment, including habituation to the looming box on the 7th day and receive three times upper visual looming stimuli on the 8th day.

Looming Stimulation Test

The behavioral arena was a 40 cm × 40 cm × 30 cm closed plexiglass box with a shelter nest in the corner and an LCD monitor on the ceiling to present the looming stimulus (LS). The LS was a black disk that expanded from 2 to 20 degrees in diameter; it was presented 15 times in 5.5 s. The stimulation was manually triggered by the experimenter when the mice were in the farthest area away from the shelter. The handling group of the mice have experienced the recurrent 7 days gentle handling. On the 7th day, the mice in gentle handled group and non-handled group have 10 min to habituation to the looming box. Then on the 8th test day, after 3 min pre-test session, all the mice have subjected three times of the upper visual black looming stimulation. And the duration between each of the stimulation is no less than 3 min.

Fiber Photometry

A fiber photometry system (ThinkerTech, Nanjing) was used to record the calcium signals of CaMKII α -positive neurons in the IL of the SC in response to upper visual field looming stimulation. Two to 3 weeks following AAV9-CaMKII α -GCaMP6-EGFP injection into the IL, an optical fiber (220 μ m O.D., 0.37 NA; Newdoon, China) was placed above the IL of the SC. One week after the surgery, mice took 10 min to habituation to looming box the day before the test day. In the test day, mice received three times upper visual field black looming stimulations and three times upper visual field white looming stimulations. The upper visual field white looming here is used as a control which does not evoke the flight behavior.

To record the fluorescence signals, a 480 nm excitation light from a LEDs (CREE XPE), reflected of a dichroic mirror with a 435–488 nm reflection band and a 502–730 nm transmission band (Edmunds Inc.) and coupled to a long optical fiber (220 μ m O.D., 0.37 NA, 2 m long, Thorlabs, Inc.). The laser intensity at the fiber tip was 20–30 μ W to minimize GCaMP bleaching. GCaMP fluorescence was filtered with a GFP bandpass (Filter 525/39; Thorlabs, Inc., United States), detected by the sensor of a CMOS camera (Thorlabs, Inc. DCC3240M). A Lab view program is developed to control the CMOS camera and acquire calcium signal in about 50 Hz. The behavior event signal is recorded by a DAQ card (NI, usb-6001) in 1000 Hz using the same program.

Statistical Analysis

All data values are presented as the mean \pm SEM. Mann-Whitney *U* test was used to analyze statistical differences using GraphPad Prism 7 software. Statistical significance was set at **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. Functional network analyses of the *c*-Fos dataset were computed with

the Rubinov and Sporns (2010) Brain Connectivity Toolbox¹ in MATLAB R2016 (The Mathworks Inc.) Graph theoretical analysis of *c*-Fos data between gentle handling and non-handling conditions was used to calculate the relationship among the 16 ROIs. Correlations among the 16 ROIs arranged by rank-order were conducted using Kendall's Tau correlation coefficient. Community correlation network and participation coefficients were analyzed according to previous studies, and a threshold value of 0.35 was set (Newman, 2006; Power et al., 2013; Vetere et al., 2017; Rogers-Carter et al., 2018).

RESULTS

Gentle Handling Attenuates Defensive Responses to Overhead LS

After 1 week habituation in the laboratory, mice underwent 7 consecutive days of gentle handling. To determine whether gently handling affects the innate defensive responses of mice, we used a behavioral assay with a rapidly expanding dark disk stimulus that mimicks an approaching predator (Yilmaz and Meister, 2013; Shang et al., 2015; Wei et al., 2015; De Franceschi et al., 2016; Huang et al., 2017; Vale et al., 2017; Salay et al., 2018). In response to repeated LS (three trials, intervals longer than 3 min), gently handled mice had an increased latency to onset flight behavior (**Figure 1B**), increased latency of flight to nest (**Figure 1C**), decreased duration hiding in nest (**Figure 1D**), and decreased locomotion speed (**Figure 1E**) compared with the non-handled group (*n* = 12 in each group, Mann-Whitney *U* test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001). Taken together, these results demonstrate that long-term repeated handling attenuates innate defensive responses to visual threat stimuli. These data suggest that instinctive behavioral output is sensitive to tactile stimulation.

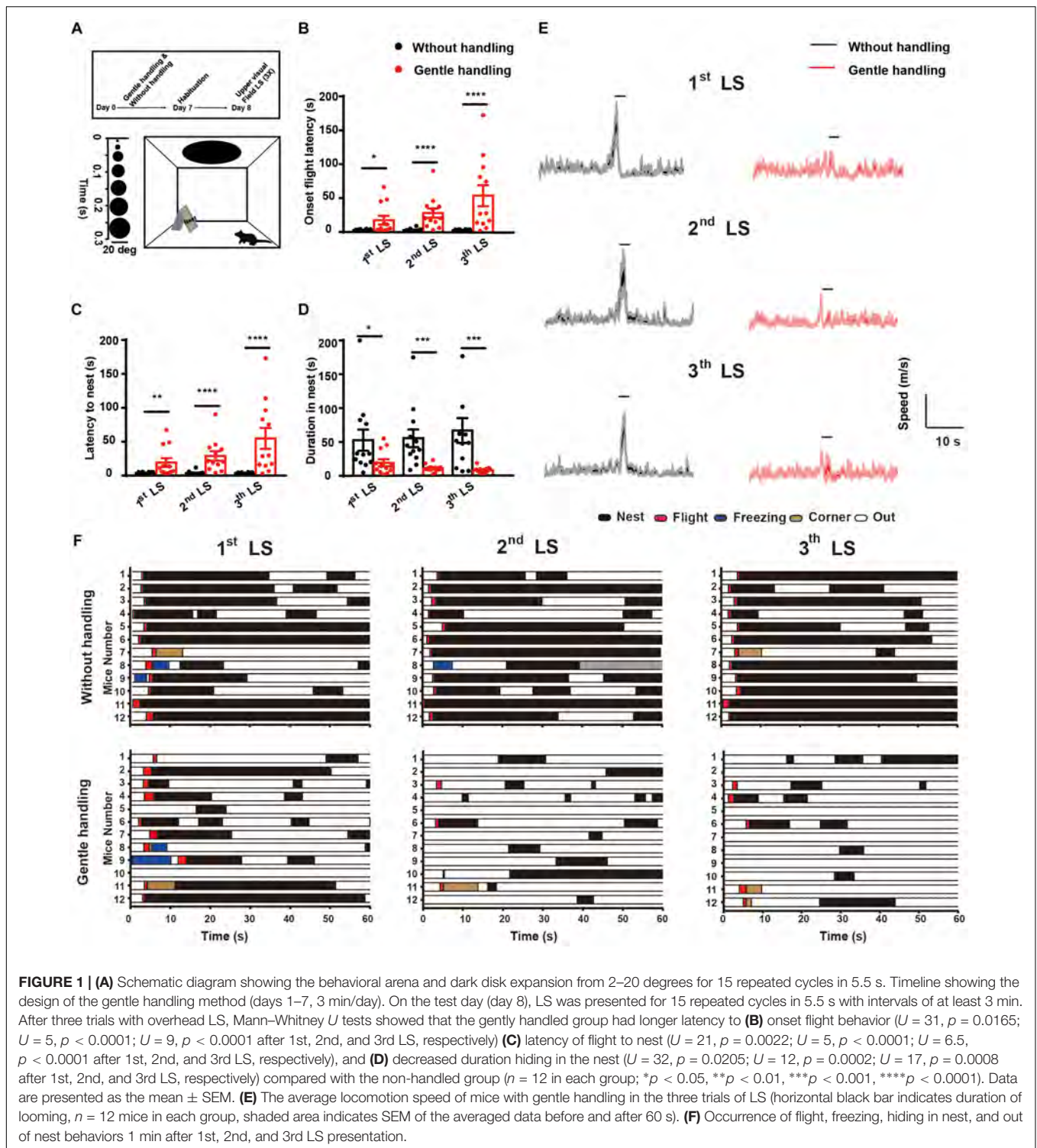
Gentle Handling Reduces the Activities of SC Neurons in Response to Overhead Looming Stimulus

To test whether gentle handling affects SC responses to LS, we performed *c*-Fos labeling in the gently handled and non-handled groups after the behavioral test. The gently handled group had attenuated innate defensive behavior (**Figure 1**) accompanied by a decrease of *c*-Fos expression in the IL and DL of the SC, but not in the SL of the SC (**Figure 2**; *n* = 6 mice in each group, Mann-Whitney *U* test, *U* = 8, *p* = 0.132, *U* = 2, ***p* = 0.0087, *U* = 4, **p* = 0.0260). These data indicate that gentle handling reduced the activities of the IL and DL of the SC in response to the black looming in the upper visual field.

Analyses of the *c*-Fos Functional Network in Response to Overhead Looming Stimulus After Gentle Handling

To determine the functional network connectivity, we performed *c*-Fos mapping in 16 ROIs in response to overhead LS between

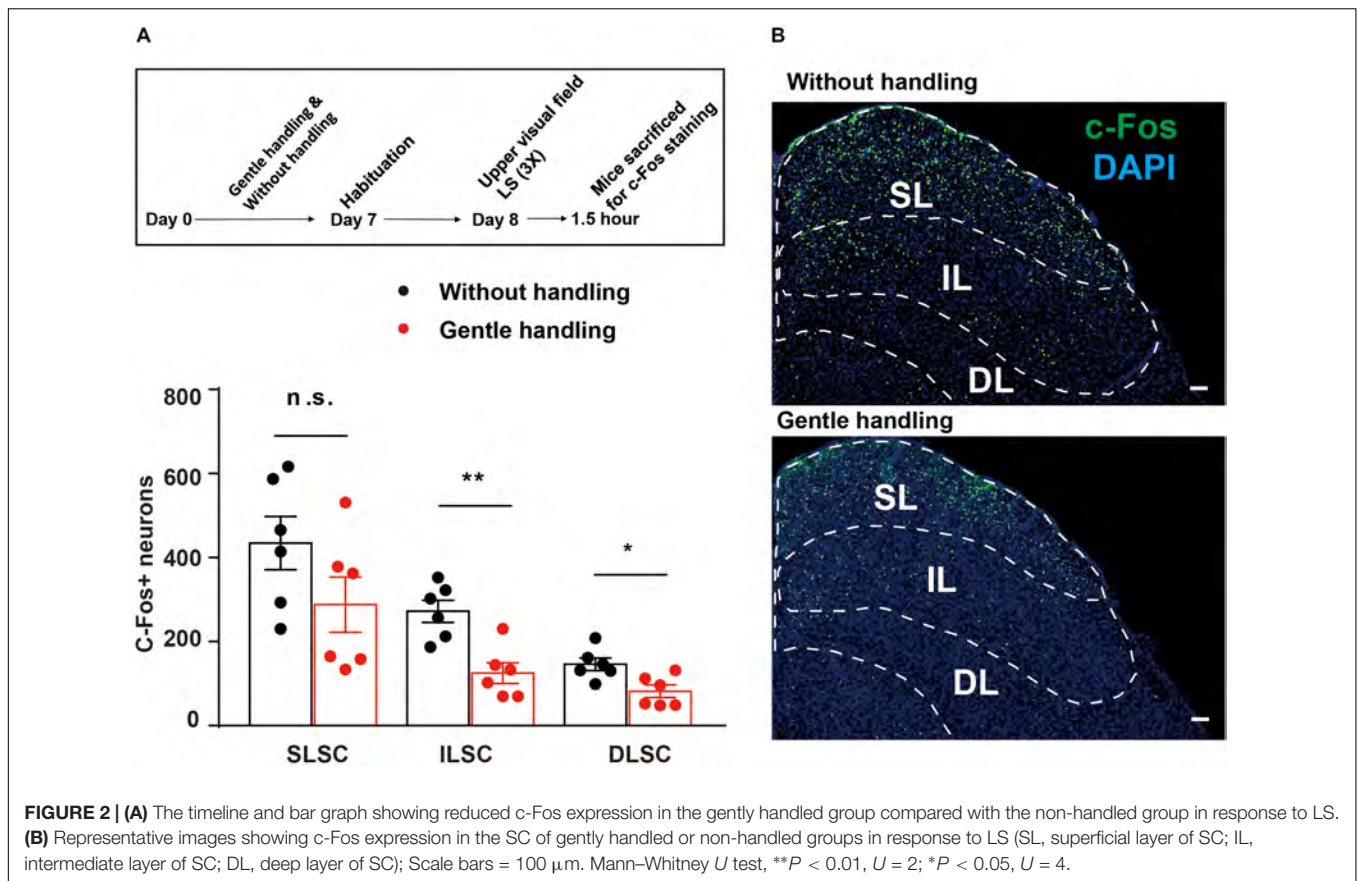
¹<https://sites.google.com/site/bctnet/>



gently handled and non-handled mice. The 16 ROIs are selected by the expression of *c-Fos* which is comparatively higher than some other brain regions, also the brain regions what we choose, is most like generally functionally associated with the “fear” and “sensory” processing. Graph theoretical analysis was used to divide the 16 ROIs into two modules, in which the functional

correlation of ROIs in each module was higher than that between modules (Figure 3A).

Our paradigm used in the current study is overhead life-threatening looming stimulation which mimic the predatory in the upper visual field. And most ROIs in module 1 (Figure 3D, purple dots) maybe generally functionally associated



with “fear” related, such as different layer of superior colliculus, ventral tegmental area, basolateral amygdala, anterior cingulate cortex, pontine gray, piriform cortex. And the recurrent long-term (7 days continuously stimulation) gentle handling should transmit sensory input signal. And most ROIs in module 2 (Figure 3D, green dots) maybe generally maybe generally functionally associated with “sensory” related, such as primary somatosensory cortex, barrel field; primary somatosensory cortex, trunk region; primary visual cortex. After gentle handling, the correlation was decreased in module 1 and increased in module 2, and the correlation across modules 1 and 2 was increased (Figure 3B). The participation coefficients were calculated to determine the hubs in the functional correlation-based networks (Power et al., 2013; Vetere et al., 2017; Rogers-Carter et al., 2018). The nodes with the highest participation coefficients across two modules included brain areas such as the IL and DL of the SC, S1BF, V1, PVT, and PAG (Figure 3C). Functional network analysis indicated that compared to the SL and DL, the IL of the SC was more densely connected with other nodes related to innate fear and sensory perception.

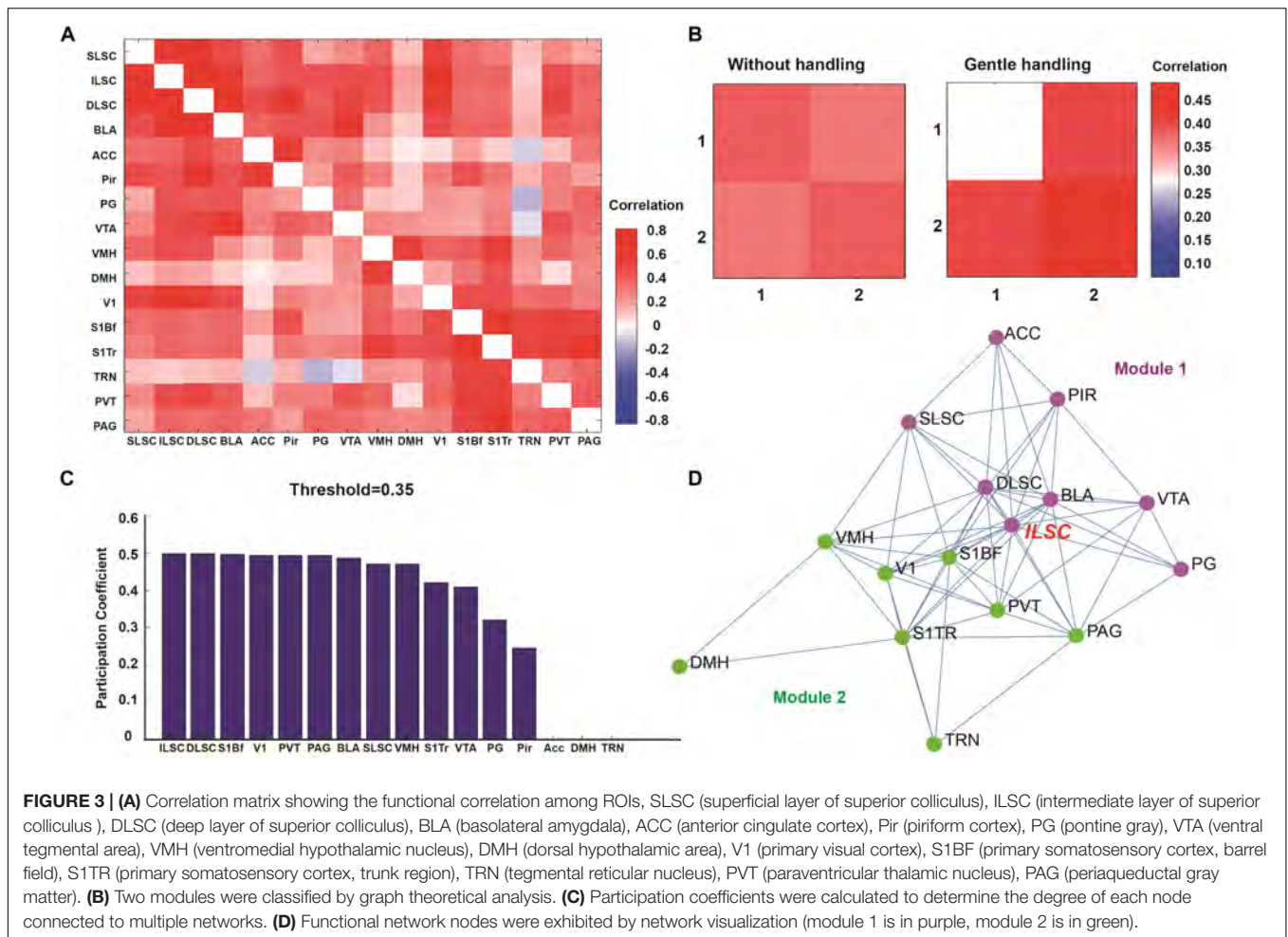
Dynamic Activation of the IL of SC in Response to Looming Overhead Stimulus

The SC, especially the SL of the SC, receives direct retinal input and mediates visual threat processing (Shang et al.,

2015, 2018). To investigate whether the IL of the SC receiving somatosensory input reflects visual threat signals, we injected AAV9-CaMKII α -GCaMP6s into the SC and optical fibers were implanted into the IL. Fiber photometry was used to record calcium transients in CaMKII α -positive neurons in the IL in response to a black looming in the upper visual field (Figure 4A). A white looming in the upper visual field was used as a control, as it does not trigger flight-to-nest behavior (Figures 4B,C). The IL of SC CaMKII α -positive neurons exhibited a significant increase in the activity after presentation of a black LS in the upper visual field ($9.52 \pm 2.13\%$, $\Delta F/F$ mean) compared with the white LS in the upper visual field ($0.98 \pm 0.74\%$, $\Delta F/F$ mean). Calcium signal onset to looming overhead stimulus rapidly increased (15% of peak signal as the onset time point) with a latency of 0.71 ± 0.18 s and peak signal of $19.08 \pm 3\%$, $\Delta F/F$ (Figures 4D,E). Taken together, these data demonstrate the IL of SC CaMKII α -positive neurons encode visual threat processing.

Retrograde Labeling of Input to the IL of the SC

To visualize inputs to the IL, the CTB Alexa Fluor 594 Conjugate CTB was used for tracing. Two weeks after microinjection of CTB into the IL, the brain was sectioned to 40 μm, and every third section was processed for subsequent analysis. Across the entire brain, the most abundant labeling was found in the cortex (Aux, V1, S1, ACC, TeA, EcT, PRh, EnT), thalamus (LD, LP, AV),



and hypothalamus (LH, DMH, VMH, ZI), brainstem (LC, DRN) (Figure 5). These data suggest that the IL of the SC receives broad cortical inputs from visual cortex, auditory cortex, and somatosensory cortex, as well as autonomic inputs from LC, LH, and thalamic nuclei (Saper, 2002).

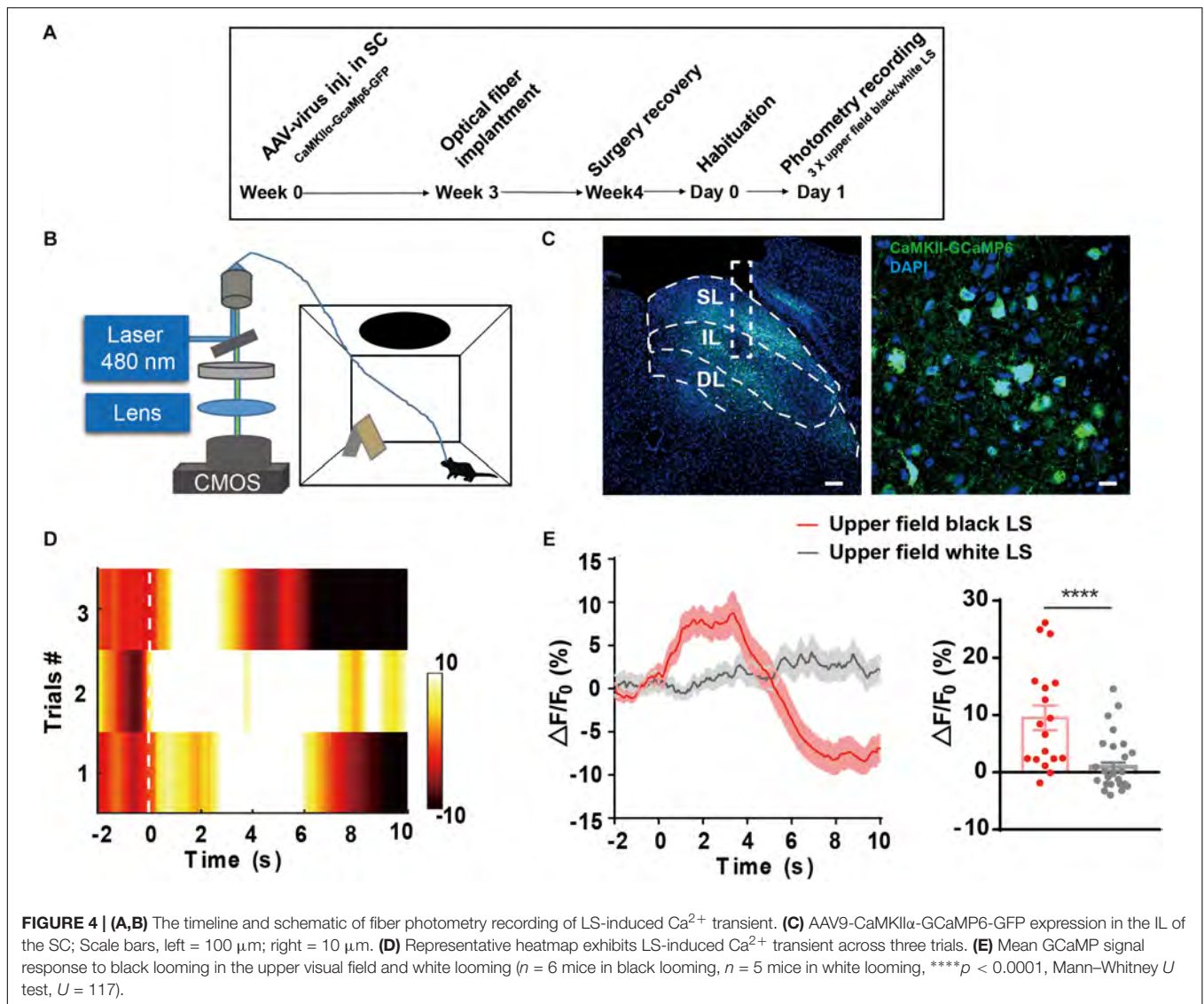
DISCUSSION

The innate defensive reactions to threats are vital for survival. Although innate fear is instinctive and unconditional, the expression of innate fear can be affected by the circuits of motivation, arousal, emotional state, and reinforcement (LeDoux, 2012; Anderson and Adolphs, 2014). Tactile stimuli is a fundamental form of sensory perception conserved across species (Ratcliffe, 2012). Gentle handling has been shown to reduce stress, modify cognitive behavior, and improve the relationship between handlers and animals (Harlow and Suomi, 1970; Antoniazzi et al., 2017). Furthermore, gentle handling has been proven to decrease depression and reduce anxiety-like behavior in laboratory mice (Grandin, 1997; Madruga et al., 2006). Visually evoked defensive responses to overhead looming stimulus are initiated from the SC and lay downstream of the SC

(Wei et al., 2015; Shang et al., 2018), but whether gentle handling affects innate visual threat processing is largely unknown.

In the current study, we demonstrated that gentle handling attenuates innate defensive reactions to visual threat stimuli, consistent with findings from previous studies (Grandin, 1997; Madruga et al., 2006). These studies provide a possibility that gentle handling might change the social interaction with experimenter and animal to improve the animal's performance. Moreover, it's possible that gentle handle induced the neurotransmitters such as oxytocin and dopamine release or lower corticotropin releasing hormone (CRH) to reduce emotionality, promote calm, and increase the ability to address visual threat situation. (Harlow and Suomi, 1970; Fenoglio, 2006; Ellingsen et al., 2014; Scheele et al., 2014; Peled-Avron et al., 2016).

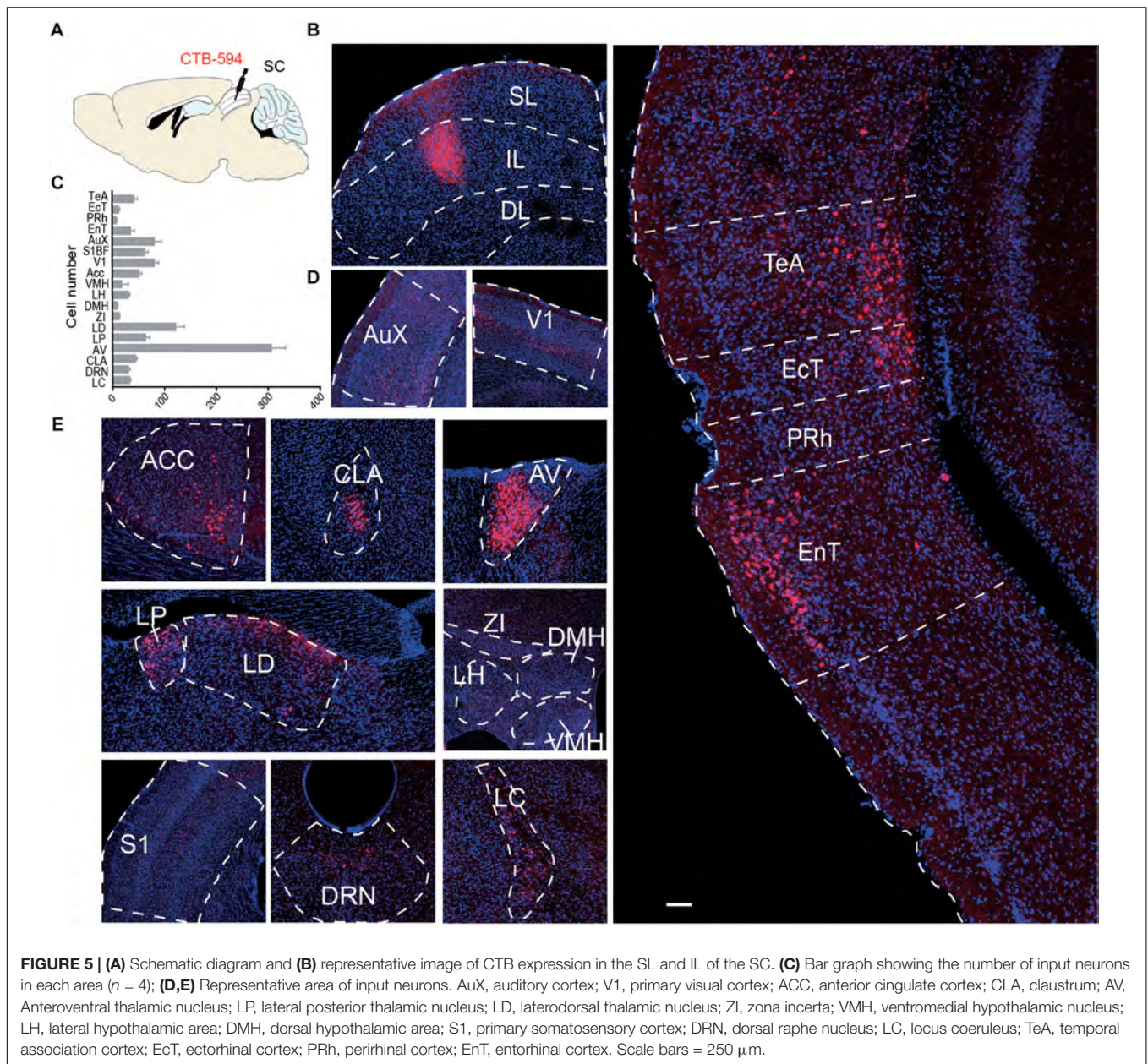
Our c-Fos network data analysis revealed that after gentle handling, the correlation became weaker in module 1, but was stronger in module 2, and the functional connection across modules 1 and 2 became much stronger, as seen in Figure 4B. This suggests that gentle handling reduces activity in fear emotional-related regions but enhances the sensory connection response to visual threat. Functional network analysis demonstrated the IL and DL of the SC, S1BF, V1, PVT, and



PAG, these brain areas have the highest participation coefficients across two modules. IL and DL of SC are thought to be involving in defensive behavior and are also functionally as the multisensory integration center (Sahibzada et al., 1986; Mooney et al., 1992). And in our study, IL and DL of SC are vital hubs to process both the sensory and visual threatening signals. S1BF and V1 are a part of primary sensory cortex, previous studies have defined the S1BF encode the representation of a sensory stimulus shaped by associative fear learning and V1 inputs to SC increase the response magnitude to looming (Gdalyahu et al., 2012; Zhao et al., 2014). Our recurrent gentle handling supposed to enhance the cortical plasticity and change the connection between cortex and subcortex which could lead to a change of defensive responses respond to overhead looming. PVT is a part of thalamus, which is recognized as relay station to transfer the sensory information, has proved to contribute to threatening events and fear memory (Penzo et al., 2015). PAG has defined to orchestrates sensory and motor (Koutsikou et al., 2015) and

initiation of escape to looming stimuli (Evans et al., 2018). Taken together, all these brain regions functionally as hubs in powerful way in sensory and fear related networks.

The SC is a laminar sensory-motor structure. The SL of SC is thought to process primarily visual signal, whereas the IL and DL of SC exert sensory responses and multimodal integration, contributing to saccadic eye movements and head movements (Wurtz and Goldberg, 1971; Stein, 1984). Our CTB retrograde tracing data indicated that the IL of the SC receives broad cortical and autonomic inputs. Another possible mechanism may be multimodal integration of the IL-SC, in which recurrent somatosensory stimuli enhance visual discrimination (Macaluso et al., 2000, 2002). Our *c-Fos* mapping data demonstrated that, gentle handling affects visual threat signals by preferentially influencing different layer of SC. That's *c-Fos* expression in gently handled mice was significantly higher in the IL and DL of the SC but not in the SL of the SC compared with the non-handled group respond to overhead looming stimuli.



In addition, our *c-Fos* functional network analysis confirmed that the IL has more intensive connections with the other regions than the DL and SL of the SC. However, the different functions of IL and DL have not been well defined. Thus, the neural mechanisms underlying interlaminar modulation of the SC needs further investigations (Mooney et al., 1992; Saito and Isa, 2005; Cohen and Castro-Alamancos, 2010; Ghitani et al., 2014).

The results of this study indicate that innate defensive responses are sensitive to external conditions, which are vital factors that influence animals' behaviors by affecting the internal state of brain. Our data consistent with the previous studies suggest that the environmental condition (e.g., housing, husbandry, habituation, handling, transport) and standard of

behavioral protocol are crucial for animal behavioral tests, especially the innate behavioral test (Grandin, 1997; Poole, 1997; Deacon, 2006; Baumans, 2009; Hurst and West, 2010; Clarkson et al., 2018).

Our current study revealed that gentle handling changes the activation of key brain regions that respond to danger signaling, indicating that these regions are vital for processing the interaction between sensory perception and fear emotion. The fact that recurrent sensory stimuli affect innate fear processing suggest that related neural circuits may be causally responsible for psychiatric diseases such as autism, of which the core characteristics are sensory and emotion deficits (Pernon et al., 2007; Cascio et al., 2012; Robertson and Baron-Cohen, 2017).

AUTHOR CONTRIBUTIONS

XL, LW, and FW designed the experiments. CC performed the behavioral tests. YL conducted the fiber photometry recording. CC, YL, and XL performed the histological studies. XL, CC, YL, ZW, and KH analyzed the data. XL, LW, and FW wrote the manuscript.

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Behavioral Effect of Plant Volatiles Binding to *Spodoptera littoralis* Larval Odorant Receptors

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Phytophagous insects use volatile organic compounds (VOC) emitted by plants to orient towards their hosts. In lepidopteran pests, crop damages are caused by larval stages—the caterpillars—that feed extensively on leaves or other plant tissues. However, larval host plant choice has been poorly studied, and it is generally admitted that caterpillars feed on the plant where the female laid the eggs. The mobility of caterpillars has been generally overlooked even though several studies showed that they can orient towards odors and change host plant. Recently, a large number of odorant receptors (ORs) tuned to plant volatiles have been characterized in the model pest moth *Spodoptera littoralis* (Noctuidae). In the present work, we identified nine of these orphanized ORs as expressed in *S. littoralis* caterpillars. In order to understand whether these ORs are involved in host searching, we tested the behavioral significance of their ligands using a larval two-choice assay. This OR-guided approach led to the identification of nine plant volatiles, namely 1-hexanol, benzyl alcohol, acetophenone, benzaldehyde, (Z)3-hexenol, (E)2-hexenol, indole, DMNT and (Z)3-hexenyl acetate, which are active on *S. littoralis* caterpillar behavior, increasing our knowledge on larval olfactory abilities. To further explore the link between OR activation and behavioral output induced by plant volatiles we used a modeling approach, thereby allowing identification of some ORs whose activation is related to caterpillar attraction. These ORs may be promising targets for future plant protection strategies.

Keywords: insect, olfaction, olfactory receptor, volatile organic compound, crop pest, caterpillar, Lepidoptera, Noctuidae

INTRODUCTION

Holometabolous insects are characterized by two mobile developmental stages with drastically different morphologies and physiologies. The larval stage constitutes a period of active feeding and growth, while the adult stage is a period devoted to reproduction and dispersal. Larvae and adults thus have different life styles, are not in competition for the same resources, and develop independent adaptations in response to different selective pressures. This distinction between adults and larvae is particularly striking in Lepidoptera. While larvae (or caterpillars) are actively feeding on their host plant, the adults generally live only a few days and feed on the nectar of flowers (Powell, 2009). Almost all plant species are damaged by caterpillars, many of which are pests of both crops and stored products (Stehr, 2009).

Host plant choice is a crucial task for phytophagous insects, and it is highly dependent on the sense of smell. The detection of plant-emitted volatile organic compounds (VOC) has been the subject of intense research, notably in crop pest insects (Bruce and Pickett, 2011; Bruce et al., 2015). In a number of lepidopteran pests, VOCs have been identified as attractants towards host plants, as repellents towards non-host or damaged plants or as oviposition stimulants (Saveer et al., 2012; Borrero-Echeverry et al., 2015). However, despite the impact of caterpillars on crop production, most studies focused on the adults and little is known about larval olfaction. A well-admitted theory, referred as “mother knows best,” assumes a strong selective pressure for females to lay their eggs on the plant where the larvae will have the highest performance (Jaenike, 1978; Carrasco et al., 2015). However, in some species it has been demonstrated that the caterpillars can leave the plant on which they hatched to select another host plant (Soler et al., 2012; Gamberale-Stille et al., 2014). Consistently, caterpillars exhibit attraction or repulsion behaviors towards VOCs of ecological significance (Carroll and Berenbaum, 2002; Huang and Mack, 2002; Singh and Mullick, 2002; Carroll et al., 2006, 2008; Castrejon et al., 2006; Becher and Guerin, 2009; Mooney et al., 2009; Piesik et al., 2009; Poivet et al., 2012; Zhu et al., 2016; Di et al., 2017) and are even able to perform associative learning (Blackiston et al., 2008; Salloum et al., 2011). This indicates that olfaction may play a more prominent role than initially expected in host plant choice of caterpillars, which could lay foundation for the development of novel pesticide-free strategies for fighting against those insects.

The peripheral olfactory system of caterpillars is generally composed of three olfactory sensilla located on the antennae, and four to five olfactory sensilla located on the maxillary palps (Grimes and Neunzig, 1986; Laue, 2000; Vogt et al., 2002; Roessingh et al., 2007; Poivet et al., 2012; Zielonka et al., 2016). These sensilla house the olfactory sensory neurons that express transmembrane odorant receptor (OR) proteins, which bind odorants and allow signal transduction (Leal, 2013). The repertoires of ORs expressed in caterpillar tissues have been identified only in a few species, such as the silkworm *Bombyx mori* (Tanaka et al., 2009), the cotton bollworm *Helicoverpa armigera* (Di et al., 2017) and the cotton leafworm *Spodoptera littoralis* (Poivet et al., 2013). In this latter species, 15 ORs (further referred as SlitORs) tuned to plant VOCs have been recently deorphanized (de Fouchier et al., 2017), i.e., their ligands have been identified (**Supplementary Figure S1**). These VOCs are mainly short-chain alcohols, aldehydes or esters (also referred as green leaf volatiles, abundantly released from damaged leaves), aromatics and terpenes (most of them being ubiquitous odorants, present in high amounts in floral bouquets). However, the effect of these SlitOR ligands on the behavior of *S. littoralis* larvae remains largely unknown. Among them, only 1-hexanol (a green leaf volatile) has been shown to be attractive at high dose toward 2nd and 3rd-instar larvae (Rharrabe et al., 2014).

In the present work, we first re-examined the expression pattern of the 15 deorphanized SlitORs in adult and larvae olfactory organs, and identified nine as expressed at the larval

stage. We then used a simple bioassay to carry out a systematic behavioral analysis of 14 VOCs previously identified as ligands of these nine SlitORs. Using this OR-guided approach, we found 1-hexanol, benzyl alcohol, acetophenone, benzaldehyde, (Z)3-hexenol, (E)2-hexenol, indole, DMNT and (Z)3-hexenyl acetate as active on the behavior of *S. littoralis* caterpillars, increasing our knowledge on larval olfactory abilities. Building on the results of these behavioral assays and on our previous knowledge of SlitOR response spectra (de Fouchier et al., 2017), we used a modeling approach in order to identify possible correlations between the activation of SlitORs and the behavioral response of caterpillars. By doing so, we highlighted ORs whose activation may be critical for larval attraction towards plant volatiles.

MATERIALS AND METHODS

Insects and Chemicals

S. littoralis larvae were reared on a semi-artificial diet (Poitout and Bues, 1974) at 22°C, 60% relative humidity and under a 16 h light: 8 h dark cycle. The panel of odorants tested was composed of 14 synthetic molecules (**Supplementary Table S1**) previously shown to be active on SlitORs expressed at the larval stage (de Fouchier et al., 2017). Odorants were diluted in paraffin oil (Sigma-Aldrich, St. Louis, MO, USA), except indole that was diluted in hexane (Carlo-Erba Reagents, Val de Reuil, France). The odorants were used at concentrations of 100, 10, 1, 0.1 or 0.01 µg/µl.

RNA Isolation and Reverse-Transcription PCR

Fifty *S. littoralis* male and female adult antennae and 50 pairs of 4th-instar larvae antennae and maxillary palps were dissected and immediately placed in TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) for total RNA extraction. After isolation using phenol-chloroform, RNA was purified using the RNeasy Micro Kit (Qiagen, Venlo, Netherlands), including a DNase I treatment. RNA purity and quantity were measured on a NanoDrop™ ND-2000 spectrophotometer (Thermo Fisher Scientific). cDNA synthesis was performed using 1 µg of total RNA as template, with the iScript Reverse Transcription Supermix (BioRad, Hercules, CA, USA). PCRs were performed using the LightCycler® 480 SYBR Green I Master mix (Roche, Basel, Switzerland) under the following conditions: 95°C for 5 min, followed by 40 cycles of denaturation (95°C for 10 s), hybridization (58–62°C—depending on primer pairs—for 15 s) and elongation (72°C for 15 s). Primer pairs were designed from SlitOR nucleotide sequences using Primer3Plus¹. All primer sequences, annealing temperatures and expected product sizes are listed in **Supplementary Table S2**. Orco, the obligatory OR co-receptor (Malpel et al., 2008; Leal, 2013), was used as control for the four tissues. For each amplification, negative controls consisted of amplifications run on DNase-treated RNAs and water templates. The amplification products were loaded on 1.5% agarose gels and visualized using GelRed™

¹<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>

Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). Tissue dissections, RNA extractions and RT-PCR experiments were repeated three times at different periods, to serve as biological replicates.

Behavioral Experiments

Two-choice behavioral assays were performed using *S. littoralis* 3rd and 4th-instar larvae, starved for 16–22 h prior to experiments. The behavioral assay consisted in placing 10 caterpillars in the center of a Petri dish. Filter papers were placed at two opposite sides of the dish. One was loaded with 10 μ l of an odorant solution and the other with 10 μ l of the corresponding solvent. Each odorant concentration was tested 10–15 times. For each experiment, 10 Petri dishes (containing 10 different odorants) and one control dish with solvent on both sides were recorded during 15 min. In each dish, two zones were defined around the filter papers, an “odorant” zone and a “solvent” zone (the layout of the zones are visible in **Figure 1**). The number of caterpillars in each zone was counted 2.5, 5, 10 and 15 min after the beginning of the experiment.

Data Analysis and Modeling

For each time point, a preference index (PI) was calculated using the following formula:

$$PI = (N_{\text{odorant}} - N_{\text{solvent}}) / (N_{\text{total}})$$

N_{odorant} being the number of larvae in the odorant zone, N_{solvent} being the number of larvae in the solvent zone and N_{total} being the total number of larvae in the assay. As this

PI varies between -1 and 1 , a positive value means that the odorant is attractive and a negative value indicates repellency. To test for the statistical significance of the observed PI, we compared the value to a theoretical value of 0 with a Wilcoxon two sided unpaired test using R (Package stats version 3.3.2).

In order to compare observed PIs with responses of the SlitORs (in spikes. s^{-1}) when expressed in the *Drosophila* empty neuron system (de Fouchier et al., 2017), we performed multiple linear regressions using the “step” and “lm” function of R (Package stats version 3.3.2). To obtain the most efficient equation, we performed stepwise linear regressions relating PI with all possible interactions between the larval SlitOR responses (SlitOR7, 14, 19, 24, 25, 27, 28, 29 and 31). As odorant stimulus quantities used in electrophysiology experiments cannot be directly related to quantities used in the present behavior experiments, we built models for different electrophysiology-behavior odorant quantity relationships (1:1, 1:1/10, 1:1/100 and 1:1/1,000). We selected the equation with the highest R^2 and refined it performing another stepwise multiple linear regression. This model relates the PI with all the interactions between the factors with an impact significantly different from zero ($\Pr(>t) p \leq 0.05$) in the previously selected model. To further simplify the model, we performed a last multiple linear regression relating PI with only additive interactions of the previously used variables.

We also built some models to further test the importance of the different SlitORs in predicting larval PI. One using all possible interactions between the responses of SlitOR14, 19, 28, 29 and 31, and four other models using linear regressions of the PI explained by the response from only SlitOR7, 24, 25 or 27.

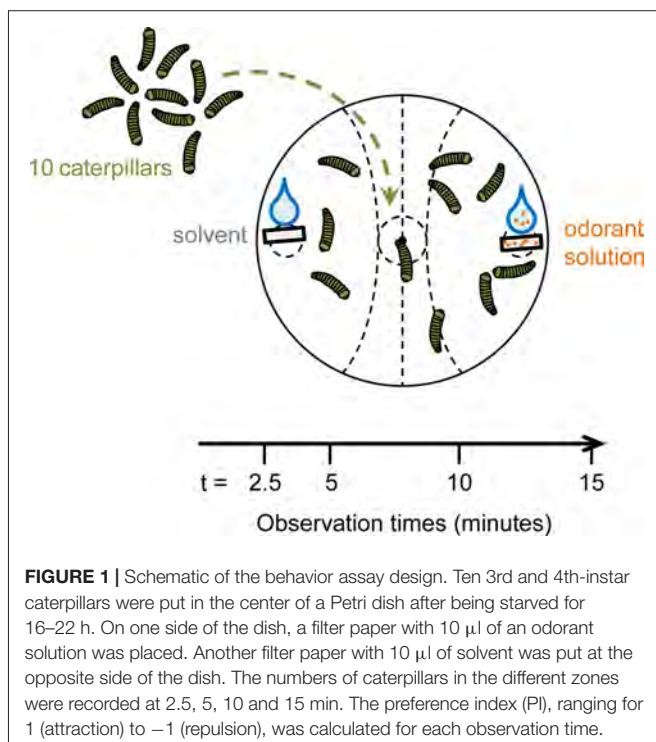
RESULTS

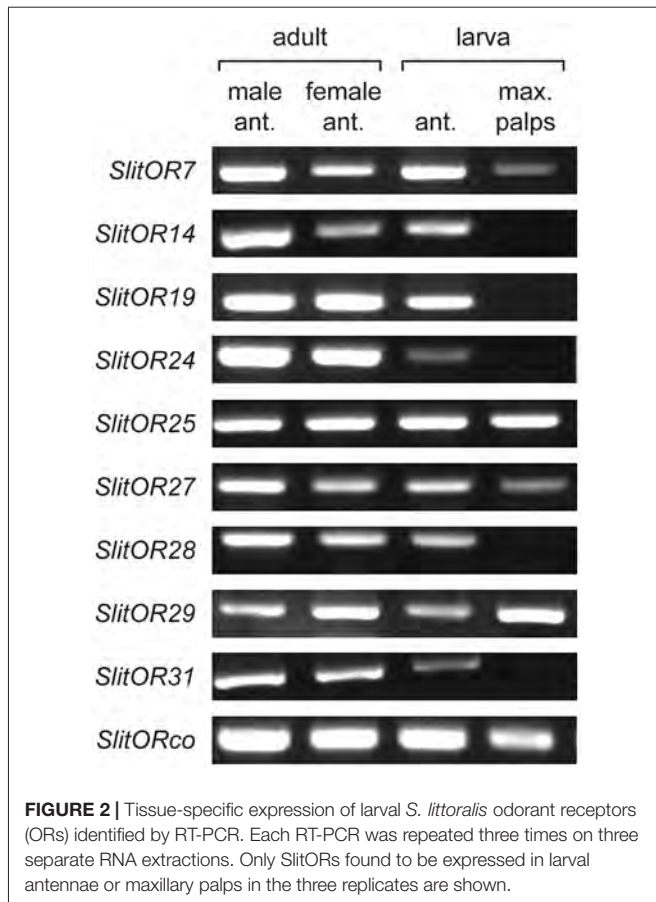
Expression of SlitORs at the Larval Stage

The expression pattern of 15 previously deorphanized SlitORs in male and female adult antennae, larval antennae and larval maxillary palps (4th-instar larvae) was re-investigated using RT-PCR. As found previously, all SlitORs were expressed in male and female antennae. Among them, nine SlitORs were also expressed in larval tissues (**Figure 2**). Five ORs were expressed in larval antennae (SlitOR14, 19, 24, 28 and 31), and four ORs were expressed in both larval antennae and maxillary palps (SlitOR7, 25, 27, 29). Altogether, these nine ORs were previously found to detect 20 plant VOCs (**Supplementary Figure S1**) among a panel of 50 molecules from different chemical classes, when expressed in the *Drosophila* empty neuron system (de Fouchier et al., 2017). We then selected a panel of 14 of these odorants, chosen based on the distinct OR activation patterns they elicit, in order to test their effect on larval behavior.

Behavior of *S. littoralis* Caterpillars Toward SlitOR Ligands

We assessed the valence of plant VOCs for *S. littoralis* caterpillars by describing their repartition in a two-choice





bioassay (Figure 1) using a PI over a period of 15 min. Figure 2 reports the PIs measured at 2.5 min for the different VOCs at different doses. PIs measured for other time points are presented in Supplementary Figure S2. For 2-phenyl acetaldehyde, 1-indanone, (E)-ocimene and eugenol, we observed no significant attraction ($PI > 0$) or repulsion ($PI < 0$), at any dose and any time. Benzyl alcohol, acetophenone, benzaldehyde, indole, 1-hexanol, (Z)3-hexenol and (E)2-hexenol were attractive at least at one dose, with the highest PI measured at 2.5 min (Figure 3). 1-hexanol displayed the strongest attraction, with a mean PI of 0.50 at 100 μg , and 0.44 at 10 μg . Benzyl alcohol was attractive over the wider range of doses, from 100 down to 1 μg per filter paper. Benzaldehyde elicited attraction at 100 and 10 μg , and acetophenone only at 100 μg . Indole was attractive at 10 and 0.1 μg only and (E)2-hexenol

was attractive only at 1 μg . For most of these VOCs, the PI tended to decrease over time (Supplementary Figure S2), which suggests that sensory adaptation occurred. The only stimulus that remained attractive over time was acetophenone, when presented at the highest dose (100 μg). (Z)3-hexenyl acetate differed from the previous VOCs as doses of 100 and 10 μg were found to be attractive after 5 min of experiment, and not after 2.5 min (Supplementary Figure S2).

At 2.5 min, benzaldehyde (at 0.1 μg) was the only VOC found to be repulsive (Figure 3). (Z)3-hexenyl acetate (1 μg) was repulsive after 5 min, and (E)2-hexenal and DMNT also induced a negative PI (for 0.1 and 100 μg , respectively) at 15 min of observation (Supplementary Figure S2).

Modeling of the Relationship Between SlitOR Activation and Behavioral Activity Induced by Their Ligands

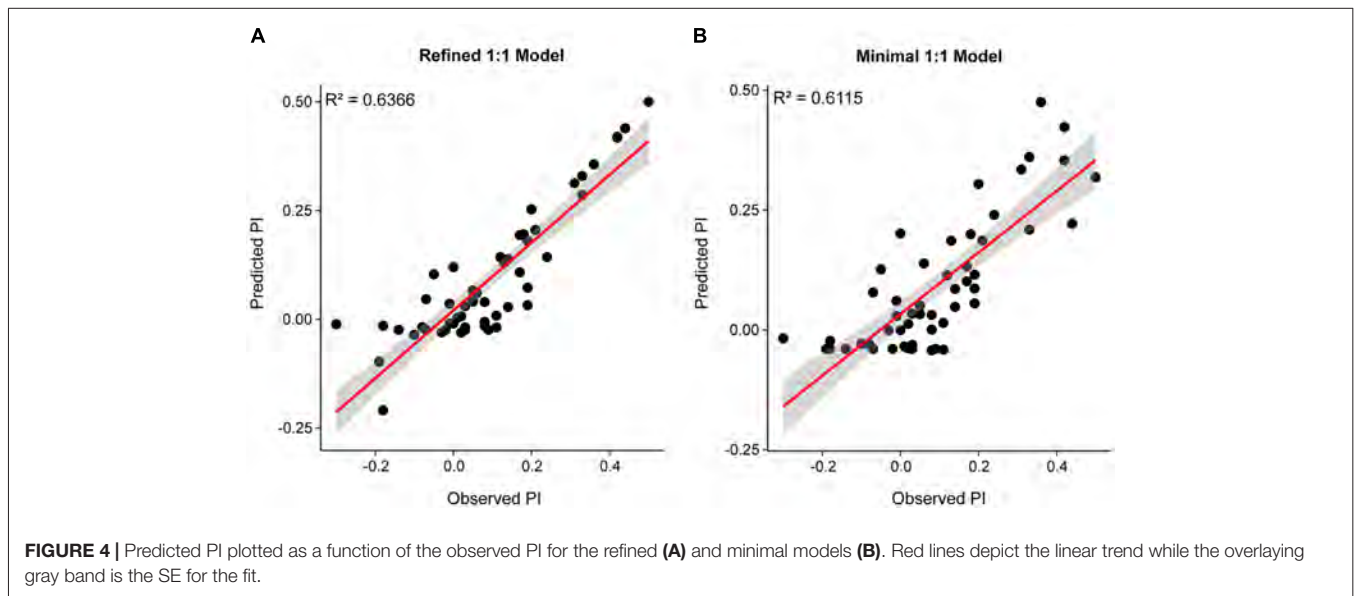
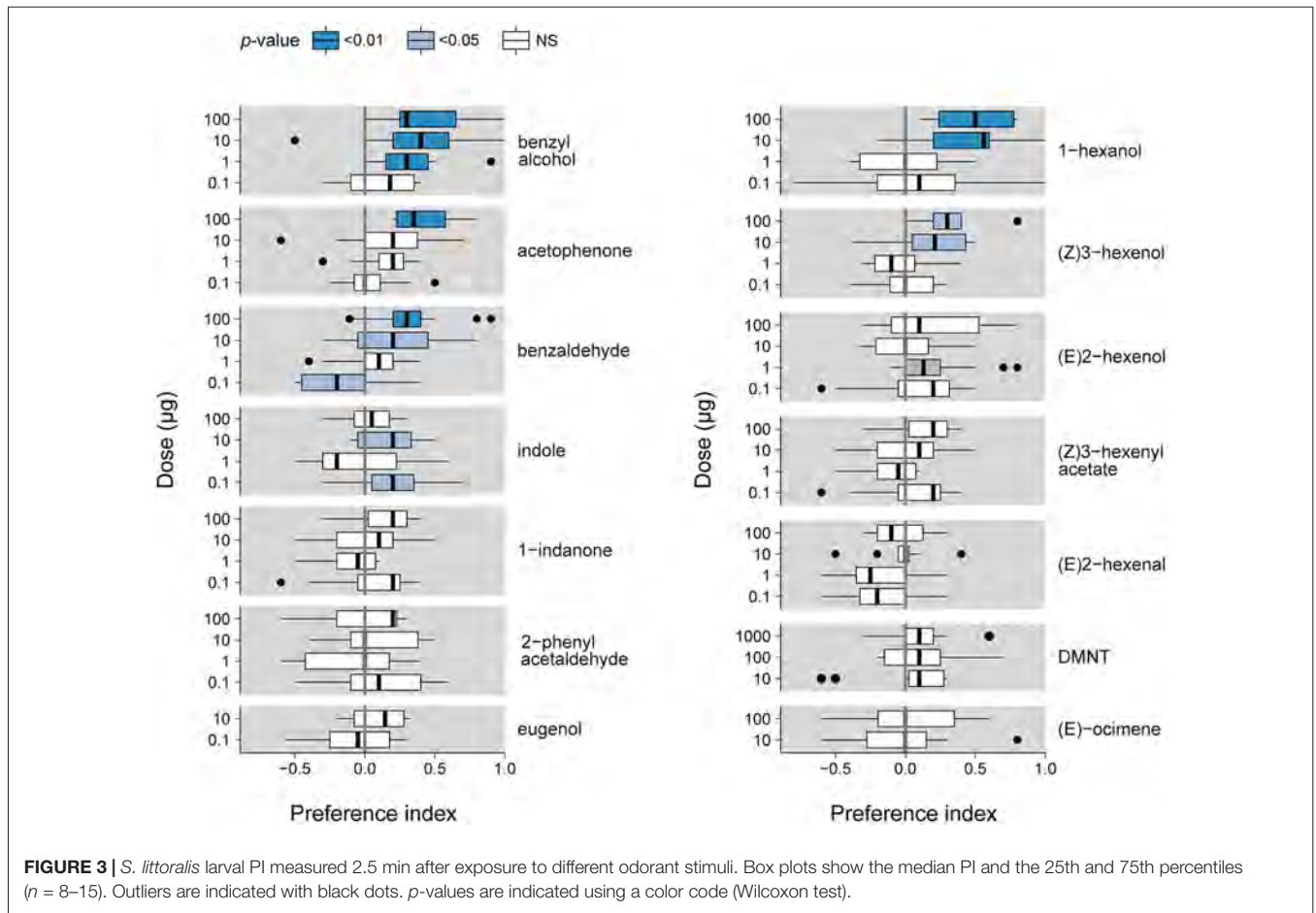
We next aimed to identify which of the SlitORs could be linked to attraction or repulsion towards plant VOCs. To assess the correlation between the valence of odorants and their activation pattern of ORs, we built models relating caterpillar PIs measured here with larval SlitOR responses to the same odorants (previously characterized in de Fouchier et al., 2017). We used stepwise multiple linear regressions, taking into account all possible interactions between the variables. The equations of the first models built are available in Supplementary Datasheet 2. The multiple linear regression giving the highest adjusted R^2 (0.6861) was the one using a 1:1 relationship between quantities used in behavior and electrophysiology experiments (Table 1).

To identify the SlitORs whose activation is the most critical to the valence of plant odorants for caterpillars, we refined the equation of the 1:1 model. For this, we performed stepwise multiple linear regressions taking into account all possible interactions between the factors with an effect significantly different from zero in the 1:1 model ($\text{Pr}(> t) p \leq 0.05$). This model was able to describe the variation of PIs from the responses of 5 SlitORs (SlitOR7, 14, 24, 25 and 27; F -Test, $p \leq 0.001$, $R^2 = 0.6366$, Table 1, Figure 4A and Supplementary Figure S3). The equation of the refined model is given in Supplementary Datasheet 2. The intercept value of this model was not different from 0 ($\text{Pr}(> t) p \geq 0.05$), which predicts that an absence of SlitOR activation would result in an absence of behavioral output. In this refined model, activation of SlitOR24 was predicted to have a positive effect by itself on PIs ($\text{Pr}(> t) p \leq 0.05$), whereas activations of SlitOR7, 25 and 27 were predicted to have an

TABLE 1 | SlitOR/behavior multiple linear regression model statistics.

Model	Adjusted R^2	Residual standard error	F-test	Shapiro test
Model 1:1	0.6861	0.09647	***	***
Model 1:1/10	0.6225	0.1048	***	NS
Model 1:1/100	0.5795	0.1106	***	*
Model 1:1/1000	0.3061	0.142	***	NS
Refined 1:1 model	0.6366	0.1038	***	**
Minimal 1:1 model	0.6115	0.1073	***	NS

Statistics associated with the models of *S. littoralis* caterpillars PIs. The Shapiro Test column indicates the p -value of a normality test for the distribution of the model residuals. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, NS: $p > 0.05$.



effect on PIs only through OR co-activation. SlitOR14 associated coefficients were not different from 0 ($\text{Pr}(> t) p \geq 0.05$).

As the refined model had a complicated equation (20 terms), we then built a simpler model to predict the behavior using only

additive interactions. The equation of this minimal model is:

$$PI = a + b \times SlitOR7 + c \times SlitOR24 + d \times SlitOR25 + e \times SlitOR27$$

with *SlitORx* as the ORx responses to the considered odorant in spikes. s^{-1} and *a-e* as coefficients. The values of these coefficients (available in **Supplementary Datasheet 2**) were all different from 0 ($\text{Pr}(> t) p \leq 0.05$), except for the intercept. The R^2 value for this model was 0.6115 (**Table 1**, **Figure 4B** and **Supplementary Figure S3**), which is comparable to the performances of the refined 1:1 model. SlitOR24 had the highest coefficient (2.6070×10^{-3} , $p \leq 0.001$), which further supports a link between this receptor and neuronal circuits driving attraction in *S. littoralis* larvae. It is interesting to note that the coefficient associated with SlitOR7 was negative (-5.0528×10^{-3} , $p \leq 0.05$). This predicts that activation of SlitOR7 has a negative effect of the PI of *S. littoralis* caterpillars.

To further confirm the importance of those four SlitORs for models performance in predicting the observed PI, we tried to build a model using all interactions between all the SlitORs except SlitOR7, 24, 25 and 27. The stepwise multiple linear regressions method was unable to produce a model from these variables, thus highlighting the importance of these receptors for the response of caterpillars to the VOCs tested. We also built models using the responses from only SlitOR7, 24, 25 or 27. The R^2 values for these models were respectively: 0.15, 0.48, 0.19 and 0.04. The values of the coefficients of the intercept and of the SlitOR response were different from 0 ($\text{Pr}(> t) p \leq 0.05$), except for the intercept of the model based on SlitOR24. These observations support that SlitOR24 is the most important receptor to predict the PI observed for the plant volatiles we tested.

DISCUSSION

Building upon the previous identification of ligands for a large number of *S. littoralis* ORs, we aimed at identifying behaviorally active odorants for caterpillars, which are pests feeding on a wide range of plants, notably economically important ones (Salama et al., 1971; Cabello, 1989; Thöming et al., 2013; von Mérey et al., 2013; Proffit et al., 2015). Nine *S. littoralis* ORs were confirmed to be expressed in larval chemosensory organs, namely the antennae and the maxillary palps. Our “OR-guided” strategy, by which we tested molecules active on these larval SlitORs, appeared as a good strategy as we could identify plant VOCs being behaviorally active when presented alone, most of them being attractive to caterpillars. Following that work, it will be of interest to test the effect of blends of these VOCs. It has been shown in *H. armigera* that a mixture of the best ligands of four ORs was the most attractive stimulus for first-instar larvae (Di et al., 2017), and one would expect that the same holds true for *S. littoralis*.

Our study complements a former study (Rharrabe et al., 2014) that investigated 11 odorants commonly emitted by plants, identifying only a small part of them as behaviorally active. In this previous work, eugenol was found to be repellent and 1-hexanol attractive. Here, attraction towards 1-hexanol could be reproduced in our assay but eugenol was inactive. This discrepancy could be explained by the fact that odorants and controls were presented together with food pellets in the aforementioned study while we used only filter papers as odor source. Hence, it is likely that repellent VOCs for *S. littoralis*

caterpillars may be identified only when given the choice between food sources (or food odors) with or without the VOC.

Another interesting difference between these two types of behavioral assays is that the presence of food will make the larvae stay on the food source once they have made a choice. In our experiments, larvae resumed foraging after their initial choice, which enabled to observe a decrease of the PI in most cases, likely due to sensory adaptation. Another possible explanation for this PI decrease would be that the volume of the Petri dish has been rapidly saturated with the odor, leading to a loss of the odor gradient necessary for larval orientation.

A similar OR-guided approach was recently used on another species of pest caterpillars, *H. armigera*, and led to the identification of several OR ligands that were active on the behavior of first-instar larvae (Di et al., 2017). Even if *S. littoralis* and *H. armigera* both belong to the same family (Noctuidae) and are both highly polyphagous herbivores, their larval OR repertoires seem to differ drastically. Indeed, the orthologs of only three of the nine larval SlitORs were also found to be expressed in *H. armigera* larvae (Di et al., 2017). The same holds true when comparing with the more distantly related species *B. mori* (Tanaka et al., 2009). Accordingly, a limited number of odorants identified as active on *S. littoralis* larvae are also active on other species, and vice versa.

The most attractive VOC (i.e., with the highest PI) was 1-hexanol, an ubiquitous plant volatile (Knudsen et al., 2006), which has been observed to be attractive for caterpillars of the Tortricidae *Lobesia botrana* (Becher and Guerin, 2009). Among other attractive compounds for *S. littoralis* larvae, (Z)3-hexenol was also observed to be attractive to *L. botrana* and *H. armigera* (Di et al., 2017), but not to *B. mori* (Tanaka et al., 2009). (Z)3-hexenyl acetate is a volatile released by plants that suffered attacks from insects and it has been reported to serve as a chemical message between plants (Frost et al., 2008; Helms et al., 2014). It has been observed to be attractive for the larvae of *S. littoralis* (this study), *H. armigera*, *L. botrana*, and *B. mori*. This suggests that (Z)3-hexenyl acetate is an important cue for a large spectrum of lepidopteran species. However, at a lower dose (1 μg), it is also the most repulsive VOC for *S. littoralis* caterpillars. Further experiments specially designed for the identification of repellents would be necessary to confirm this repulsive effect, but *S. littoralis* might use (Z)3-hexenyl acetate to detect and avoid damaged plants. Indeed, it has been demonstrated previously that *S. littoralis* larvae are able to discriminate between different leaves of a host plant and show a preference for young leaves, this preference being modified by herbivore damage (Anderson and Agrell, 2005). (Z)3-hexenyl acetate is detected via the activation of several ORs (de Fouchier et al., 2017). Their differential activation pattern relative to the dose may encode the concentration, as previously hypothesized for pheromone receptors detecting the same pheromone component in adults (de Fouchier et al., 2015).

From the comparison of behavior results with our previous results on SlitOR deorphanization (de Fouchier et al., 2017), we built models that can predict PI values for odorants based on their OR activation pattern. Results of this modeling approach suggest that larval attraction depends on the activation of a

particular subset of ORs (i.e., circuit-based) rather than on the summed response of the entire OR repertoire. This will be possible to confirm this hypothesis only when the complete larval OR repertoire will be characterized. In *D. melanogaster*, similar linear regression-based approaches allowed to predict larval behavior from the responses of only five ORs (Kreher et al., 2008). Still in *D. melanogaster*, a strong link has been identified between larval attraction and activation of two larval ORs, DmelOR42a and DmelOR42b (Kreher et al., 2008; Asahina et al., 2009; Grewal et al., 2014). Here, models supported that SlitOR24, 25 and 27 are involved in pro-attraction neuronal circuits, while SlitOR7 activation would antagonize attraction. Activation of the first three receptors, especially SlitOR24, seems to be sufficient to trigger attraction of *S. littoralis* toward different concentrations of odorants. This will need further experimental validation, notably by identifying new ligands for these receptors and testing their behavioral effect, but it could be a promising way to identify new compounds that could impact the behavior of this important crop pest.

AUTHOR CONTRIBUTIONS

AF, EJ-J and NM designed the study. AF, XS and ST performed behavioral experiments. GC-V performed molecular biology

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnbeh.2018.00264/full#supplementary-material>

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Visual Contrast Modulates Operant Learning Responses in Larval Zebrafish

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The larval zebrafish is a promising vertebrate model organism to study neural mechanisms underlying learning and memory due to its small brain and rich behavioral repertoire. Here, we report on a high-throughput operant conditioning system for zebrafish larvae, which can simultaneously train 12 fish to associate a visual conditioned pattern with electroshocks. We find that the learning responses can be enhanced by the visual contrast, not the spatial features of the conditioned patterns, highlighted by several behavioral metrics. By further characterizing the learning curves as well as memory extinction, we demonstrate that the percentage of learners and the memory length increase as the conditioned pattern becomes darker. Finally, little difference in operant learning responses was found between AB wild-type fish and *elavl3:H2B-GCaMP6f* transgenic fish.

Keywords: zebrafish larvae, behavioral neuroscience, learning, vision, high-throughput imaging, automated image analysis

INTRODUCTION

In operant conditioning, an animal learns to correlate its behavioral responses with consequences. Responses leading to satisfying consequences are reinforced whereas those leading to negative consequences are weakened or discarded. This form of associative learning has been intensively studied on behavioral, cellular, and molecular levels (Freund and Walker, 1972; Brembs et al., 2002; Nargeot and Simmers, 2011; Ishikawa et al., 2014), and many factors, such as dopaminergic signaling (Wise, 2004; Wassum et al., 2011; Steinberg et al., 2013) and Hebbian plasticity (Bi and Poo, 1998; Cassenaer and Laurent, 2007; Froemke et al., 2010), are known to play critical roles. Nevertheless, it remains largely elusive how *in vivo* learning rules, by which local synaptic plasticity and reward signaling must be integrated across distributed brain circuits, subserves adaptive animal behaviors. To make progress, it would be illuminating to measure neural activity of defined cell types at the whole-brain scale during the entire learning process.

The larval zebrafish is a promising vertebrate model to identify brain-wide mechanisms underlying learning and memory: its small brain is a great compromise between system complexity and simplicity. Recently, it has become possible to perform whole-brain imaging of calcium activity in freely behaving larval zebrafish (Cong et al., 2017; Kim et al., 2017). Whereas, fish are well-established animal models to study learning and memory (Davis and Agranoff, 1966; Agranoff and Davis, 1968), few associative learning paradigms have been developed for zebrafish larvae. Li (2012) reported operant learning in head-fixed larvae, in which fish learned to correlate the relief of

aversive heat stimulus with biased tail turning. Valente et al. (2012) showed that 1-week larvae were unable to perform an operant learning paradigm, in which fish must learn to swim to the other half of an arena to avoid electroshocks. Other reports demonstrated that larval zebrafish could be classically conditioned: they could associate the conditioned stimulus (CS)—a moving spot with the unconditioned stimulus (US)—a touch of the body (Aizenberg and Schuman, 2011). Social reward, such as visual access to conspecifics, could also be paired with a distinct visual environment cue during classical conditioning in larval zebrafish (Hinz et al., 2013).

Zebrafish have sophisticated vision. Adult zebrafish can distinguish colors (Colwill et al., 2005; Zimmermann et al., 2018) and visual patterns with different orientations (Colwill et al., 2005). Spatial and non-spatial visual learning tasks have been studied in adult zebrafish (Arthur and Levin, 2001). The visual system of zebrafish develops rapidly. 70–80 hpf (hour after post-fertilization) larval zebrafish can respond to abrupt light intensity change (Easter and Nicola, 1996), and exhibit optokinetic responses to rotating illuminated stripes (Huang and Neuhaus, 2008; Portugues and Engert, 2009; Mueller and Neuhaus, 2010). Seven-days-old larval zebrafish show strong optomotor responses to sophisticated motion stimuli (Orger et al., 2000; Roeser and Baier, 2003; Orger and Baier, 2005). However, much less is known about how properties of visual stimuli would modulate learning process in larval zebrafish.

Here, we report a modified operant conditioning paradigm (Valente et al., 2012) in freely swimming larval zebrafish, a system that combines a high-throughput automated training process and a toolkit for post-data analysis and storage. We use our new paradigm to investigate how visual contrast modulated the operant learning responses in larvae, characterized by both the positional and turning metrics. The measurements of learning curves provide a way to investigate memory extinction in larvae zebrafish. Moreover, we compare the differences between wild-type and transgenic fish in operant learning responses and memory extinction. We demonstrate that learning responses can be enhanced by a visual conditioned pattern that is darker than the background and that the percentage of learners as well as the memory length increase with darker conditioned patterns.

MATERIALS AND METHODS

Ethical Statement of Animals-Using

Handling and care of all animals were conducted in strict accordance with the guidelines and regulations set forth by University of Science and Technology of China (USTC) Animal Resources Center, and University Animal Care and Use Committee. Both raising and training protocols were approved by the Committee on the Ethics of Animal Experiments of the USTC (permit number: USTCACUC1103013).

Abbreviations: dpf, days post-fertilization; fps, frames per second; SEM, standard error of the mean; BLITZ, behavioral learning in the zebrafish; ABLITZER, the analyzer of BLITZ results.

Animals and Raising

Zebrafish (*Danio rerio*) of the genotype *elavl3:H2B-GCaMP6f* and AB wild-type fish were used in all experiments. All tested fish were 7–10 dpf (day past fertilization) larvae. They were bred, raised, and housed in the same environment. Fish were fed two times per day from 6 dpf with paramecium in the morning (8–9 a.m.) and evening (6–7 p.m.) before being used in experiments. Water was replaced with E2 medium (Cunliffe, 2003) in the morning (8–9 a.m.) and evening (6–7 p.m.). Water temperature was maintained at 28.5°C. Light was turned on at 08:00 a.m. and off at 10:00 p.m.

Experimental Setup

The behavioral system with custom software suites and supporting hardware was built to achieve an end-to-end high-throughput experimental workflow (Figure 1A).

Hardware

Zebrafish swam freely in custom-built acrylic containers with transparent bottoms. Each container was divided into four arenas separated by opaque walls. The arena's size is 3 cm × 3 cm × 1 cm, with water filled (Supplementary Figure 7). Each arena held one fish. Three CMOS cameras (Basler aca2000-165umNIR, Germany) with adjustable lens (Canon, Model EF-S 18-55mm f/3.5-5.6 IS II, Japan) simultaneously captured swimming behaviors at 10 frames per second. Three infrared LEDs (Kemai Vision, China, model HF-FX90, wavelength 940 nm) illuminated each container from below. A 700 nm long-pass filter (Thorlabs FEL0700, US.) was positioned in front of each camera to block visible light and to facilitate online imaging processing with custom software BLITZ. Visual stimuli were projected onto three containers (PIQS Projector S1, 14.6 × 7.85 × 1.75 cm, 854 × 480 pixels). Electroshocks (100 ms, 9 Volt/3 cm) were delivered via two platinum filaments, one on each side of the arena. Shock delivery at each arena was controlled by custom software BLITZ via a 16-channel relay (HongFa JQC-3FE, China). Room temperature was controlled by an air-conditioner at 27°C.

Software Suites

Custom C++ software BLITZ (Behavioral Learning In The Zebrafish), which inherited the coding style from MindControl (Leifer et al., 2011), processed three video streams in parallel to obtain real-time head, center, tail positions and heading angle by using the Pylon library (Basler AG, Germany) and the open source computer vision library (OpenCV) (Bradski, 2000). The program also rendered visual pattern and programmable electroshocks delivery based on the timeline and real-time fish motion parameters. Experimental information (e.g., experiment start time, visual pattern index, electroshock delivery information, and fish motion parameters) were recorded in YAML files. Raw videos were recorded.

The BLITZ software is available at <https://github.com/Wenlab/BLITZ>.

Another custom MATLAB (The MathWorks, Inc.) software ABLITZER (the Analyzer of BLITZ Results) was used to import YAML files, to visualize data, as well as to perform the behavioral and statistical analysis.

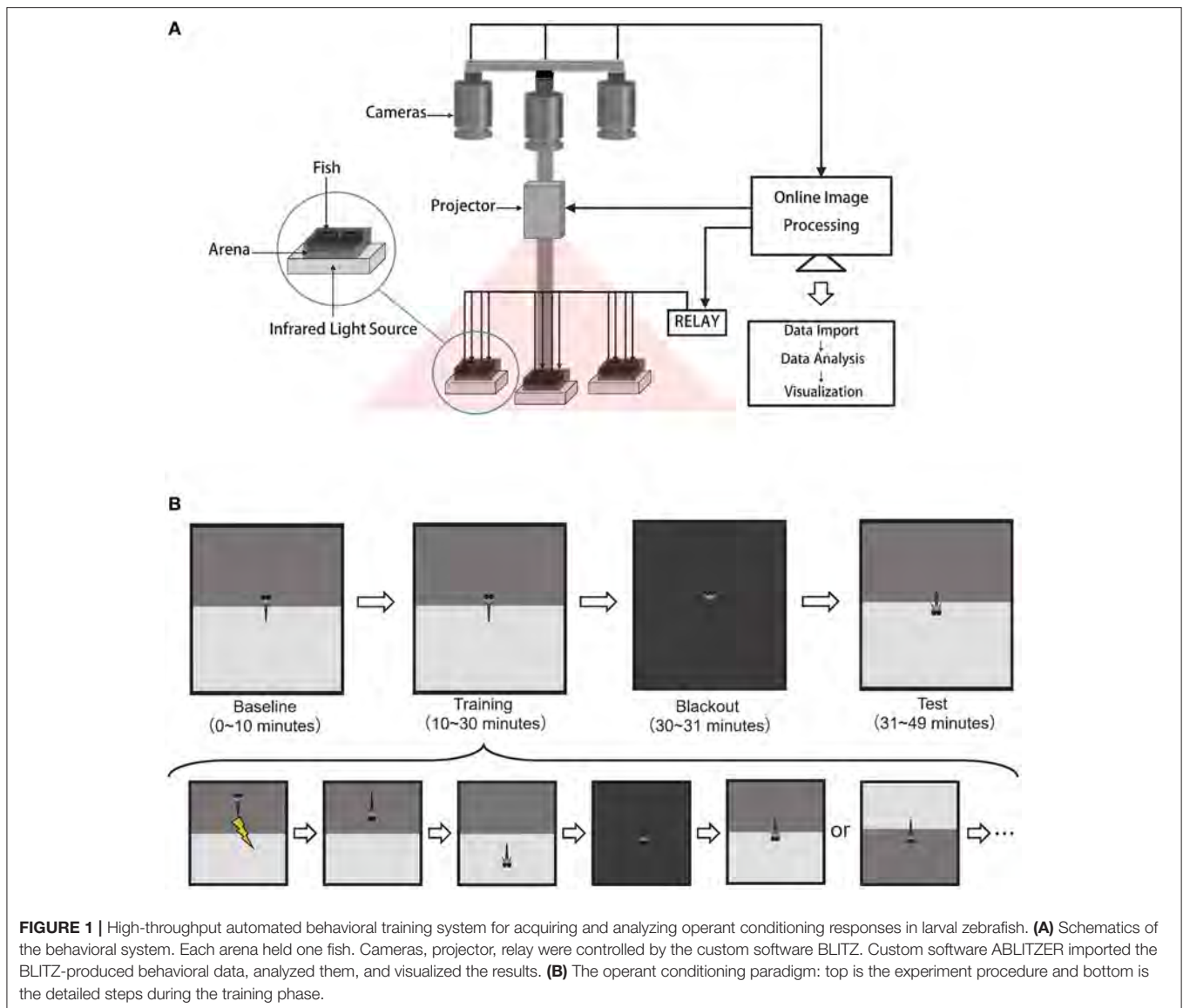


FIGURE 1 | High-throughput automated behavioral training system for acquiring and analyzing operant conditioning responses in larval zebrafish. **(A)** Schematics of the behavioral system. Each arena held one fish. Cameras, projector, relay were controlled by the custom software BLITZ. Custom software ABLITZER imported the BLITZ-produced behavioral data, analyzed them, and visualized the results. **(B)** The operant conditioning paradigm: top is the experiment procedure and bottom is the detailed steps during the training phase.

The ABLITZER software is available at <https://github.com/Wenlab/ABLITZER>.

Experimental Procedure

Fish were fed at least an hour before being used in the experiment. Fish were placed via a Pasteur pipette (Nest, US) from the raising tank to the experimental arenas. The behavioral experiment would not run until fish started moving around to avoid startle responses to novel stimuli. Fish in the paired-group were trained first with the self-control protocol (see below), and then with the operant conditioning protocol. Fish in the unpaired-group were trained first with the self-control protocol, and then with the unpaired operant conditioning protocol (see below).

Fish used in the paired-group and unpaired-group were all naive fish.

Operant Conditioning Protocol

This operant conditioning protocol was modified from Valente's learning paradigm (Valente et al., 2012). Here, fish would experience four different phases in order: baseline phase, training phase, blackout phase, and test phase (Figure 1B).

First, in the 10-min baseline phase, the visual pattern beneath each arena would flip between the CS at the top (Supplementary Figures 5A,C,E) and CS at the bottom (Supplementary Figures 5B,D,F) with a random duration that was uniformly sampled from 30 to 45 s.

Second, in the 20-min training phase, both the update of visual patterns and the delivery of electroshocks were dependent upon fish's behavior. After the visual pattern was updated (including the first visual pattern in the training stage), there was a 7-s delay for fish to make decisions before electroshocks were delivered based on their positions. If fish were in the CS zone after the delay time, whole-arena shocks would be delivered every 3 s until fish

TABLE 1 | Visual contrasts of all conditioned patterns.

	Mean RGB value	Grayscale value	Visual contrast	Light irradiance ($\mu\text{W}/\text{cm}^2$)
Grayscale 0	(0, 0, 0)	0	-128	9.9
Grayscale 32	(32, 32, 32)	32	-96	28.9
Grayscale 43	(43, 43, 43)	43	-85	30.8
Grayscale 64	(64, 64, 64)	64	-64	36.9
Grayscale 96	(96, 96, 96)	96	-32	53.7
Red-black checkerboard	(128, 0, 0)	43	-85	40.7
White-black checkerboard	(128, 128, 128)	128	0	102.4
Background (grayscale 128)	(128, 128, 128)	128	0	108.1

In the table, the first column lists all conditioned patterns used in the experiments. Visual contrast = grayscale value of conditioned pattern—grayscale value of background (128).

escaped from the CS zone. After fish stayed in the Non-CS zone for 48 s, the visual pattern (CS zone at the top or bottom) would update with equal probability. The whole procedure would repeat (Figure 1B bottom).

After the training phase, there was a 1-min blackout phase to deprive all visual stimuli.

Finally, in the last 18-min test phase, to ask whether fish could develop the association between the CS and US, the visual pattern interchanged every 2 min between the CS at the top and CS at the bottom.

Self-Control Conditioning Protocol

All phases were identical to the operant conditioning protocol, except for no electroshock delivery.

Unpaired Operant Conditioning Protocol

All phases were identical to the operant conditioning protocol except for the training phase, in which electroshocks, without pairing with visual patterns, were randomly delivered across the 20-min duration.

Behavioral Analysis

Visual Contrast

The visual contrast was defined as the grayscale value difference between the conditioned pattern and the background pattern (pure-gray) (see Table 1 for more details). The light irradiance was measured with a power meter (PM16-130, Thorlabs) that averaged the received light over 50 s.

Pre-screening

We defined data quality as the percentage of non-frozen frames. Frames were considered frozen when fish did not move for over 1 s. Fish with data quality lower than 0.95 were excluded from the analysis since those fish did not swim spontaneously and frequently. Fish with poor data quality were considered unhealthy.

The positional index was defined as the percentage of frames when fish were in the non-CS zone.

Turning Analysis

We scored a turning event when the heading-angle-change between two consecutive frames exceeded 15 degrees. Fish would get +1 score when performing an escape turn, and -1 score

when returning to the CS zone. Fish in the Non-CS zone executed an escape turn when they approached the midline (within twice body length) and then turned back (Supplementary Figure 6). The turning index was defined as

$$\text{turning index} = \frac{1}{2} + \frac{s(+)+s(-)}{(|s(+)|+|s(-)|)\cdot 2}$$

where, $s(+)$ and $s(-)$ are positive and negative scores, respectively. In this way, the turning index would fall between 0 and 1, the same range as the positional index.

Distance to the Mid-line

This was defined as a signed Euclidean distance from the fish head position to the mid-line. The sign was -1 when fish were in the CS zone and +1 when fish were in the non-CS zone.

Learning Analysis

To evaluate whether fish learned the operant conditioning task, we divided the entire operant conditioning protocol time into 24 2-min-epochs. The learning responses diminished in the absence of electroshocks during the test phase, which is known as memory extinction (Myers and Davis, 2007). The extinction point was computed as the first time when the positional index within an epoch dropped below the baseline. The recall period was defined from the starting time of test phase to the extinction point. Here we use memory length or recall period interchangeably. If the positional indices in the recall period were significantly higher than those in the baseline phase, fish were classified as learners (The unpaired t -test was applied). The extinction rate was defined as

$$\text{extinction rate} = \frac{PI_1 - PI_2}{\text{Memory Length}}$$

where, PI_1 is the positional index of all learners of the first peak at the beginning of test phase, PI_2 is the positional index of all learners of the nearest valley to the extinction point.

The positional index increase is the difference between the mean positional index in the recall period and the mean index in the baseline period; the turning index increase is the difference between the mean turning index in the recall period and the mean index in the baseline period.

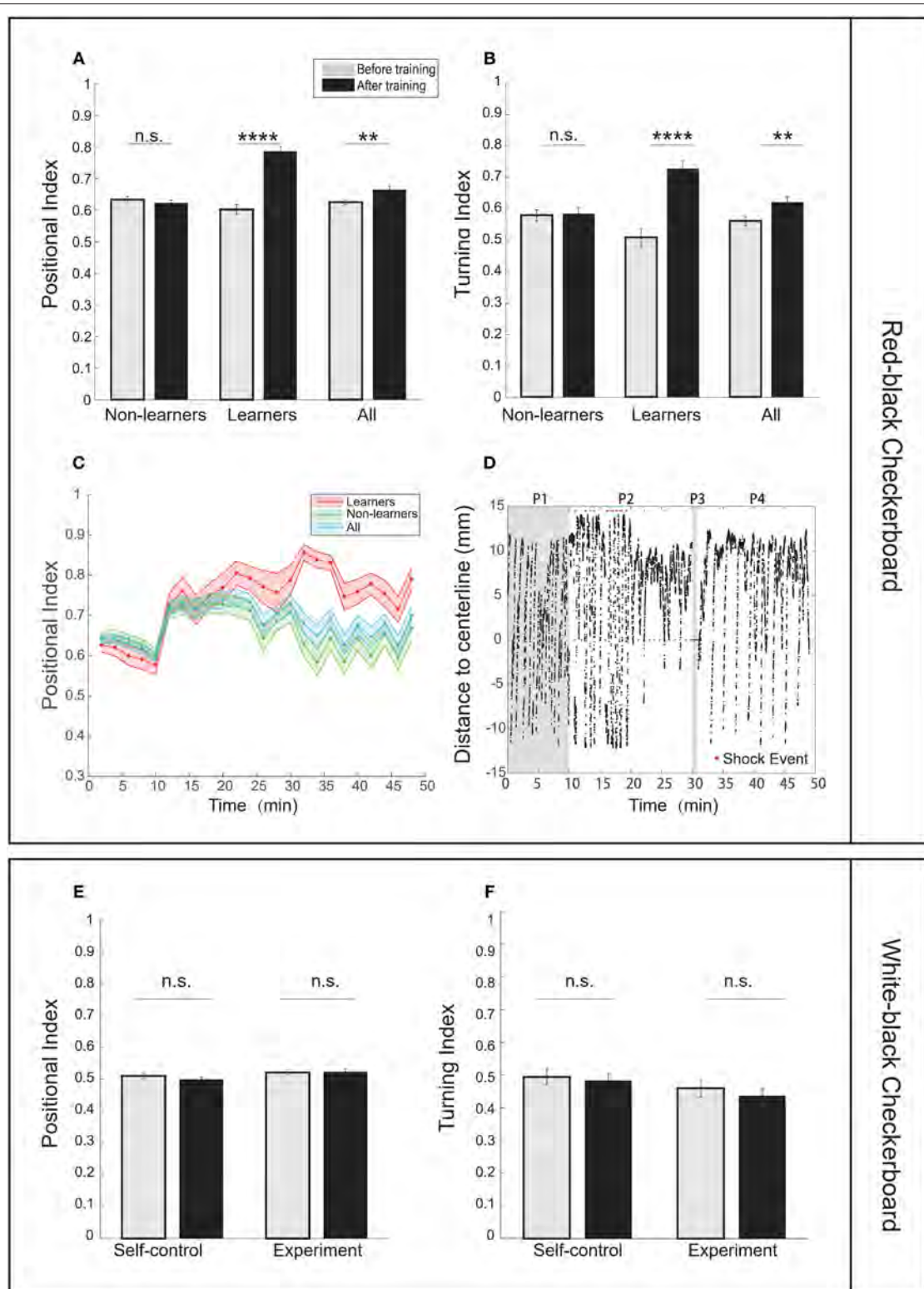


FIGURE 2 | learners, $p = 0.3849$ for non-learners, and $p = 0.0020$ for all fish). **(B)** Learners also showed significant differences in the turning index (t -test, $p = 2.28e-6$ for learners, $p = 0.8606$ for non-learners, and $p = 0.0099$ for all fish). **(C)** Learning curves using the positional index in the experiment group. Fish were classified as learners (red shaded line) and non-learners (green shaded line). The entire training process was divided into 2-min epochs (CS zone: red-black checkerboard). **(D)** A typical learner's behavioral trace, characterized by the relative position to the midline. The red-black checkerboard conditioned pattern was presented to the animal. A positive distance implies fish staying in the non-CS zone (also see section Materials and Methods). Each red dot represents the delivery of one electroshock. (P1: baseline phase; P2: training phase; P3: blackout phase; P4: test phase). **(E)** Analysis of the positional index suggests that fish did not show significant learning responses when the white-black checkerboard was presented as the conditioned pattern (t -test, $p = 0.8832$ for the experiment group, $p = 0.2493$ for the self-control group). We found that only one fish could be classified as learner. Because no significant learning response was found in the experiment group, we did not carry out unpaired-control experiments. **(F)** Analysis of the turning index suggests that fish did not show significant learning responses (t -test, $p = 0.3750$ for the experiment group, $p = 0.7089$ for the self-control group. There was no unpaired-control group because no significant learning response was found in the experiment group). All error bars are SEM. ** $p < 0.01$, **** $p < 0.0001$.

TABLE 2 | Percentages of learners in AB wild-type and transgenic fish.

	Grayscale 0	Grayscale 32	Grayscale 43	Grayscale 64	Grayscale 96	Red-black checkerboard	White-black checkerboard
<i>elavl3</i>	21/44 (50%)	11/39 (28%)	9/39 (23%)	8/38 (21%)	1/33 (3%)	27/104 (26%)	1/37 (3%)
AB/WT	22/44 (50%)	8/36 (22%)	10/46 (22%)	11/44 (25%)	1/41 (2%)	17/68 (25%)	1/16 (6%)

The numerator is the number of learners and the denominator is the total number of fish.

Statistical Analysis

The paired t -tests were used to compare the difference of the same fish between different phases in the same conditioning protocol; whereas the unpaired t -tests were used for the comparison between fish trained with the unpaired operant conditioning protocol and those with operant conditioning protocol. The sample size exceeded 20 for all tests.

RESULTS

Larval Zebrafish Show Significant Learning Responses in an Operant Conditioning Task

In our modified operant conditioning task (**Figure 1B**), larval zebrafish Tg (*elavl3:H2B-gcamp6f*) freely swam in an arena divided by two distinct patterns, each of which was projected onto one half of a transparent floor. In all cases, a pure-gray (grayscale-128) visual pattern was presented on the non-CS zone, and the CS patterns were presented on the other half. The CS was paired with the US—moderate electroshocks. The delivery of US and the update of visual patterns depended upon fish's positions and time (see Materials and Methods for detailed experimental procedures).

To scale up the training process, we developed a high-throughput operant conditioning system (**Figure 1A**) with custom supporting software suites BLITZ and ABLITZER (see Materials and Methods) that allowed training twelve fish simultaneously. BLITZ provided a fully automated workflow from video capture, online image processing, to visual stimuli presentation and electroshocks delivery for all behavioral protocols. Raw experimental data were then imported, analyzed and visualized by ABLITZER.

First, we tested several stimulus patterns in our operant conditioning task. When the red-black checkerboard was used as the conditioned pattern, 7–10 dpf zebrafish larvae showed significant learning responses (**Figures 2A,B**,

Supplementary Videos 1, 2), evaluated based on two metrics—the positional index and turning index (see Material and Methods). By analyzing individual fish behavior, we classified 27 out of 104 (26%) fish as learners (**Table 2**) (see section Materials and Methods for criteria). Learners showed significant increase in the positional and turning indices after training, whereas non-learners did not (**Figures 2A,B**).

Larval zebrafish have an innate positive light preference. Therefore, we developed two control settings: the self-control conditioning protocol in which no electroshock was delivered and the unpaired operant conditioning protocol in which electroshocks were randomly delivered (see Materials and Methods). Results from the two control settings were compared with those from the operant conditioning protocol to determine whether fish established the association (**Supplementary Figures 1I,J**). **Figure 2C** shows how the learning curves of learners, non-learners, and all fish, characterized by the positional index, changed during the entire learning process. **Figure 2D** shows a typical trajectory of a learner who tended to avoid the conditioned visual pattern after training.

We asked whether spatial features of the checkerboard alone could induce learning responses. However, when using the white-black checkerboard as the conditioned pattern, we found that fish showed little learning response after training (**Figures 2E,F**).

Visual Contrast Modulates the Percentage of Learners in the Operant Conditioning Task

We asked whether visual contrast—the grayscale value difference between a conditioned pattern and background (grayscale-128)—would modulate learning. Because light irradiance from the projector increases with the grayscale value of an image (**Table 1** and **Supplementary Figure 4**), we tested whether conditioned patterns with varying grayscale values (0, 32, 43, 64, and 96) would modulate operant learning responses.

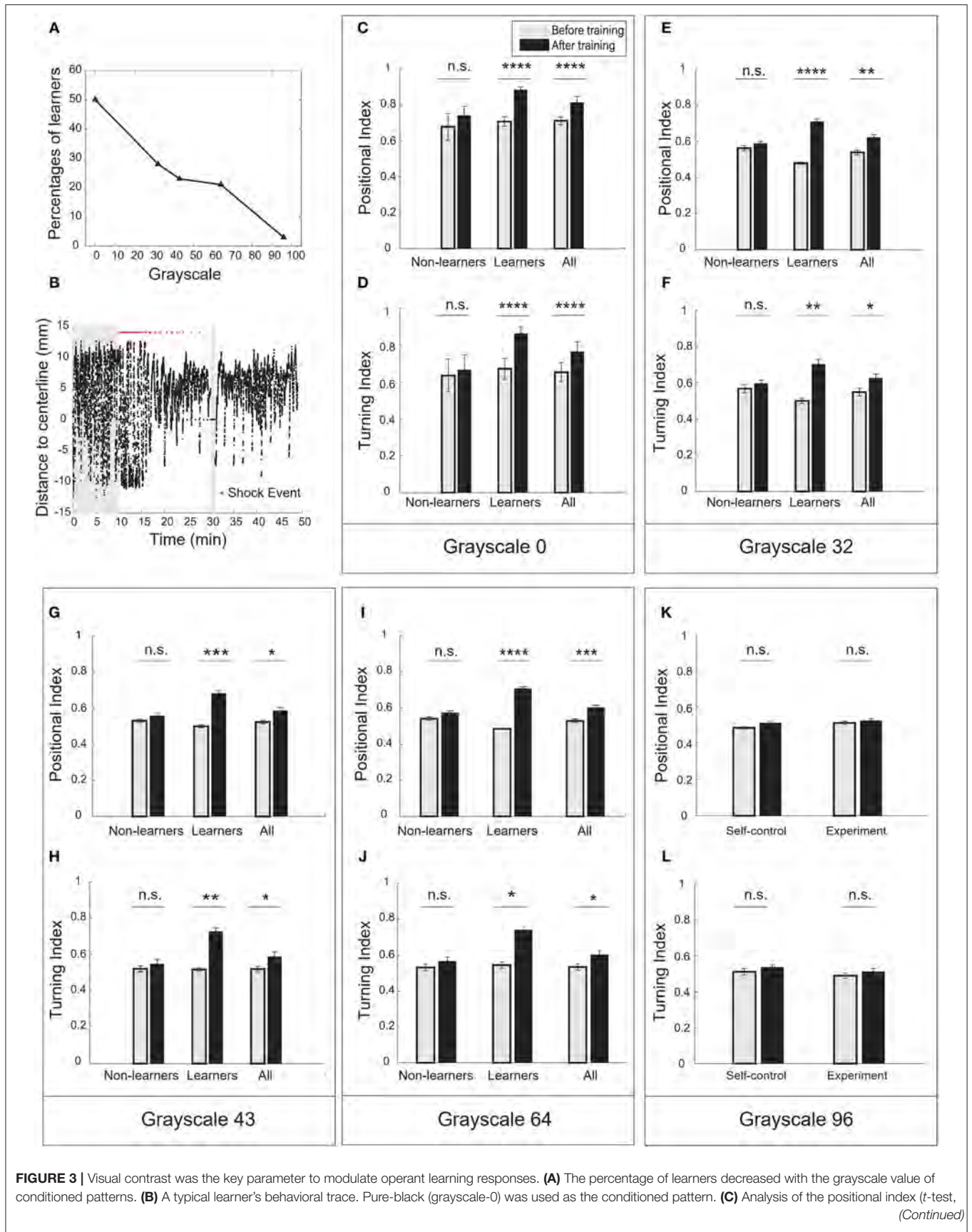


FIGURE 3 | Visual contrast was the key parameter to modulate operant learning responses. **(A)** The percentage of learners decreased with the grayscale value of conditioned patterns. **(B)** A typical learner's behavioral trace. Pure-black (grayscale-0) was used as the conditioned pattern. **(C)** Analysis of the positional index (*t*-test, *(Continued)*

FIGURE 3 | $p = 1.98 \times 10^{-11}$ for the learners, $p = 0.9492$ for the non-learners, and $p = 2.03 \times 10^{-6}$ for all fish). Pure-black (grayscale-0) was used as the conditioned pattern. **(D)** Learners also showed significant increase in the turning index (t -test, $p = 1.22 \times 10^{-5}$ for learners, $p = 0.6491$ for non-learners, and $p = 0.0057$ for all fish). **(E)** Analysis of the positional index (t -test, $p < 0.00001$ for learners, $p = 0.3252$ for non-learners, and $p = 0.0018$ for all fish). Grayscale-32 was the conditioned pattern. **(F)** Learners also showed significant increase in the turning index (t -test, $p = 0.0049$ for learners, $p = 0.4923$ for non-learners, and $p = 0.0331$ for all fish). **(G)** Analysis of the positional index (t -test, $p = 0.0005$ for learners, $p = 0.3182$ for non-learners, and $p = 0.0128$ for all fish). Grayscale-43 was the conditioned pattern. **(H)** Learners also showed significant increase in the turning index (t -test, $p = 0.0022$ for learners, $p = 0.5067$ for non-learners, and $p = 0.0498$ for all fish). **(I)** Analysis of the positional index (t -test, $p < 0.0001$ for learners, $p = 0.0927$ for non-learners, and $p = 0.0009$ for all fish). Grayscale-64 was the conditioned pattern. **(J)** Learners also showed significant increase in the turning index (t -test, $p = 0.0108$ for learners, $p = 0.3694$ for non-learners, and $p = 0.0480$ for all fish). **(K)** Analysis of the positional index (t -test, $p = 0.3130$ for the experiment group, $p = 0.0749$ for the self-control group). Grayscale-96 was the conditioned pattern. **(L)** Analysis of the turning index suggested that fish did not show significant learning responses (t -test, $p = 0.3360$ for the experiment group, $p = 0.3019$ for the self-control group). All error bars are SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

In the case of pure-black (grayscale-0), half of the fish population (21 out of 42, **Table 2**) can be classified as learners. They all showed significant increase in the positional index and the turning index after training (**Figures 3C,D**, **Supplementary Figures 1A,B**). **Figure 3B** showed a typical behavioral trace of a learner (see also **Supplementary Videos 3, 4**).

In the case of grayscale-96, however, fish showed little increase in the positional and turning indices (**Figures 3K,L**). We found only one fish can be classified as learner. In the other cases, a fraction of fish population exhibited learning responses (**Figures 3E–J**, **Supplementary Figures 1C–H**). The percentage of learners decreased with grayscale value (**Figure 3A**). Reversing the conditioned pattern and the background pattern in our paradigm, however, failed to elicit learning responses.

The red-black checkerboard and the grayscale-43 conditioned pattern had the same mean grayscale value (see **Table 1**). Consistently, similar percentages of fish (26 vs. 23%, **Table 2**) could learn the two conditioned patterns, respectively. Together, these results suggest that the visual contrast, not spatial checkerboard features, contributed to the operant learning responses.

Visual Contrast Modulates Memory Extinction in the Operant Conditioning Task

To investigate how the operant conditioning behavior changed over time, we divided the entire process into epochs (excluding the blackout phase). Every 2-min interval is one epoch. The baseline phase has five epochs; the training phase has 10 epochs; and the test phase has nine epochs.

We defined the memory extinction point as the first time when the positional index within an epoch dropped below the mean index in the baseline phase; we defined the duration from the start of the test phase to the extinction point as the memory length. Memory length shorter than two epochs (e.g., fish may stay still in the non-CS zone) were excluded (see Materials and Methods).

We plotted the learning curves—the positional index vs. time—for learners and non-learners (**Figures 4A–D**). In the case of pure-black, the learning curve of learners rose and approached the maximum near the end of training; during the test phase, the learning curve remained high across the entire test phase (**Figure 4A**). Note that a large percentage of fish (16/21) did not show memory extinction (**Figure 4G**).

In the cases of other grayscale conditioned patterns, the learning curves of learners also reached their peaks near the end

of training. However, all curves decayed after several epochs in the test phase (**Figures 4B–D**). The memory extinction points were similar (grayscale-32 at 45 min, grayscale-43 at 41 min, grayscale-64 at 41 min). **Figures 4E,F** illustrate a typical animal that learned the association and then experienced memory extinction in the test phase. The fish started swimming more in the CS-zone near 43 min.

In **Figure 4G**, we compared the distribution of memory lengths across all grayscale conditioned patterns. The mean memory length was the highest (970 s, **Table 4**) and the rate of extinction (see Materials and Methods) was the lowest in the case of pure-black conditioned pattern (**Table 3**).

We also found that the mean memory lengths (756 vs. 813 s) were similar when the red-black checkerboard and grayscale-43 pattern were used as the CS (**Table 4**). The percentages of learners that did not show memory extinction were also similar (12/27 vs. 5/9) in the two groups.

Wild-Type Fish Show Similar Learning Responses in the Operant Conditioning Task

To test whether the learning effect was strain-specific, we also performed the operant conditioning task in AB wild-type fish. Like transgenic fish, the percentage of learners in AB fish also decreased with the grayscale value of conditioned patterns (**Figure 5H**, **Supplementary Figures 2C–H, 3**): half of the fish population could be classified as learners in the case of pure-black (**Figures 5A–C**, **Supplementary Figures 2A,B**); whereas only one in the case of grayscale-96 (**Supplementary Figures 2I,J**). Some AB fish can learn the association between the red-black checkerboard and US (**Figures 5D–F**, **Supplementary Figures 2K,L**), but not between the white-black checkerboard and US (**Supplementary Figures 2M,N**).

We note that in the case of pure-black CS, more wild-type fish exhibited memory extinction (**Figure 5I**). **Figure 5G** shows a learner with memory extinction in the test phase. Fish started to swim more in the CS zone at 41 min.

DISCUSSION

Operant Learning in Larval Zebrafish

Operant learning allows animals to avoid danger or to find potential reward in a complex environment (Skinner, 1984). An earlier study (Li, 2012) demonstrated an operant learning paradigm for head-fixed zebrafish larvae, where ~75% larvae

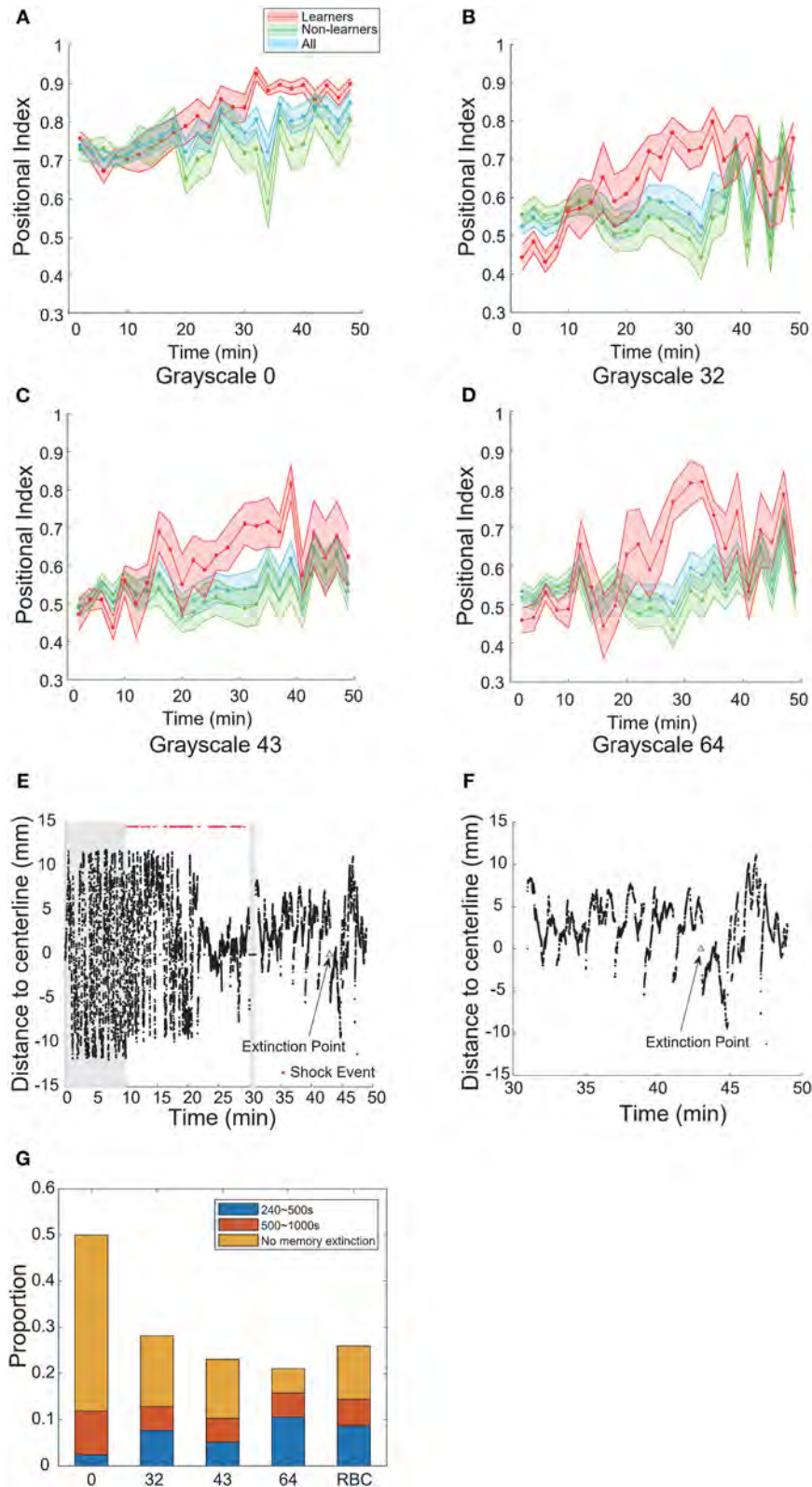


FIGURE 4 | Visual contrast modulated memory extinction. **(A–D)** Learning curves for different grayscale conditioned patterns. **(E)** A typical learner’s behavioral trace. Grayscale-32 was the conditioned pattern. The blue triangle indicates the extinction point. **(F)** A magnification of the test phase in **(E)**. **(G)** Memory length distribution for each conditioned pattern (grayscale values 0, 32, 43, 64, 96; RBC: red-black checkerboard). Blue: short-term memory fish (240–500 s); orange: mid-term memory fish (500–1,000 s); yellow: fish that did not show memory extinction during the entire test phase. All error bars are SEM.

TABLE 3 | Memory extinction rates.

	Grayscale 0	Grayscale 32	Grayscale 43	Grayscale 64	Red-black checkerboard
<i>elavl3</i> (min^{-1})	3.83e-3	4.68e-3	1.00e-3	3.00e-2	8.11e-3
<i>AB/WT</i> (min^{-1})	9.82e-3	8.33e-3	1.01e-2	2.01e-2	2.04e-2

TABLE 4 | Mean memory lengths of learners.

	Grayscale 0	Grayscale 32	Grayscale 43	Grayscale 64	Red-black checkerboard
<i>elavl3</i> (s)	970	807	813	570	756
<i>AB/WT</i> (s)	725	690	744	665	691

learned to correlate a positive consequence—the relief of heat exposure—with biased tail turning in the absence of conditioned cue.

In the current work, we demonstrated that a significant proportion of 7–10 dpf larval zebrafish showed significant operant learning responses when a darker-than-background conditioned pattern, such as a pure-black pattern, was paired with a punishment—moderate electroshock. In an earlier study (Valente et al., 2012), it was reported that 1-week larvae showed no significant learning response. Several factors may explain the discrepancy.

First, we observed little learning response when the white-black checkerboard or grayscale-96 pattern was paired with the US (only one fish learned the contingency), consistent with Valente's result. Enhancement of learning was observed, however, when a darker visual pattern, such as a pure-black pattern, was paired with the US.

Second, in our modified paradigm, fish possessed more opportunities to learn the contingency between the CS and US during the training period: when fish stayed in the non-CS zone for more than 48 s, the positions of CS and non-CS patterns would update. In Valente's paradigm, however, no visual pattern updates were implemented when fish stayed within the non-CS zone.

Visual Contrast Is the Key Parameter to Modulate Operant Learning Responses

Larval zebrafish can detect light intensity change at a very early age (Easter and Nicola, 1996; Emran et al., 2008). Opsins that are sensitive to long and short wavelength light start to be expressed in cone photoreceptors at 50 hpf (Raymond et al., 1995). During optomotor behaviors, 7 dpf larvae can detect motion stimuli by computing the spatiotemporal correlation of light intensities from nearby pixels in a visual pattern (Orger et al., 2000; Orger and Baier, 2005). Here, our study shows that the visual contrast, rather than spatial features in a visual pattern, is critical in modulating larval zebrafish operant learning responses.

Many studies have demonstrated that larval zebrafish exhibit positive phototaxis (Steenbergen et al., 2011; Chen and Engert, 2014; Guggiana-Nilo and Engert, 2016). In our behavioral paradigm, the behavioral metric baselines

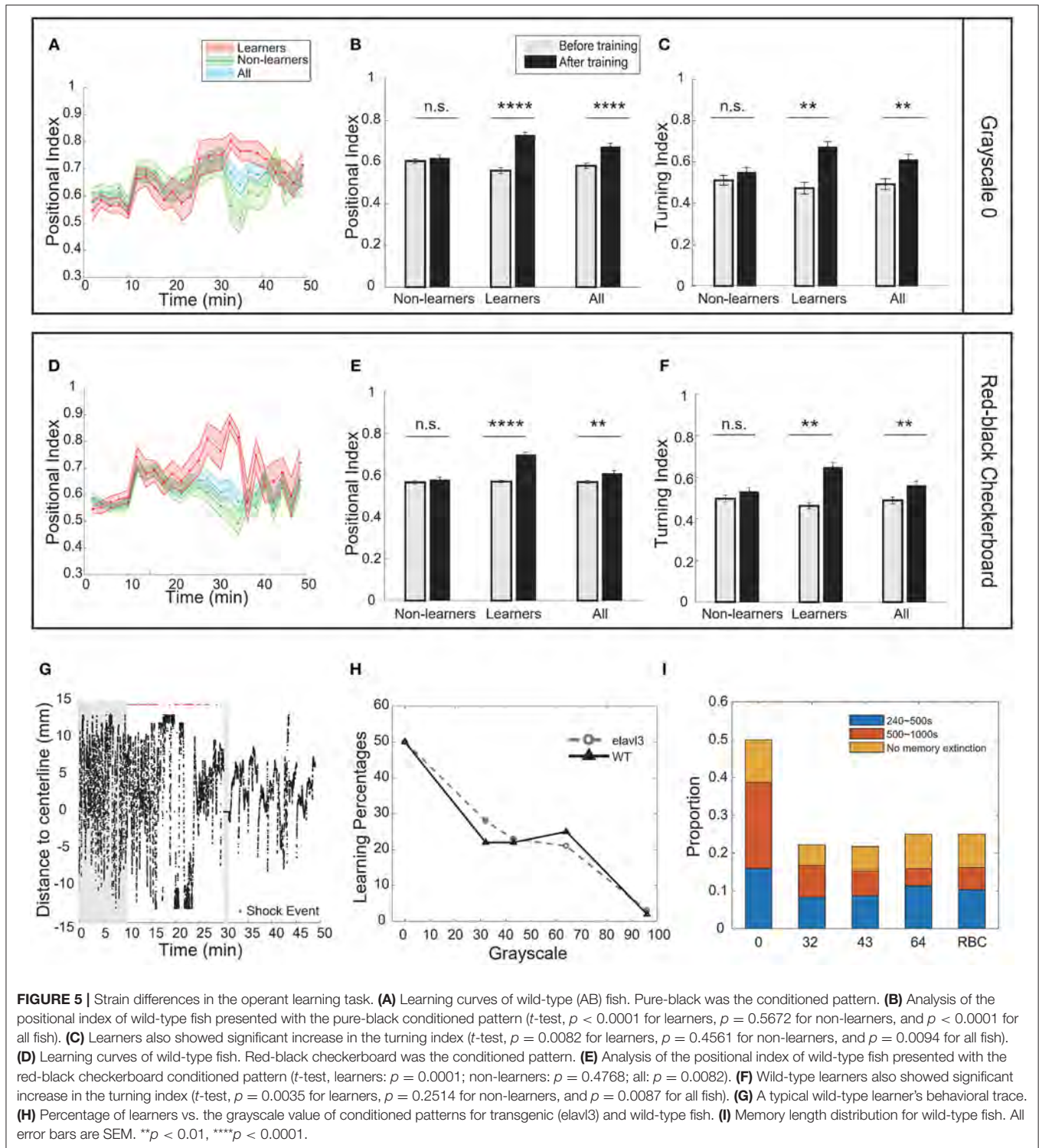
(e.g., positional index) were computed first before the training phase (see Materials and Methods). Light intensity could shift the baselines due to an innate illuminance bias. Significant changes in the behavioral metrics during and after operant conditioning (see **Figure 2**), however, require an explanation that goes beyond the innate avoidance response.

Here we speculate that this visual-contrast-dependent learning may arise from the crosstalk between phototaxis and fear conditioning circuits. Both phototaxis and US-triggered fear responses involve habenula (Agetsuma et al., 2010; Zhang et al., 2017), a specialized brain region where a direct association between a CS and fear may occur through synaptic plasticity. According to this model, a CS would trigger fear responses, and learning leads to a stronger association between visual-related inputs and escape responses. These predictions can potentially be tested by combining our behavioral system with whole brain calcium imaging in freely behaving larval zebrafish (Cong et al., 2017).

Memory Extinction in the Operant Conditioning Task

Memory extinction is an active learning process where an animal learns to dissociate the conditioned response and CS in the absence of US (Myers and Davis, 2007). In our assay, the extinction point was defined as the first epoch whose positional index dropped below the mean index of the baseline. In addition, fish that did not keep a high level of positional index for at least two epochs were not counted as learners (see Materials and Methods). When the pure-black pattern was used, few learners showed memory extinction before the test phase ended. The distribution of memory length (**Figure 4G**) is consistent with that in a recent classical conditioning paradigm in larval zebrafish (Aizenberg and Schuman, 2011). When other grayscale patterns were used as the CS, mean memory lengths were further reduced (**Table 4**).

We also computed the rate of memory extinction (see Materials and Methods) for different CS patterns. We found that the extinction rate increased with the grayscale value of conditioned patterns for both wild-type and transgenic strains (**Table 3**). Also, the extinction rate in wild-type strain was higher



than that in the transgenic strain when grayscale-0 was the CS (Figures 4G, 5I).

The above observations suggest that the dissociation between conditioned patterns and behavior could also be modulated by the visual contrast. Recent studies in *Drosophila* (Felsenberg

et al., 2017, 2018) showed that aversive memory formation and extinction involved different neural circuits and they competed with each other during decision-making. Identifying the neuronal circuitries underlying memory representation and extinction might help us test the hypothesis.

Strain Differences in the Operant Conditioning Task

We performed operational conditioning for both *elavl3:H2B-GCaMP6f* (in the *casper* mutant background) transgenic fish and wild-type (AB) fish and found that visual contrast modulated learning behaviors in similar ways (Figure 5). Nevertheless, more wild-type fish exhibited memory extinction when the pure-black conditioned pattern was used. A previous study (Parker et al., 2013) has systematically characterized the behaviors of wild-type (TU) fish and *casper* mutants. No difference was found in adult fish, yet wild-type fish showed a significant increase in anxiety when treated with 1-phenyl-2-thiourea (PTU), a drug that is used to maintain the transparency of embryos. Whether differences in the internal state of the two strains led to the difference of memory extinction in our behavioral paradigm requires further investigation.

High-Throughput Behavioral Assays for Learning and Memory in Larval Zebrafish

Larval zebrafish are amenable to high-throughput screen due to their transparency, small size and high permeability to small molecules (Kokel et al., 2010; Rihel et al., 2010). Though most systems are designed for drug or genetic screens (Rihel et al., 2010; Gehrig et al., 2018; Yang X. et al., 2018), here we have developed a high-throughput behavioral training system with custom supported software suites. Compared with previous work (Pelkowski et al., 2011; Hinz et al., 2013), the BLITZ software has enabled a fully automatic control of video capture, online image processing, visual pattern presentation and electroshocks delivery, making it an easily adaptable system for various purposes. Our complementary ABLITZER software also allows users to import, analyze and visualize data with well-structured classes and functions.

In its current version, our system cannot deal with situations of overlapping larvae, whose identities are hard to assign based on the current tracking algorithm. An earlier work (Mirat et al., 2013) showed that accurately tracking multiple larvae in groups over long periods of time were feasible. Integration of their algorithm with BLITZ may allow the study of social interactions of larval zebrafish in the future (Buske and Gerlai, 2014).

In conclusion, we have developed a high-throughput operant conditioning system for larval zebrafish. When using

electroshocks as the US and the red-black checkerboard or pure-black pattern as the CS, we found that a significant portion of larval zebrafish population could acquire operant learning. We also compared the learning responses between AB wild-type fish and transgenic fish. The percentage of learners and memory length strongly depended upon the visual contrast. Future work that combines imaging and genetic tools should help identify neural mechanisms underlying the visual-contrast-dependent learning behavior.

AUTHOR CONTRIBUTIONS

WY conceived the study, designed, and built the behavioral setup, developed the software suites, designed, carried out the experiments, wrote the manuscript, and conceived the figures. YM helped carry out the experiment and conceive the figures. DL helped design, build the behavioral setup, and conceive the figures. QW helped design the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnbeh.2019.00004/full#supplementary-material>

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