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RESEARCH ARTICLE

Associative learning between odorants and mechanosensory punishment in larval *Drosophila*

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SUMMARY

We tested whether *Drosophila* larvae can associate odours with a mechanosensory disturbance as a punishment, using substrate vibration conveyed by a loudspeaker (buzz: ◂). One odour (A) was presented with the buzz, while another odour (B) was presented without the buzz (A◂/B training). Then, animals were offered the choice between A and B. After reciprocal training (A/B◂), a second experimental group was tested in the same way. We found that larvae show conditioned escape from the previously punished odour. We further report an increase of associative performance scores with the number of punishments, and an increase according to the number of training cycles. Within the range tested (between 50 and 200 Hz), however, the pitch of the buzz does not apparently impact associative success. Last, but not least, we characterized odour–buzz memories with regard to the conditions under which they are behaviourally expressed – or not. In accordance with what has previously been found for associative learning between odours and bad taste (such as high concentration salt or quinine), we report that conditioned escape after odour–buzz learning is disabled if escape is not warranted, i.e. if no punishment to escape from is present during testing. Together with the already established paradigms for the association of odour and bad taste, the present assay offers the prospect of analysing how a relatively simple brain orchestrates memory and behaviour with regard to different kinds of 'bad' events.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/23/3897/DC1

Key words: *Drosophila*, learning, memory, olfaction, punishment, mechanosensation.

INTRODUCTION

Drosophila melanogaster larvae can learn the association between odorants and gustatory reinforcement. Pairing either an odour (Scherer et al., 2003) or a light (Gerber et al., 2004) with appetitive substances such as fructose induces appetitive memory, while aversive memory is formed after pairing an odour with either a bad taste [quinine or highly concentrated salt (Gerber and Hendel, 2006)] or an electric shock (Aceves-Piña and Quinn, 1979; Pauls et al., 2010a). In odour–reward learning, for example, larvae are rewarded in the presence of one odour but not in the presence of another (A+/B), and then are tested for their choice between A and B. A second group of larvae undergoes the same test, but after reciprocal training (A/B+). Thus, differences in test performance indicate an effect of the odour–reward contingency, or in other words, associative learning.

In terms of psychological mechanism, such conditioned behaviour can be understood in terms of the expected outcome of tracking down the learnt odour: conditioned search for reward in the appetitive case, and conditioned escape from punishment in the aversive case (Gerber and Hendel, 2006) (see also Schnaitmann et al., 2010). This interpretation is based on the observation that conditioned search is disabled if the sought-after reward is already present, and that conditioned escape is disabled if an escape-inducing punishment is not present (Schleyer et al., 2011).

In terms of neurobiological mechanism, odour–taste learning in the *Drosophila* larva has been analysed to some extent (Michels et al., 2005; Kaun et al., 2007; Zeng et al., 2007; Selcho et al., 2009; Pauls et al., 2010b; Michels et al., 2011; Saumweber et al., 2011), based on the fairly detailed previous knowledge of the chemosensory pathways of *Drosophila* in particular (for reviews, see Scott, 2005; Hallem et al., 2006; Gerber and Stocker, 2007; Vosshall and Stocker, 2007; Olsen and Wilson, 2008; Gerber et al., 2009), as well as the progress in understanding olfactory learning in insects in general [see reviews regarding *Drosophila* (e.g. Heisenberg, 2003; Keene and Waddell, 2007), honey bee (e.g. Menzel, 2001; Giurfa, 2007; Schwärzel and Müller, 2006) and cricket (e.g. Mizunami et al., 2009)]. In brief, sensory neurons target the antennal lobes, a first-order brain region where lateral connections shape olfactory representations. Antennal lobe output neurons have two target areas. One collateral conveys olfactory information directly towards the lateral horn and further on towards premotor circuitry. The second branch involves a detour *via* the mushroom bodies and only then towards premotor circuitry. In contrast, gustatory information bypasses the actual central brain, and is conveyed from gustatory

sensory neurons towards the suboesophageal ganglion and then to premotor centres in the ventral nerve cord. Notably, modulatory neurons ascending from the suboesophageal ganglion branch off towards the brain and in particular the mushroom bodies to signal internal reinforcement. Indeed, the mushroom bodies are the likely site of coincidence of olfactory and reinforcement information (Akalal et al., 2010; Gervasi et al., 2010). Notably, internal reinforcement is dissociated according to valence, such that the net training effect of octopaminergic neurons, as defined by the TDC2-Gal4 expression pattern, is rewarding, and the net training effect of dopaminergic neurons, as defined by the TH-Gal4 expression pattern, is punishing (Schroll et al., 2006) (see also Selcho et al., 2009).

Here, we extended the scope of larval olfactory learning paradigms by using mechanosensory disturbance as a punishment. This seems timely as mechanosensation is rather well analysed (Jarman, 2002; Kernan, 2007; Lumpkin et al., 2010; Yin and Kuebler, 2010; Wu et al., 2011), including attempts to identify first- and second-order interneurons (Smith and Shepherd, 1996; Diegelmann et al., 2008; Cardona et al., 2009). Also, from a practical point of view, temporal control over mechanosensory stimulation can be much finer grained than is the case for gustatory reinforcement in the larva, where tastants have to be added to the substrate and therefore changes in substrate necessarily involve translocation of the animals.

Following the experimental rationale referred to above, one odour (A) was presented together with mechanosensory disturbance (a 'buzz': ◀), while another odour (B) was presented without such a disturbance (A◀/B training). Then, animals were offered the choice between A and B. A second experimental group was tested in the same way, but after reciprocal training (A/B◀). We found that larvae show conditioned escape from the reinforced odour, indicating the punishing nature of the mechanosensory stimulus employed. We characterized basic parametric features of this paradigm, including the movement kinematics with respect to the punishment, the temporal dynamics of retention during the test, the dependence of associative success on the number of punishment pulses within a trial, as well as on the number of training cycles, and on the amplitude of the mechanosensory disturbance. Last, but not least, we exploited this paradigm to ask for the rules of the behavioural expression of the memory trace.

MATERIALS AND METHODS

Larvae, apparatus and stimuli

Larvae of the Canton-S wild-type strain (Universität Würzburg) were raised in groups of ~200 at 25°C, 60–70% relative humidity, on a 14 h:10 h light:dark cycle. We used third instar feeding-stage larvae throughout, aged 5 days after egg laying.

Larvae in all experiments were free to crawl on a relatively large Petri dish (145 mm diameter; Sarstedt, Nümbrecht, Germany), the bottom of which was covered with 1% agarose (electrophoresis grade; Roth, Karlsruhe, Germany) on the eve of the experiment. This Petri dish was fixed on top of a loudspeaker (MC GEE 201847 CON Elektronik, Greußenheim, Germany, impedance 8 Ω, diameter 16 cm, acoustic pressure: 89.2 dB W^{-1}, power 150 W r.m.s.) in a 50×50×75 cm box covered on the inside by silencing foam (Fig. 1A). The loudspeaker could be activated *via* a computer and was set to produce a vibration with a speed of displacement of 1.1 m s^{-1}, at a frequency of 100 Hz, unless stated otherwise. For punishment, 200 ms pulses of such vibrations were delivered once per second, unless mentioned otherwise (this stimulus is defined as a 'buzz': ◀). A webcam (5 frames s^{-1}) mounted above the Petri dish allowed recording of the larvae for offline analyses; to facilitate image acquisition, a ring of 30 red

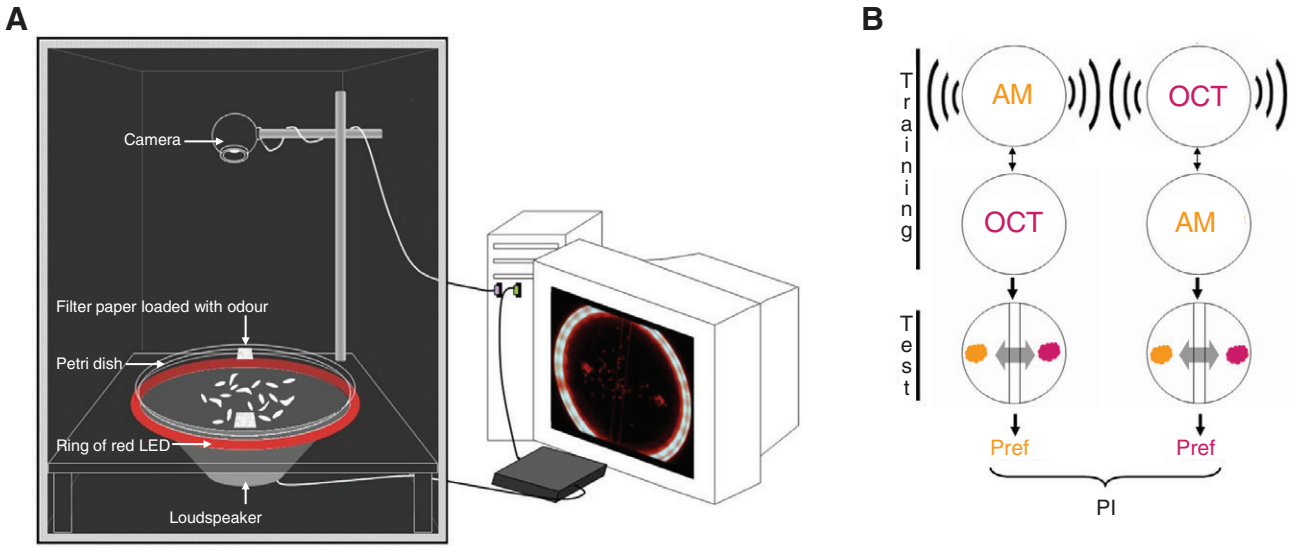

Fig. 1. Protocol for odour–buzz associative learning. (A) Experimental arena: inside a dark box illuminated with red LEDs, the larvae were free to crawl on a Petri dish with an odour emanating from odorant-soaked filter papers taped on the Petri dish lid. The Petri dish was fixed on top of a loudspeaker to deliver mechanosensory disturbances ('buzzes'). Larval behaviour was recorded by a webcam for offline analyses. (B) Experimental design: during training, a first group of larvae received the buzz during the presentation of *n*-amylacetate (AM) while 1-octanol (OCT) was presented alone (AM◀/OCT). A second group received the reverse contingency (OCT◀/AM). These training cycles were repeated three times, unless specified otherwise. For the test, larvae were free to crawl on a test Petri dish for 5 min, with AM and OCT presented on opposite sides to create a choice situation. For both reciprocally trained groups, the preference (Pref) for AM was calculated. The associative performance index (PI) quantifies the difference in preference between the reciprocally trained groups, and thus associative learning (for details, see Materials and methods), such that negative PIs indicate aversive memory, positive PIs appetitive memory. Please note that throughout this study the sequence of trials was as indicated in half of the cases (i.e. AM◀/OCT and OCT◀/AM), whereas it was reverse in the other half (i.e. OCT/AM◀ and AM/OCT◀, not shown).

light emitting diodes (624 nm LED; Conrad Electronics, Hirschau, Germany) was arranged around the Petri dish. To ensure even dispersion of light, a 1 cm thick ring of opaque Perspex was inserted between these LEDs and the Petri dish. The overall design of this set-up corresponds to that reported earlier (Wu et al., 2011).

As olfactory stimuli, we used 1-octanol (OCT, purity 99%, CAS: 111-87-5) and *n*-amyl acetate (AM, purity 98%, CAS: 628-63-7, diluted 1:50 in paraffin oil, CAS: 8012-95-1) (Merck, Darmstadt, Germany). We applied 10 µl of odour substance onto each of two 7×7 mm filter papers that were pasted inside the lid of the Petri dish, 5 cm from its edge and ~8 cm apart from each other along the equator of the dish. For better aeration, we used custom-made Petri dish lids perforated in the middle by 10 holes of 0.5 mm diameter each.

For gustatory punishment, we used either 4 mol l^{-1} of sodium chloride (NaCl, purity 99.5%, CAS: 7647-14-5; Roth) or 0.20% of quinine hemisulfate (QUI, purity 92%, CAS: 57-48-7; Sigma-Aldrich, Munich, Germany) in agarose for preparing the Petri dishes.

Learning paradigm

We compared cohorts of 50 larvae that received reciprocal associative conditioning (Fig. 1B): for the first group, AM was presented together with the buzz, whereas OCT was presented alone (AM◀/OCT); for the second group, OCT was presented with the buzz and AM was presented alone (OCT◀/AM). After such training, larvae were tested for their choice between the two odours. A difference in AM–OCT preference between the reciprocally trained groups thus indicates associative learning.

Specifically, ~50 larvae were taken from their rearing vials, gently washed in tap water and placed on a 145 mm diameter plastic Petri dish. Immediately before the beginning of each trial, odour (e.g. AM) was loaded and the lid of the training Petri dish was closed. Throughout the subsequent 5 min training trial, the buzz was applied (AM◀). Then, larvae were gently removed with a wet brush and placed on a fresh training Petri dish, this time loaded with OCT; during this trial, no buzz was presented (OCT). This AM◀/OCT training cycle was repeated three times. Between trials, the training Petri dish was discarded, while the odour-loaded filter papers were removed from the perforated lid, which was then cleaned with alcohol, and equipped with freshly loaded filter paper for the following trial with that odour.

For testing, the larvae were transferred to the middle of a test Petri dish containing agarose as usual, but offering a choice between AM on one side and OCT on the other side; unless mentioned otherwise, testing was carried out in the presence of the training reinforcer, as this is required to reveal conditioned escape (Gerber and Hendel, 2006) (see also Schnaitmann et al., 2010). Larvae were allowed to wander in the test Petri dish for 5 min. At the time points given in the Results, we counted the number of larvae on either side of the Petri dish, and on a 1 cm-wide middle strip (@AM, @OCT, @middle). We calculated a preference index as:

$$\text{Pref} = (@\text{AM} - @\text{OCT}) / (@\text{AM} + @\text{OCT} + @\text{middle}) . \quad (1)$$

This preference index thus varies between 1 (indicating preference for AM), and –1 (indicating preference for OCT), while a preference index of 0 would indicate that the larvae were distributed equally between the odours.

After one such preference value was obtained, a second cohort of 50 larvae was trained reciprocally (i.e. OCT◀/AM), and the choice behaviour was described by the preference index score as detailed above. This allowed calculation of an associative performance index (PI), quantifying the difference in preference indices between the reciprocally trained larvae:

$$\text{PI} = (\text{Pref}_{\text{AM◀/OCT}} - \text{Pref}_{\text{OCT◀/AM}}) / 2 . \quad (2)$$

PI thus also varies between 1, indicating conditioned approach, and –1, indicating conditioned avoidance.

Please note that in half the cases the sequence of training trials was as indicated (i.e. AM◀/OCT and OCT◀/AM for the reciprocal groups), but in the other half the sequence of trials was reverse (i.e. OCT/AM◀ and AM/ OCT◀, respectively). The sequence of training trials had no significant influence on test behaviour (supplementary material Fig. S1).

For odour–taste learning, experiments were performed in the same way as detailed above, except that either NaCl or QUI was used instead of the buzz.

Kinematics of larval movement

We used custom-designed tracking software in LabVIEW (National Instruments, Austin, TX, USA) to detect larvae by luminosity contrast. For each frame (frame rate, 5 frames s^{-1}), we determined the position of the centroid of the larva and the orientation of the longitudinal axis going through it (Fig. 2A). From this information, we characterized the kinematics of the behaviour of the larvae upon presentation of a buzz, as follows. (1) We calculated the speed (mm s^{-1}) of the larvae by considering their centroid during each of the respective 1 s periods as the frame-to-frame sum of the distances

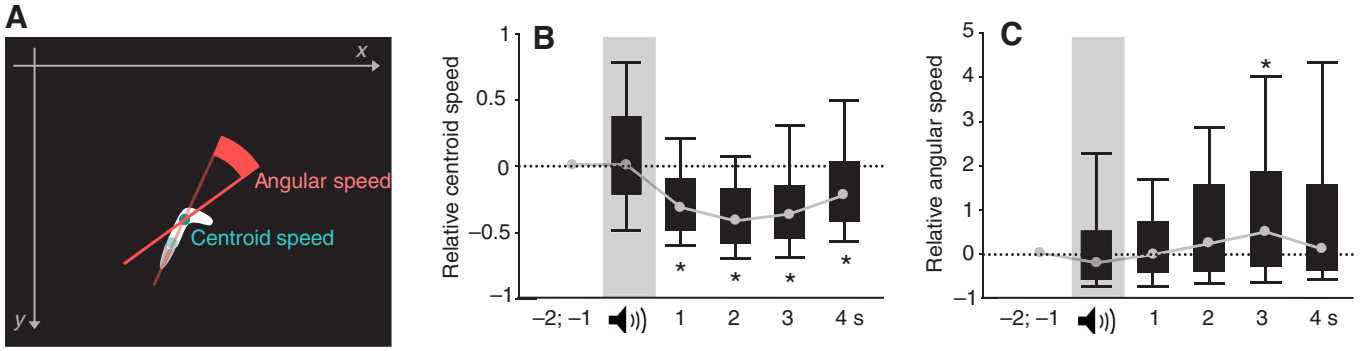

Fig. 2. Unconditioned behaviour regarding the buzz. (A) Sketch of the kinematic measurements taken. We determined the speed of the centroid (B) and the turning propensity (C) of individual, experimentally naïve larvae for the 4 s following a buzz, relative to baseline [the median of, respectively, speed (0.76 mm s^{-1}) and angular speed (15.3 deg s^{-1}) for the 2 s preceding the buzz]. Apparently, larvae slow down and then turn in response to a buzz. Asterisks indicate a significant difference of the scores [one-sample sign (OSS) test: $P<0.05/5$] from baseline.

covered by the centroid during that second. (2) We calculated the angular speed of the larvae (deg s^{-1}) as the frame-to-frame sum of the orientation changes of the longitudinal axis during that second.

For display purposes (Figs 2 and 5), we consider the relative speed and turning propensity, using the median value of the 2 s preceding the buzz of the considered individual as baseline. The absolute baseline values of median speed and median turning propensity are given in the figure legends.

Statistics

Given the definition of the preference and PI scores, and given the fact that often these scores are not normally distributed, we opted for non-parametric statistics and display throughout. We used Kruskal–Wallis tests (K–W tests) for comparisons across multiple groups, followed in cases of significance by pair-wise comparisons with Mann–Whitney *U*-tests (M–W *U*-tests). One-sample sign tests (OSS tests) were used to compare scores with zero. When multiple comparisons were made within one experiment, we applied a Bonferroni correction; that is, the criterion of significance (0.05) was adjusted by dividing it by the number of comparisons performed, such that the experiment-wide error remained below 5%. All statistical tests were performed with Statistica 7.1 (Statsoft, Tulsa, USA) on a PC. Data are presented as box–whisker plots, with the middle bold line indicating the median, the box boundaries indicating the lower and the upper quartile, and the whiskers the 10% and the 90% percentiles.

RESULTS

Behaviour of experimentally naïve larvae regarding the buzz

We first describe the unconditioned behaviour of the larvae upon presentation of the buzz. Larvae were placed onto a Petri dish, and after 1 min a single, 200 ms buzz was presented. As parameters for analysis we chose the speed of the centroid of the larva (mm s^{-1}) and the larva's turning propensity (deg s^{-1}) (Fig. 2A; see also supplementary material Movie 1). As shown in Fig. 2B, the buzz induced the larvae to slow down within the ensuing second (OSS tests, $P>0.05/5$ during the buzz and $P<0.05/5$ for the four 1 s periods after the buzz, $N=122$); with additional delay, larvae then increased turning propensity (Fig. 2C; OSS tests, $P>0.05/5$ during the buzz and during the first, second and fourth 1 s period after the buzz; $P<0.05/5$ for the third 1 s period after the buzz). These results replicate those reported earlier (Wu et al., 2011) using a similar experimental set-up. We interpret such buzz-induced behaviour [which is similar to what has been described in response to light 'touch' (Kernan et al., 1994)] as a startle response followed by reorientation, together comprising a behavioural 'escape' module. We therefore reasoned that the buzz may be effective as a punishment.

Establishing odour–buzz memories, and translating them into conditioned behaviour – or not

Larvae were trained either as AM◀/OCT or reciprocally as OCT◀/AM, and then were tested for their choice between AM and OCT (Fig. 3A). In Fig. 3B, we display the resulting preference indices of these reciprocally trained larvae, for each minute of the 5 min tests. When tested in the absence of the buzz, odour preferences were equal for the reciprocally trained groups (Fig. 3B left; M–W *U*-tests, $P>0.05/5$, $U=248, 242, 271.5, 260, 287$ for the five test periods, $N=24$). In contrast, larvae tested in the presence of the buzz revealed associative memories between odours and buzz: we observed significant escape from the previously punished odour by the end of the second minute (Fig. 3B right; M–W *U*-tests, $P>0.05/5$, $U=212$ for the first, and $P<0.05/5$, $U=103.5, 63.5, 78, 62.5$, for the second to the fifth test minute; $N=24$).

Considering Fig. 3B as well as the previous literature on odour–tastant learning, we decided to use the data from the end of the third test minute for calculation of the associative performance indices. It turned out that associative performance indices of larvae tested in the absence of the buzz were not different from chance (Fig. 3C; OSS test, $P>0.05/2$), but when tested in the presence of the buzz, we observed significantly negative associative performance indices (Fig. 3C; OSS test, $P<0.05/2$) (a direct comparison between the performance indices with a M–W *U*-test yields $P<0.05$, $U=146$).

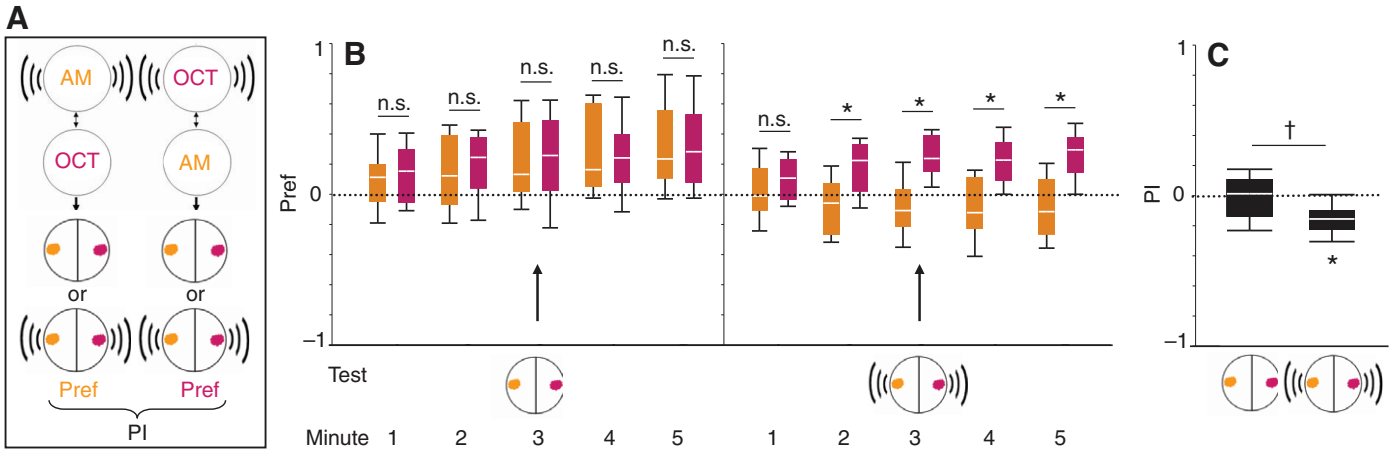

Fig. 3. Odour–buzz associative learning. (A) Sketch of the experimental protocol. Two groups of larvae underwent reciprocal odour–buzz training, and the difference in preference between them was quantified by the associative performance index (PI). Testing was carried out in either the absence or the presence of the training buzz. (B) For both reciprocally trained groups, the preference for AM is displayed separately for each minute of the 5 min test period. A difference in AM preference between groups needed at least 2 min to appear, and was observed only when the test was carried out in the presence of the training buzz. Statistically significant differences between preference scores are indicated by asterisks [Mann–Whitney (M–W) *U*-test: $P<0.05/5$]. These preference scores did not vary according to the sequence of trials during training (e.g. whether training followed the sequence AM◀/OCT or OCT/AM◀, supplementary material Fig. S1). (C) Associative performance indices obtained from the preference scores in B, using the data from the third minute of the test (arrow). Only when testing was carried out in the presence of the training buzz were aversive memories uncovered, as indicated by negative performance indices (*OSS test: $P<0.05/2$, †M–W *U*-test: $P<0.05$).

Given that the larvae tested in the presence *versus* absence of the buzz had undergone the same training and thus must have stored the same odour–buzz memories, these results not only argue that odour–buzz associative memories are formed but also mean that, dependent on the test situation, these memories can be 'translated' into conditioned behaviour – or not. Specifically, and as was previously reported for odour–bitter and odour–high salt associations (Gerber and Hendel, 2006), aversive memories are behaviourally expressed in the presence of punishment but not in its absence, and in this sense are embedded into a conditioned 'escape routine' which is employed only when escape indeed is warranted.

More buzzes per trial – better learning

To parametrically characterize odour–buzz associative learning, we varied the number of punishment pulses by changing the interval between the buzzes from 0.4 s (corresponding to a total of 750 pulses per 5 min trial) to 126 s (2 pulses per trial, Fig. 4A). Independent groups of larvae were tested either in the absence or in the presence of the respective training buzz.

Confirming the previous results, associative performance indices were zero when the larvae were tested in the absence of the buzz (Fig. 4B; left-most plot; a K–W test across all groups tested in the absence of the buzz yields $P>0.05/2$, $H=12.76$, d.f.=6, N=22, 25, 25, 29, 25, 25, 25; for the pooled data, the OSS test yields $P>0.05/8$, $N=176$: supplementary material Fig. S2). In contrast, aversive memories were revealed when testing in the presence of the buzz, and more importantly in the current context, the associative performance indices observed depended on the number of punishment pulses (Fig. 4B; for the groups tested in the presence of the buzz, K–W test: $P<0.05/2$, $H=15.82$, d.f.=6, N=22, 25, 25, 25, 25, 25, 25). Specifically, performance indices remained below statistical cut-off as long as fewer than 60 pulses per trial were used (Fig. 4B; OSS tests: $P>0.05/8$ in all three cases), but aversive memories were revealed for 60 or more pulses per trial (Fig. 4B; OSS tests: $P<0.05/8$ in all four cases) (for the underlying preference scores of this experiment, see supplementary material Fig. S3).

Interplay: behaviour towards the buzz during test

At this point, we wondered whether the behaviour of the larvae towards the buzz would be associatively altered by the training regimen and/or would change across the 3 min test period. We focused on two time points: the very first buzz delivered during the test (Fig. 5, left, N=452) and the very last buzz delivered during test (Fig. 5, right, N=432). For either time point, we separated the data according to whether the observed larva was located on the side of the previously punished odour or on the side of the previously non-punished odour. It turned out that locomotor kinematics appear uniform regardless of experimental history of the ambient odour (not shown). Further, although the buzz induced a decrease in speed and an increase in turning both at the beginning and at the end of the test period (Fig. 5; OSS tests with $P<0.05/5$ as criterion), speed decreased less and turning increased less at the end of testing (Fig. 5; all M–W U-tests: $P<0.05/5$ for beginning *versus* end). Also, we noted that the effect of the buzz on locomotion appeared slightly diminished from what we had observed before for experimentally naïve larvae (compare Fig. 2 with Fig. 5, right). This suggests that buzz-induced escape behaviour, in terms of the slowing-down-and-turn behavioural components, although sensitive to non-associative changes, is in principle robustly observed even after up to 3×60 buzz presentations during training, after odour exposure as entailed by the training regimen as well as experimental handling, plus the 48 buzzes received during testing. This, we believe, underscores its predominantly unconditional, reflexive character.

More training cycles – better learning

Returning to the parametric analyses of odour–buzz associations, we next asked whether associative performance indices would increase with extended training. To this end, we trained larvae with one, two or four training cycles (Fig. 6A). Relatively mild punishment (60 buzzes per trial) revealed an increase in associative effect (Fig. 6B; K–W test: $P<0.05/2$, $H=8.34$, d.f.=2, N=16, 16, 16), such that at least two training cycles were needed to reach significance (Fig. 6B; OSS tests: $P>0.05/3$, after one training cycle, $P<0.05/3$ after two as well as after four training cycles). Interestingly, this incremental effect of the number of training cycles was obscured if more severe punishment

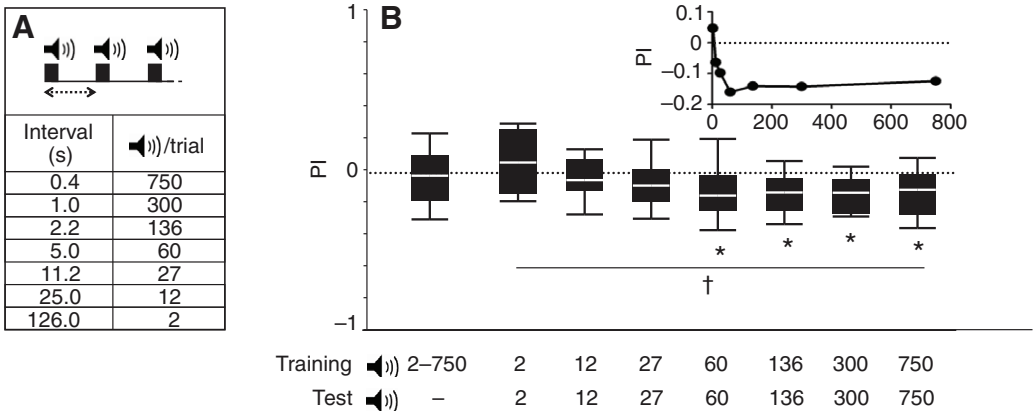

Fig. 4. Increasing the number of punishment pulses per trial increases the associative effect. (A) The parameters of punishment. (B) Irrespective of the number of punishment pulses per trial, odour–buzz memories are not behaviourally expressed when tested in the absence of the training buzz (left-most plot). The data were thus pooled between groups (for non-pooled data, see supplementary material Fig. S2). Testing in the presence of the training buzz, however, reveals odour–buzz memories; notably, the associative effect increases with the number of punishment pulses per trial. The difference across groups is indicated by the dagger [Kruskal–Wallis (K–W) test: $P<0.05/2$]. The inset displays the median associative performance indices plotted linearly across the number of buzzes per trial. The four right-most groups have PIs significantly different from zero (*OSS test: $P<0.05/8$). For the underlying preference scores, see supplementary material Fig. S3.

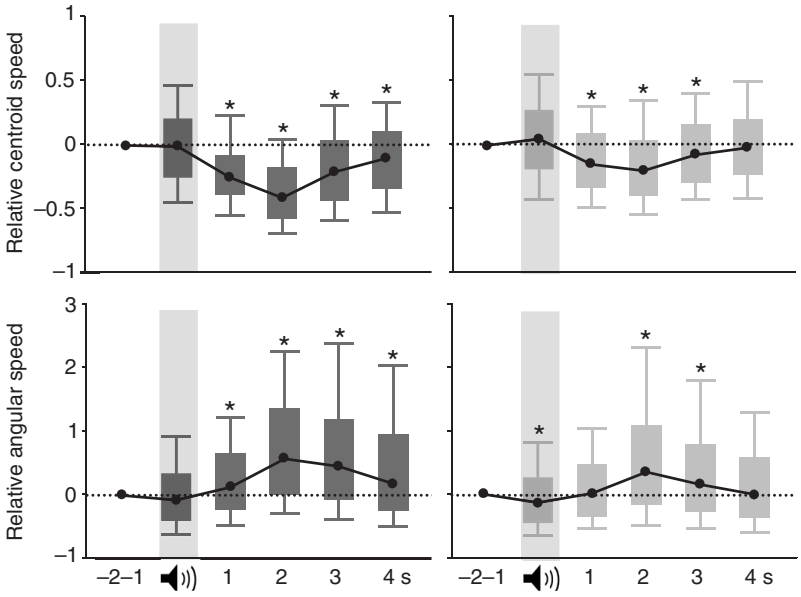

Fig. 5. Behaviour towards the buzz during the test. Larvae were trained with 60 buzzes per trial and were then individually tracked during the test, which was carried out in the presence of the buzz. For the very first as well as the very last test buzz (left and right, respectively), we determined centroid speed (top row; mm s^{-1}) and turning propensity (bottom row; deg s^{-1}). Scores are displayed from 2 s before to 4 s after the buzz. The data were normalized to the scores obtained for the respective individual during the 2 s preceding the buzz (median values for speed: 1.13 mm s^{-1} at the beginning and 0.95 mm s^{-1} at the end of the test; turning propensity: 13.7 deg s^{-1} at the beginning and 14.3 deg s^{-1} at the end of the test) (*P<0.05/5 in OSS test).

was used (300 pulses per trial) (Fig. 6C; K–W test: P>0.05/2, H=0.98, d.f.=2, N=8, 8, 8; the OSS test for the pooled data yields P<0.05) (for the underlying preference scores for this experiment, see supplementary material Fig. S4). This may reflect the fact that there is an upper limit to the punishing effect of the buzz (at least concerning the particular parameters of the buzz used in this experiment) that cannot be overcome by increasing training cycles, and/or that using too frequent pulses at the moment of testing puts a curb on performance indices: given that buzzes make the larvae slow down and turn (Fig. 2), using 300 pulses per trial during the test may 'trap' them at their starting position. Indeed, 29% of the larvae trained and tested with 300 pulses per trial were found in the middle at the moment of scoring, whereas this proportion was 15% when only 60 pulses per trial are used. Because in odour–taste learning protocols one does not need to reckon with such 'trapping' (Schleyer et al., 2011), this may partially explain why associative performance indices are smaller with the present protocol than for odour–taste protocols (e.g. Gerber and Hendel, 2006).

Testing for effects of the pitch of the buzz

Next, we varied the 'pitch' of the buzz, using 60 buzzes per trial. Specifically, we used buzzes of 50, 100 or 200 Hz and found that these variations in pitch did not alter training success (Fig. 7; K–W test: P>0.05, H=1.5, d.f.=2, N=20, 20, 20; for the pooled data the OSS test yields: P<0.05, N=60; for the underlying preference scores, see supplementary material Fig. S5).

How 'bad' is the buzz?

Given that odour–buzz training endows the odour with the capacity to direct conditioned escape from the buzz during the test, we

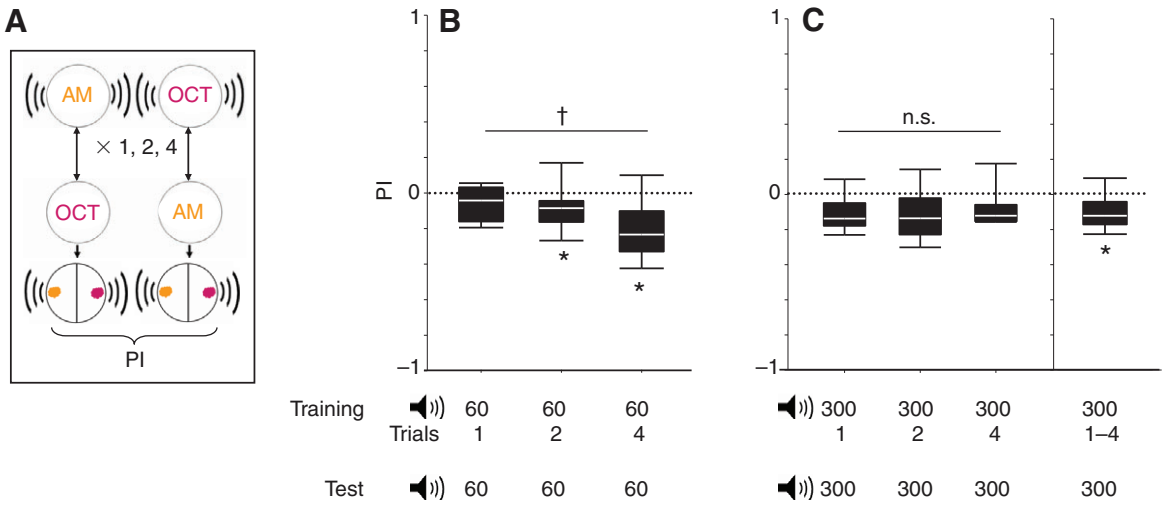

Fig. 6. Increasing the number of training cycles increases the associative effect. (A) Sketch of the training regimen used, in which the number of training cycles was varied (1, 2 or 4 training cycles). (B) Using relatively mild punishment (60 buzzes per trial), an increment in the associative effect with an increase in the number of training cycles was observed (dagger for P<0.05/2 in K–W test, asterisks for P<0.05/3 in OSS test), whereas more intense punishment (300 buzzes per trial) obscures this dependency (C) (n.s. for K–W test P>0.05/2). The right-most plot in C presents associative performance indices pooled across the number of training cycles, which are significantly different from zero (*OSS test: P<0.05). For the underlying preference scores, see supplementary material Fig. S4.

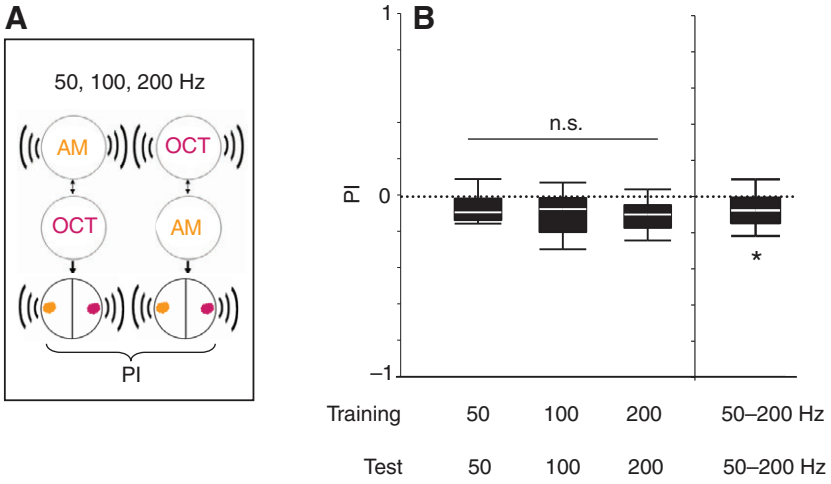

Fig. 7. Testing for an effect of the pitch of the buzz. (A) Sketch of the experimental regimen, in which the pitch of the buzz was varied (50, 100 or 200 Hz). (B) The associative performance indices are similar irrespective of the pitch used (n.s., K–W test: $P>0.05$); the right-most plot presents the pooled data (*OSS test: $P<0.05$). The corresponding preference scores are detailed in supplementary material Fig. S5.

wondered whether these odour–buzz memories would also guide escape from other kinds of unpleasant situation. Therefore, we tested the larvae in the presence of either the buzz or aversive tastants (either 4 mol l^{-1} NaCl or 0.20% quinine hemisulphate; at these concentrations, the chemical identity of the tastant is without effect in the present experiments: see supplementary material Fig. S6). Conditioned escape was seen to the same extent for the two kinds of test situation (Fig. 8B, left panel; M–W *U*-test: $P>0.05/2$, $U=1279.5$, $N=36, 78$) (for the underlying preference scores, see supplementary material Fig. S7, left panel).

Interestingly, if the experiment was reversed, that is if larvae were trained with the bad taste as punishment and were tested either in the presence of that bad taste or in the presence of the buzz, conditioned escape occurred to a lesser extent in the presence of the buzz (Fig. 8B, right panel; M–W *U*-test: $P<0.05/2$, $U=149$, $N=32, 32$); indeed, conditioned escape was seen only in the presence of the bad taste (OSS test: $P<0.05/2$, $N=32$), not in the presence of the buzz (Fig. 8B; OSS test: $P>0.05/2$, $N=32$) (for the underlying preference scores, see supplementary material Fig. S7, right panel). How can this asymmetry be understood?

The suggestion of Gerber and Hendel (Gerber and Hendel, 2006) (see also Schleyer et al., 2011) was that conditioned escape is shown as long as the test situation is at least as bad as the training reinforcer, whereas no conditioned escape should be observed if the test situation is less bad than the training reinforcer. Thus, is the buzz less bad than the bad taste? Indeed, associative performance indices tended to be smaller when the buzz was used for training and testing than when the bad taste was used for training and testing (left-most *versus* third plot of Fig. 8B; M–W *U*-test: $P<0.05$, $U=353$, $N=36, 32$). Thus, it seems that the buzz is less strong an aversive reinforcer than the bad taste, and may not be strong enough to behaviourally activate the association between odour and bad taste. Alternatively, the bad taste memory system could be specific in the sense that it is specifically the training taste that is required for taste-conditioned escape, whereas the buzz memory system may be less specific and can be engaged

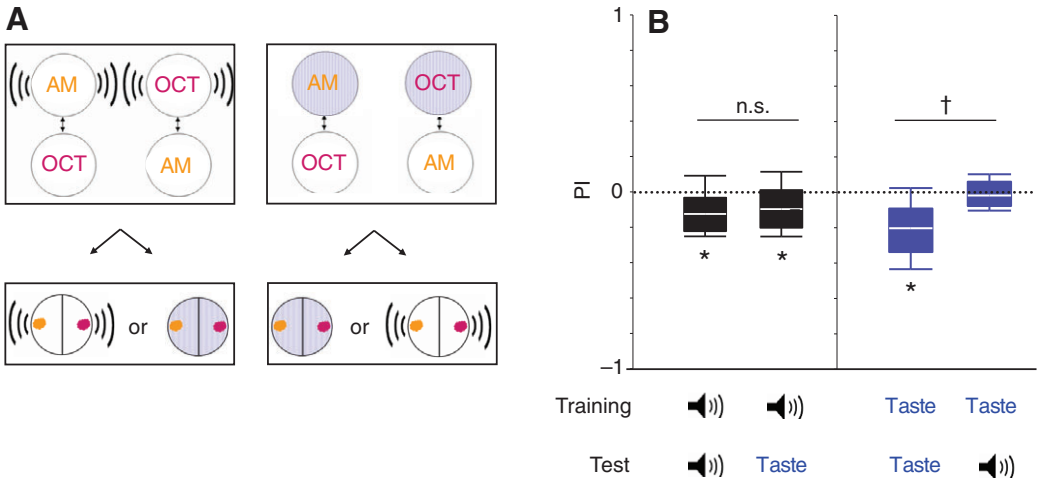

Fig. 8. How 'bad' is the buzz? (A) Sketch of the experimental design. The larvae were trained with 300 buzzes per trial and tested in the presence of either these buzzes or a disgusting bitter or salty taste (left panel). In another set of experiments, the larvae were trained with bitter or salty substances as a reinforcer and tested in the presence of either the respective taste or the buzz (right panel) (as scores obtained after quinine or salt treatment were not significantly different, the scores were pooled under a 'taste' condition [unpooled PI and preference scores are displayed in supplementary material Fig. S6). (B) The larvae trained with the buzzes show similar associative performances when tested in presence of the buzzes as when tested in the presence of taste (n.s., M–W *U*-test: $P>0.05/2$; asterisks for $P<0.05/2$ in OSS test). After training with any of the tastants, the larvae show associative performance only when tested in the presence of taste; when tested in the presence of buzzes, they do not show associative performance (dagger for M–W *U*-test: $P<0.05/2$, asterisk for $P<0.05/2$ in OSS test). For the corresponding preference scores, see supplementary material Fig. S7.

for conditioned escape by both buzz and bad taste. However, it would not be trivial to accommodate an aversive memory trace that is specific for the kind of bad stimulus used for punishment. As far as we can see, this would require the existence of (i) separate internal reinforcement systems as well as separate memory traces for buzz and bad taste, (ii) separate efferent systems to steer conditioned escape, which can be modulated by buzz or bad taste, and (iii) selective connections to allow the buzz to modulate only the buzz-related efferences, whereas the bad taste could engage both kinds of efference. We believe that, based on the available data, it is more parsimonious to propose that the bad taste is more strongly punishing than the buzzes used.

DISCUSSION

Here we report that *Drosophila* larvae can associate odours with a mechanosensory disturbance; that is, with substrate vibration conveyed by a loudspeaker (buzz), as punishment. This paradigm fulfills general expectations for classical conditioning in terms of its parametric dependencies, i.e. the increase of associative scores with the number of punishments (Fig. 4) and the increase according to the number of training cycles (Fig. 6). In contrast, we did not uncover a dependence of the associative process on the pitch of the buzz in the range between 50 and 200 Hz. However, probing a broader range of frequencies could reveal the receiver characteristics regarding the buzz (Fig. 7). This may turn out to be interesting in the context of both the sensory neurons mediating buzz perception (see below) and the kinds of signal the larvae encounter from animals foraging on their host fruit and/or from parasitoid predators (Dorn et al., 1997; Djemai et al., 2001). In any event, from the behavioural side, we note that the larvae show an unconditioned escape response to the buzz (see also Wu et al., 2011). Namely, they startle (slow down) and reorient (change direction) (Fig. 2), a behaviour that is rather robust against experience (Fig. 5) and which is observed regardless of its associative predictability (see Results). Such slow-down-and-turn behaviour has also been observed in response to other types of mechanosensory disturbance such as light touch (Kernan et al., 1994), but is qualitatively different, and apparently a level of escalation less, as compared with the 'pain' response when touched by a hot probe (Tracey et al., 2003). This pain response involves the product of the *painless* gene, namely a TRP (transient receptor potential) channel expressed in multidendritic neurons (Tracey et al., 2003; Hwang et al., 2007). Thus, given the distinct nature of unconditioned behaviour regarding heat pain *versus* the buzz, the buzz signal is probably by-passing the pathway as defined by *painless*-Gal4, and instead is received by tactile and/or proprioceptive sensory neurons (reviewed in Kernan, 2007). Indeed, at least the head-turning component of the buzz response is defective upon disruption of the function of chordotonal sensory neurons (Wu et al., 2011). It should now be possible to disentangle these sensory pathways in terms of their direct connectivity towards the motor system inducing unconditioned, reflexive behaviour on the one hand, and their connectivity to ascending modulatory circuits to signal reinforcement towards olfactory pathways on the other.

Implications regarding the nature of conditioned avoidance

In accordance with what has been suggested previously (Gerber and Hendel, 2006) (see also Schleyer et al., 2011) on the basis of odour–taste learning, conditioned behaviour after odour–buzz learning is not responsive in nature, but rather is driven by its expected outcome. That is, it is not the case that presentation of the learned odour *per se* would trigger conditioned avoidance (Fig. 3C, Fig. 4B). Also, it is not the case that the test situation *per se* would determine whether conditioned escape is expressed or not (compare left-most *versus* right-most plot in Fig. 8B). Rather, associative performance is based on an interaction of these aspects. First, the learnt odour activates its memory trace. Second, a comparison is made between the value of this memory trace and the value of the current situation. Conditioned behaviour is then expressed if the test situation is at least as bad as the memory trace suggests. This is in a sense ultra-rational, as it is only under these conditions that the larvae can substantially improve their situation by expressing avoidance of the punished odour.

Regarding the present analysis, it is noteworthy that the buzz and the bad taste memories appear to be treated according to their respective level of 'badness': the bad taste memories are more strongly negative than the buzz memories (left-most *versus* third plot of Fig. 8B), and hence conditioned escape from the buzz-associated odour is seen in the presence of the bad taste (second plot in Fig. 8B), but conditioned escape from the bad taste-associated odour is not seen in the presence of the buzz (right-most plot in Fig. 8B). Given that in all likelihood the sensory neurons to mediate bad taste *versus* the buzz are distinct, this suggests that the two kinds of punishment have access to the same kind of 'bad' value system to organize conditioned avoidance.

Outlook

Odour–buzz associative learning offers prospects both from the practical point of view, as it lends itself more readily to temporal control of reinforcement and thus to automation than odour–taste protocols, and because it allows analysis of the neuronal underpinnings of how a relatively simple brain orchestrates memory and behaviour with regard to different kinds of 'bad' events.

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