Interchangeable punishments during aversive conditioning in *Drosophila*

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Using *Drosophila melanogaster* larvae we asked whether distinct aversive stimuli have a common neural representation during associative learning. We tested the interchangeability of heat shock and electroshock punishments when used within a single olfactory associative conditioning experiment. We find that compared to animals trained with the repetitive use of a single punishment, the use of two alternating punishments results in similar associative learning. Additionally, the two punishments are shown to have different sensory origins. Therefore, while punishments are processed differently by the larvae of *Drosophila melanogaster*, the value of the stimulus is preserved.

Keywords: Associative conditioning, *Drosophila*, larvae, unconditioned stimulus.

AN understanding of associative conditioning is of fundamental importance in neuroscience. Even in socially complex and highly intelligent animals like humans, higher order learning tasks comprise combinations of basic associative conditioning, spatial learning, habituation and imitation. To fully understand the nature of associative conditioning, we need to understand the importance of the unconditioned stimulus (US) identity, the US value, and the US error or surprise signal. In this study, we used *Drosophila melanogaster* larvae as a model and sought to determine whether associative conditioning using a punishment (negative-valued US) is specific to the identity of the punishment or based on its negative value.

Olfactory associative conditioning in *Drosophila* larvae is well-established for the study of Pavlovian conditioning[1–9]. The molecular machinery, architecture and development of neurons involved in learning are conserved elements across vertebrates and *Drosophila*[10–12]. In addition, the olfactory system of insects, including *Drosophila*, shares some important similarities with mammals[13–15] and therefore olfactory conditioning in fruit flies has been extensively used as an associative learning model[1,13,16]. To behaviourally study punishment-value learning versus stimulus-specific learning, we tested the interchangeability, the effect of US pre-exposure on learning ability in larvae using modifications of heat shock (HS) conditioning[6] and electroshock (ES) conditioning[1,5,17] protocols.

Materials and methods

All the procedures employed in this study are modifications of previous studies[5,6]. Wherever there is a difference in procedure it is detailed, while replication of previous methods is summarily presented.

Fly husbandry

The Canton S strain of *D. melanogaster* was reared on standard medium at 24 ± 1°C on a 12/12 h light–dark cycle. Third instar larvae (5 days of age ± 8 h) were separated from the food media through a density separation with 30% 1500 MW polyethylene glycol. Larvae were then rested in glass petri dishes containing 0.5 ml of Ringer's solution until the onset of the experiment.

Conditioning

The basic conditioning protocol consisted of pairing 10^{-4} ethyl acetate (EA) with heat shock or electroshock. After conditioning, both the control and trained animals were tested for their response to 10^{-4} EA (refs 5, 6). For the training, we used a transfer chamber that was made by attaching a nylon mesh to the bottom of a 1 cm section cut from a 50 ml Falcon tube[6]. Larvae were placed in the transfer chamber for the training and could be easily moved to different experimental conditions (rest, heat shock, electroshock, odour alone, odour with heat shock or odour with electroshock). The chamber and larvae were rinsed with water before and after exposure to any of the conditions described. During conditioning (odour–US pairing), odour diluted in liquid paraffin was presented to the larvae by spotting 4 separate 20 µl drops on

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the surface of a 4.5 cm petri dish and then placing this as a cover of the transfer chamber. For heat shock presentation, a 10 cm glass petri dish containing 20 ml of 0.5% agar was placed on a heat block and allowed to reach a final agar surface temperature of 41°C (heat block set at 43°C). For electroshock presentation, 20 ml of 0.5% agar containing 20 mM lithium chloride was poured into 10 cm glass petri dishes and two metal plates were fastened to the edges of the dish[5]. We used a 120 V alternate current electroshock with a frequency of 60 Hz so that larvae received a shock of 1 V/mm. For all heat shock and electroshock conditioning trials, the odour alone was exposed to the larvae first for 15 sec. Subsequently US was added and the odour and US were presented for 15 sec simultaneously. Then the odour was removed and US was present alone for 15 sec. This resulted in 30 sec exposures of both the odour and US, but with the last 15 sec of odour exposure overlapping with the first 15 sec of US exposure. Following conditioning trials, the transfer chamber was returned to a rest plate. We tested all of the stimuli alone as control groups (see Figure 1), but because none of the controls significantly changed the odour response index we used an unpaired conditioned stimulus (CS)–US group as the control for all the conditioning experiments.

Olfactory testing

Olfactory testing was done using 15 cm petri dishes filled with ~ 15 ml of 2% agar as described previously[6]. Immediately following the cessation of the training or control procedures, approximately 30 larvae were placed at the centre of a plate. On one side of the plate 3 cm from the edge, 20 µl of odourant diluted in liquid paraffin was placed onto a 0.4 cm diameter filter paper disc. Diametrically opposite to this, liquid paraffin was placed on a filter disc. At the end of 3 min, the number of larvae in a 4 cm diameter zone surrounding the odour as well as the odour half of the plate were noted as well as the total number of larvae on the plate. The response index was calculated as the fraction of larvae on the plate that were in the odour zone:

$$\text{Response index} = \text{Larvae in odour zone} / \text{total participating larvae}.$$

All of our results are presented as differences in response index. Because the odour used for training is attractive to the larvae and the unconditioned stimuli are aversive, learning is seen as a decrease in response index.

Odourants for olfactory response measurements were prepared by diluting high-purity stock chemicals (99+%) in odourless liquid paraffin. Ethyl acetate (Fisher Scientific E145-1) and liquid paraffin (Acros Organics AC17140-0010) were all obtained from Fisher Scientific.

Heat avoidance assay

We assayed the larvae for their response to an aversive heat stimulus in a manner adapted from another study[18], and employed in our previous work[19,20]. A glass petri dish filled with 20 ml of 1% agar was placed so that one half of the plate was resting on a heat block set at 43°C, whereas the other half was not. This created a heat gradient across the dish. Larvae were placed in the middle of this dish and we recorded the number of larvae on each half of the plate every minute for a total of 6 min. The heat-avoidance index was calculated as

$$\text{Heat-avoidance index} = (\text{No. of larvae on cool side} - \text{No. of larvae on heated side})/\text{Total no. of larvae}.$$

Statistics and data analysis

The number of experiments is indicated in the results section and the figure legends. Values presented throughout the study are mean and the error bars presented are the standard error of the mean (SEM). For multiple comparisons, we ran an ANOVA with Bonferroni post-hoc tests where significance was indicated. An '*' is used to indicate $P < 0.05$ significance.

Results

We used two US – heat and electroshock, and a CS – EA, to explore the role of US in associative conditioning. The behavioural experiments in this study use many combinations of the two US combined with CS to produce learning. We therefore initially tested the necessary unpaired controls of the stimuli to ensure that our results were indeed representative of learning.

Unpaired, the CS and US do not affect behaviour

In flies, it has been shown that exposure to CS alone or exposure to a high-intensity US alone can alter the response of larvae to an olfactory stimulus[21–23]. We have previously established that for heat shock and electroshock conditioning, US and CS alone, if presented for a brief duration do not reduce the olfactory response[5,6]. However, because of slight changes in the protocol for this study and the use of two US, we re-explored this issue. For all cases in which CS or US was presented alone or in an unpaired manner, the response indices were statistically indistinguishable from naïve larvae (Figure 1 *a*). For the unpaired controls, larvae were exposed to eight cycles of heat shock, eight cycles of electroshock, or eight cycles of alternating heat and electroshock. These presentations were interspersed with eight cycles of odour so that 4 min separated the presentation of a US

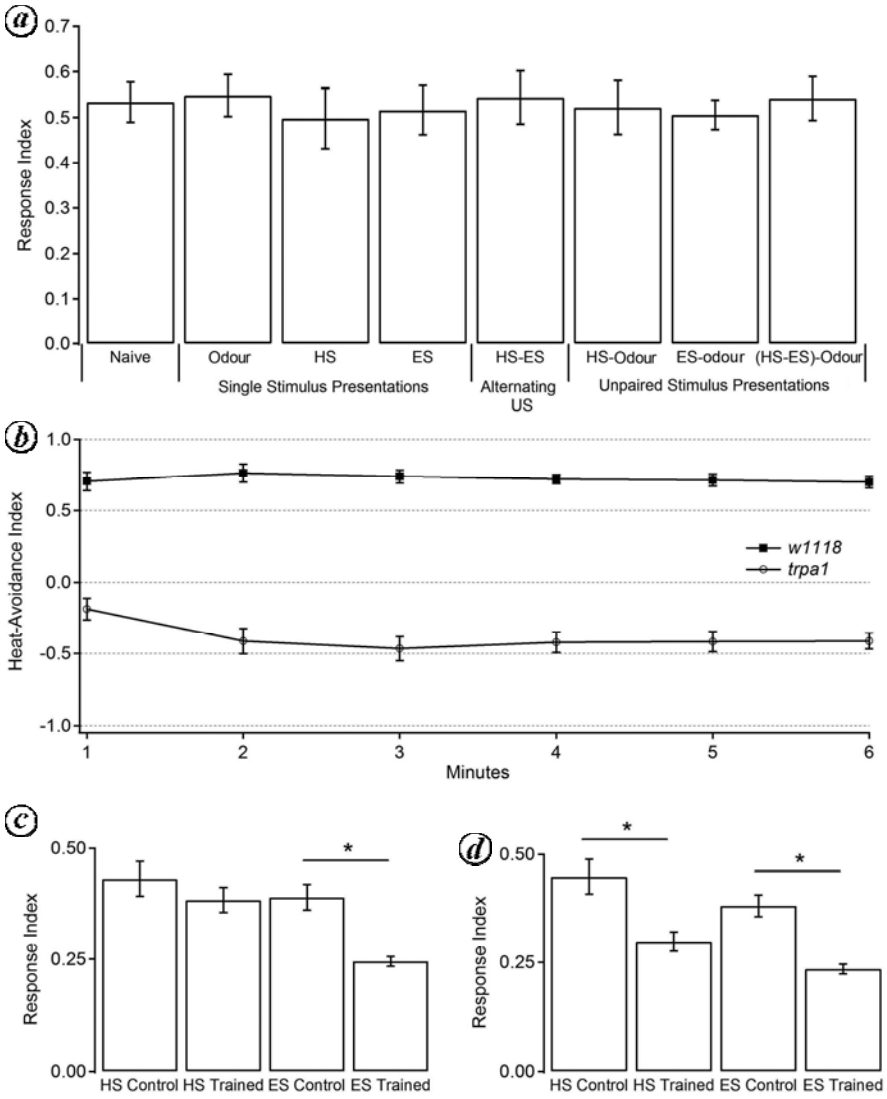

Figure 1. ***a***, All unconditioned groups show similar response indices. In this study, we employ a variety of combinations of heat shock (HS) and electroshock (ES) which are both paired with the presentation of an odour to produce associative conditioning. The odour, HS, and ES, stimuli when presented alone, did not reduce the odour response of the larvae. Additionally, two stimuli presented in the same protocol, but unpaired (i.e. their presentation is separated in time) also did not reduce the response of the larvae. Thus, any learning (reduction in odour response) we see in this study is a result of direct association of the odour (CS) and the unconditioned stimulus (US) of heat shock or electroshock (for all comparisons $P > 0.05$; $N = 8$). ***b***, In a heat avoidance assay, wild type w^{1118} larvae avoid the heat stimulus, whereas *trpa1* mutant larvae actually show a preference for the heat stimulus confirming a deficit in noxious heat sensation. ***c***, Larvae with a mutation in the *trpa1* gene show no reduction in response index following heat shock conditioning compared to controls ($P > 0.05$). This mutation however did not affect learning following electroshock conditioning as the ES-trained group had a significantly lower response index than the control ($P < 0.01$). ***d***, Wild-type larvae of the same genetic background as the mutant (w^{1118}) show significantly decreased response indices following both heat shock conditioning and electroshock conditioning compared to controls ($P < 0.01$).

and a CS. Because this treatment did not alter larval behaviour despite the large number of exposures, we chose to use this condition as the control for rest of this study.

Two distinct punishments have different sensory origins

The *trpa1* gene encodes an ion channel known to regulate thermal nociception[24]. We tested larvae with a null mutation in this gene for their ability to learn in both the heat shock and electroshock conditioning assay. The background for this mutant strain is w^{1118}. As confirmation that the mutation caused a deficiency in thermal sensation, we tested the mutant larvae in a heat-avoidance assay[18,19]. While the wild type w^{1118} consistently avoided the heated side of the agar plate, *trpa1* mutant larvae actually preferred the heated agar (Figure 1 *b*). As expected, larvae with a mutation in the *trpa1* gene do not learn well

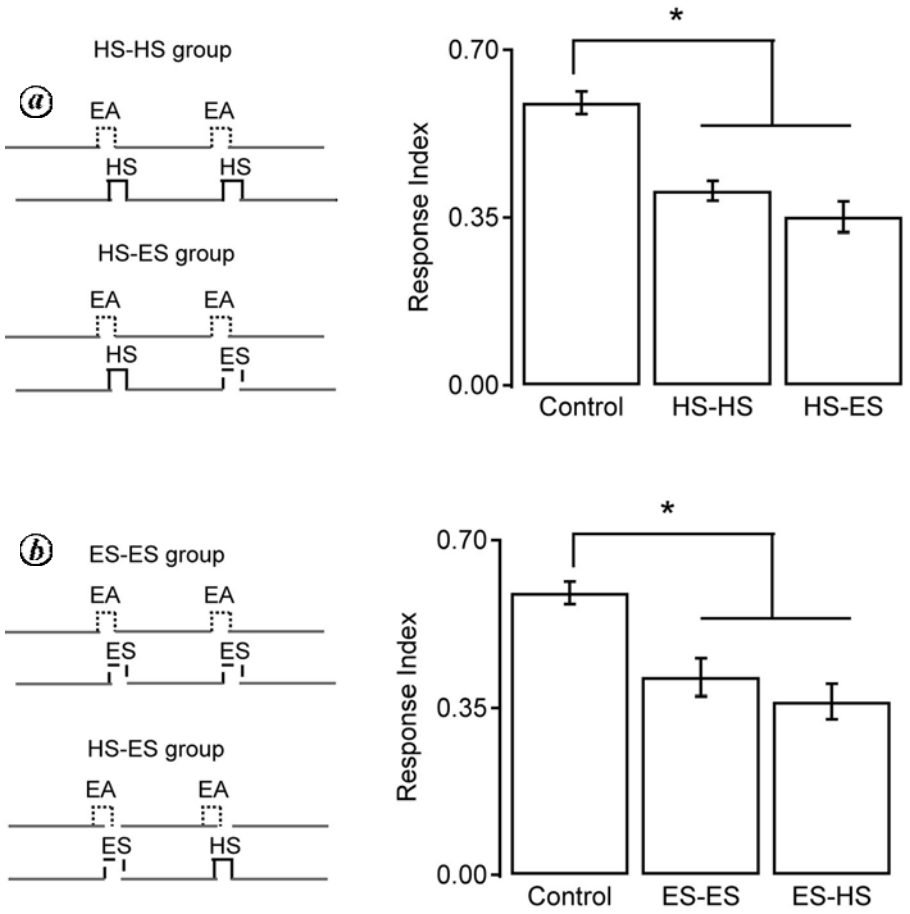

Figure 2. Different US can adequately substitute for each other. ***a***, ***b***, Two conditioning trials with either US alone significantly decreased the response index compared to the control ($P < 0.05$; $N = 8$). Performing one heat shock conditioning trial followed by one electroshock conditioning trial (***a***) and vice versa (***b***) also caused a significant reduction in response index compared to the unpaired control conditions ($P < 0.05$; $N = 8$). The reduction in response index resulting from alternating the two US was statistically indistinguishable with the drop caused by the use of either US alone.

in heat shock conditioning; that is, the trained larvae did not have a reduced response index compared to the control group (Figure 1 *c*). However, larvae trained using electroshock conditioning did have a significant reduction in response index indicating that learning was unperturbed (Figure 1 *c*). Larvae of the w^{1118} background, however, do show reduced odour-response indices when trained with either heat shock or electroshock conditioning (Figure 1 *d*).

Alternating two aversive US produces learning equivalent to either US alone

We next asked how alternating presentations of two distinct US – heat shock and electroshock – affected learning compared to the use of a single US. In this experiment we used an 8-min inter-trial interval. The alternating groups were presented with one heat shock and one electroshock (Figure 2 *a*) or one electroshock and one heat shock (Figure 2 *b*). These alternating groups were each compared with the unpaired control as well as two heat shock trials and two electroshock trials respectively. The use of two US, each presented one time in either order, caused a significant decrease in response index compared to the control group, indicating that learning occurred ($P < 0.05$). The learning produced by either US alone was identical to that produced by alternating the two US (Figure 2).

In general, repeatedly pairing a single US and CS enhances learning (decreases response indices). We wanted to see if this trend continued when two alternating US were used during training. Therefore, we alternated heat shock and electroshock conditioning for a total of eight conditioning trials (four of each) with an 8-min interval between shocks. We compared the response indices of these larvae to larvae that received eight heat shock conditioning trials and larvae that received eight electroshock conditioning trials (see schematics above the graphs in Figure 3). We observed that repeated rounds of training

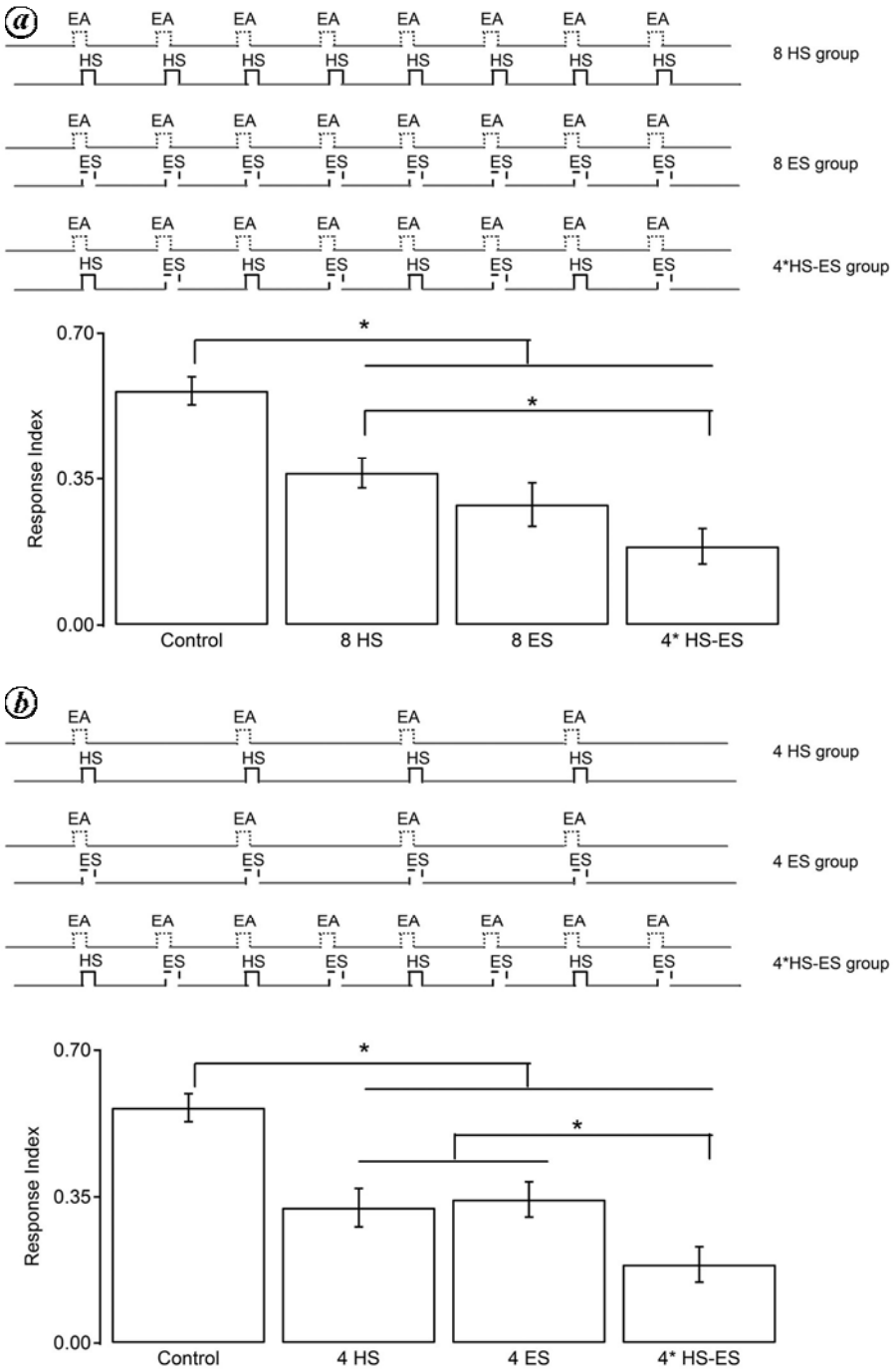

Figure 3. ***a***, Alternating heat shock and electroshock conditioning trials for a total of eight trials resulted in a significantly lower response index compared to eight trials of heat shock only ($P < 0.05$; $N = 8$). However, training using alternating heat shocks and electroshocks produced statistically indistinguishable response indices compared to training with electroshock only ($P > 0.05$; $N = 8$). ***b***, Alternating the two US produced a larger reduction in response index compared with either individual US component – four heat shock trials or four electroshock trials ($P < 0.05$; $N = 8$). As depicted in the schematic, these trials were separated by twice the inter-trial interval to mimic the role of each US in the alternating group.

using the alternating US protocol produced learning that was greater than when HS alone was used as US ($P < 0.05$; Figure 3 *a*). The learning produced using alternating US was, however, not significantly different than that produced using only electroshock as US ($P > 0.05$; Figure 3 *a*).

We have previously seen that using more than six trials of heat shock causes heat desensitization in the larvae.

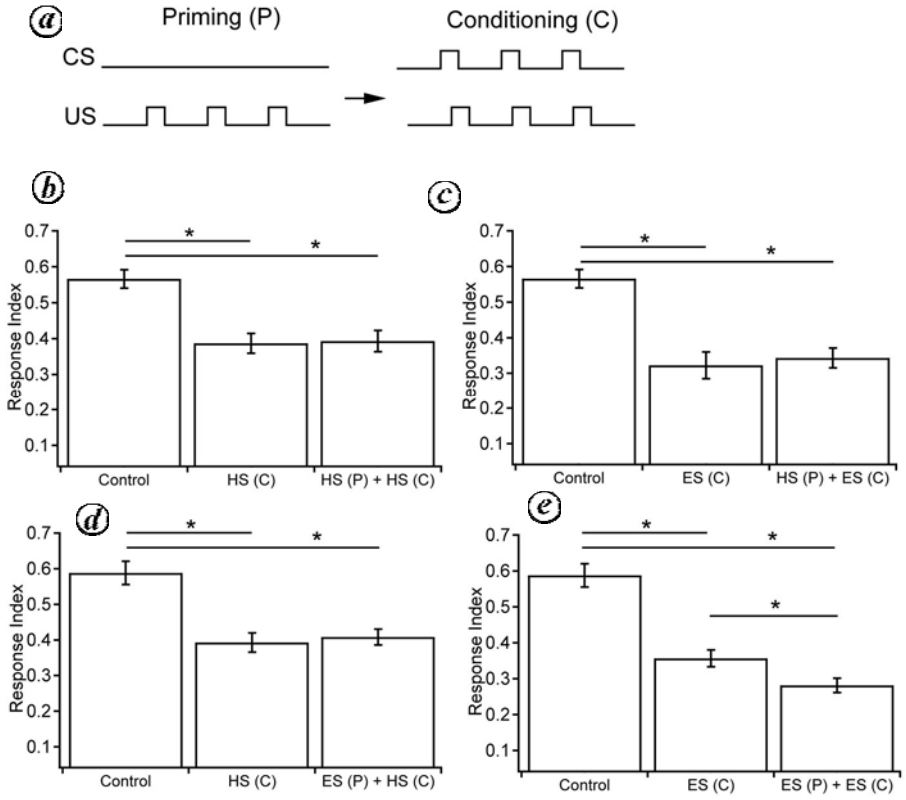

Figure 4. ***a***, Schematic of experimental protocol. Electroshock pre-exposure increases ES conditioning but heat shock pre-exposure has no effect on conditioning. We exposed larvae to US alone for three trials prior to the onset of conditioning. We tested the larval odour response following two heat shock conditioning trials and four electroshock conditioning trials. There was a significant decrease in response index of all trained groups compared to the controls ($P < 0.05$; $N = 8$). Heat shock pre-exposure had no effect on learning when (***b***) heat shock was used during conditioning ($P > 0.05$, $N = 8$) or (***c***) electroshock was used for conditioning ($P > 0.05$, $N = 8$). ***d***, Electroshock pre-exposure had no effect on conditioning using the heat shock US ($P > 0.05$, $N = 8$). ***e***, It had a significant priming effect when it preceded electroshock conditioning. This resulted in the pre-exposure group having a significantly reduced response index compared to no pre-exposure ($P < 0.05$, $N = 8$).

Using a training method with alternating US might reduce any desensitization caused by heat exposures, resulting in better learning. It is also possible that in the alternating US group, a longer interval between training trials of each US could be closer to an optimal inter-trial interval for training, which would also result in better learning. We therefore tested learning of each US component alone by measuring learning after four heat shocks only and four electroshocks only with a 16 min inter-trial interval. Alternating four of each US for a total of eight trials resulted in significantly better learning than that produced by either US component alone (four trials separated by double the standard inter-trial interval; $P < 0.05$; Figure 3 *b*).

Differential effects of heat shock and electroshock pre-exposure

Pre-exposure (or unpaired exposure) to a reinforcing stimulus has previously been shown to strongly influence subsequent conditioning. Depending on the circumstance and type of conditioning, a pre-exposure can either enhance or impede learning. In adult *Drosophila*, it has been shown that pre-exposure to a high heat stimulus caused an enhancement in associative learning that employed heat as US[25]. We wanted to see if this phenomenon was true for the larvae of *Drosophila* and whether pre-exposure with a negatively valued US that was not later used for conditioning produced the same results. We therefore exposed larvae to US alone three times prior to training. Pre-exposure experiments were done using both US and the effects were tested on training using both US (see Figure 4 *a* for schematic). We found that heat shock pre-exposure had no effect on learning regardless of whether conditioning was performed using heat shock as US (Figure 4 *b*; $P > 0.05$, $N = 16$) or electroshock as US (Figure 4 *c*; $P > 0.05$, $N = 16$). The electroshock pre-exposure did not significantly alter heat shock conditioning (Figure 4 *d*; $P > 0.05$, $N = 16$). However, pre-exposure to the electroshock US resulted in significantly lower response indices following

subsequent electroshock training compared with a group with no pre-exposure (Figure 4 *e*; $P < 0.05$, $N = 16$).

Discussion

In this study we examined associative learning using two distinct negative reinforcements or training stimuli. The training or reinforcement signal in the Pavlovian learning literature is referred to as unconditioned stimuli, a term used in this communication. We wanted to determine whether two distinct US – heat shock and electroshock – shared a common neural representation in conditioning assays.

We initially showed that the two punishments we use as US in this study have different sensory origins. We showed that a mutation in the *trpa1* gene disrupts noxious heat sensation (Figure 1 *b*) as well as learning when heat is used as US (Figure 1 *c*). This mutation, however, does not disrupt electroshock conditioning (Figure 1 *d*). It is unsurprising that during learning the processing of two aversive stimuli (heat shock and electroshock) is at least partially distinct. While it is currently unknown how electroshock is processed in larvae, the TRPA1 ion channel has been shown to be involved in the regulation of thermal nociception[24], and this processing is distinct from other types of nociception such as mechanical nociception[26–28]. Similar to these observations, we showed that larvae with a mutation in the *trpa1* gene were unable to form significant conditioned associations when heat shock was used as US. These larvae were, however, able to learn normally when electroshock was used as US (Figure 1).

To study whether there is a common underlying neural representation to punishment learning beyond the differing sensory origins, we paired an odour alternatingly with two distinct US during training. The learning produced by alternating US was equal to the use of a single US when two training trials were used (Figure 2). When eight trials were used, the alternating US group had identical learning to the electroshock group, but better learning compared with the heat shock group (Figure 3 *a*). We have previously shown that larvae become desensitized to aversive heat stimuli[6]. This likely accounts for the significantly higher learning we see when we use eight alternations of heat shocks and electroshocks compared to eight heat shocks alone.

We also wanted to ensure that the learning seen in the alternating group was resulting from a combination of the two US rather than from a single US with improved training due to an increased inter-trial interval. In the alternating group there is approximately twice the normal inter-trial interval separating exposures to one US (i.e. each HS trial is separated by 16.5 min from the nearest HS trial; see Figure 3 schematics). This could reduce any desensitization to a US and/or create an optimized learning protocol because of the different intervals between training trials. We found that the alternation of US for a total of eight trials produced better learning than that produced by either individual US within the alternating group (four trials of a single US separated by a double inter-trial interval; Figure 3 *b*).

To further behaviourally address whether the two punishments were processed in the same manner, we explored the effects that the pre-exposure, or priming, of each punishment had on learning. In this experiment we exposed the fruit fly larvae to US alone prior to training (Figure 4 *a*). Priming has been previously shown to enhance learning in an adult fly assay[25]. We found that in electroshock conditioning, larvae become sensitized by pre-exposures to electroshock resulting in better learning. This phenomenon does not occur with heat shock pre-exposure. Additionally, electroshock pre-exposure does not produce sensitization to heat-shock conditioning and pre-exposures of heat shock do not sensitize the animals for electroshock conditioning (Figure 4). This indicates that even though alternating the two US during training produces equivalent learning to the use of a single US, the processing during conditioning is at least partially distinct for the two punishments.

We propose that, despite the separate mechanisms for sensing and processing of two negative US, the negative value of US can become associated with a CS even when the identity of US changes. In this case, there would likely be a downstream convergence of the two negatively valued US signals. Another way to view this is that the neural pathways producing the conditioning from two separate US overlap downstream of the initial sensation. Therefore, when two separate US are associated with a single CS, the resulting conditioning is additive with successive trials similar to the use of a single US.

Further study is needed to determine the exact neural networks involved in heat shock and electroshock conditioning. However, due to the essential role of dopamine in conditioning, this is a likely candidate for the site of convergence of training signals coming from separate sensory peripheral pathways. Recent work on *Drosophila* has demonstrated a role for dopamine, serotonin and octopamine in coding for aspects of the US signal in conditioning[4,8,29–35]. In particular, *Drosophila* genetics has been elegantly used to dissociate the role of subsets of dopamine neurons in the coding of US[36,37]. Therefore, it may be feasible in future studies to determine whether dopamine neurons are indeed the site of convergence for two distinct aversive training signals.

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