Aspects of predictive learning in the fruit fly

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Die vorgelegte Dissertation besteht aus drei Publikationen, zwei zur Publikation vorbereiteten Manuskripten und einer zusätzlichen „Allgemeine Einleitung und Diskussion“. Die Mitwirkungen der Koautoren jeder Publikation werden auf der folgenden Seite herausgearbeitet.

Die vorliegende Arbeit wurde weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegt. Zuvor habe ich keine akademischen Grade erworben oder versucht zu erwerben.

Würzburg,

Ayse Yarali Dr. Bertram Gerber

Structure of the thesis

This thesis consists of two chapters, both of which study the predictive features of behaviour in fruit flies. Specifically, the Chapter I deals with the organization fruit fly associative learning across olfactory and visual modalities. It contains two publications, studying (1) larval and (2) adult fruit flies, respectively. Chapter II studies predictive learning of pain-relief in adult fruit flies. It contains one publication and two manuscripts prepared for publication. These three respectively analyse (1) the parametric features and psychological mechanisms, (2) the effect of the so called *white* gene and (3) the possible roles of biogenic amines. In addition, I present a 'General Introduction and Discussion' to give the reader a flavour of the thesis, without having read each chapter.

This work had not been possible without the effort of many people, and the supervision of Dr. Bertram Gerber. I take the opportunity to express my joy in this collaborative work and sincerely acknowledge the co-authors of each manuscript, whose contributions are explicated below.

Chapter I.1.

Yarali, A., Hendel, T. & Gerber, B. 2006. Olfactory learning and behaviour are 'insulated' against visual processing in larval Drosophila. *J Comp Physiol (A)*, **192,** 1133-45.
AY, TH and BG conceived the research and designed the experiments. AY and TH performed the experiments and analysed the data. AY and BG wrote the paper.

Chapter I.2.

Yarali, A., Mayerle, M., Nawroth, C. & Gerber, B. 2008. No evidence for visual context-dependency of olfactory learning in *Drosophila*. *Naturwissenschaften,* **DOI** 10.1007/s00114-008-0380-1.
AY and BG conceived the research and designed the experiments. MM, CN and AY performed the experiments. AY analysed the data. AY and BG wrote the paper.

Chapter II.1.

Yarali, A., Niewalda, T., Chen, Y., Tanimoto, H., Duerrnagel, S. & Gerber, B. In Press. 'Pain-relief' learning in fruit flies. *Anim Beh*

AY and BG conceived the research. AY, BG and TN designed the experiments. AY, TN, YC, SD performed the experiments. AY and TN analysed the data. HT introduced AY to the behavioural setup. AY and BG wrote the paper.

Chapter II.2.

Yarali, A., Krischke, M., Divya, S., Zars, T. & Gerber, B. Loss of *white* function coherently affects punishment learning and reward learning.

AY and BG conceived the research and designed the experiments. AY performed the behavioural experiments and analysed the data. AY and MK performed the amine measurements and analysed the data. TZ and DS shared their unpublished observations about the *white* mutant. AY wrote the paper.

Chapter II.3.

Yarali, A., Ritze, Y., Scholz, H. & Gerber, B. 'Pain-relief' learning in fruit flies: Testing for the roles of octopamine, tyramine, dopamine and serotonin.

AY and BG conceived the research and designed the experiments. AY performed the experiments, analysed the data and wrote the paper. YR and HS provided the SERT-Gal4 driver line.

Wuerzburg,

Ayse Yarali Dr. Bertram Gerber

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General introduction & discussion

The past, the present and the future are beautifully integrated in the choice of behaviour. First, the animals' *present* needs shape this choice; second, the upcoming *future* is considered; and third, to yield predictions about future, animals rely on their *past* experience. Experience contributes to behaviour organization mainly via associtive learning: If animals experience otherwise ordinary cues contingently with biologically significant events, they will later on behave towards these cues in anticipation of those events.

This thesis studies such predictive, associative learning, using the fruit fly *Drosophila melanogaster*. Fruit flies have a relatively simple, anatomically well-studied (Rein et al. 2002) and most importantly genetically accessible (see a review by Sokolowski [2001]) brain; combined with the available behavioural paradigms, these enable a detailed analysis of learning at the molecular and neuronal level. What *we* can learn from the fruit fly is useful in two rather different senses: First, the key moleculear machinery is often conserved through evolution and thus apply also to higher animals (e.g. molecular machinery for circadian rhythmicity, as reviewed by Yu & Hardin [2006]). Second, the neuronal architecture of the fly can be usefully implemented in 'intelligent' technical equipment (e.g. Wessnitzer & Webb 2006).

This thesis approaches fruit fly associative learning with two separate enquiries; in a general sense these complement each other, as one concerns the cues that are to be learned as predictors for an important event; whereas the other one concerns the important event itself, which is to be predicted. Specifically, Chapter I asks whether fruit flies learn that particular combinations of olfactory and visual cues predict painful or pleasant events. Chapter II in turn asks how fruit flies process and learn about two opposite aspects of a painful event, namely its beginning and its end.

I intend this 'General introduction & discussion' to give a flavour of the thesis as a whole. I summarize the relevant background and the key hypotheses; I mention the main findings and place them in the context of previous studies; and finally, I give directions for future research.

Do fruit flies learn about combinations of olfactory and visual cues?

The world is not boring: Many things happen at a time and it is vitally important to correctly choose which to ignore, respond to, bind together or learn about. This often necessitates a cross-talk between different sensory modalities. How such cross-talk is organized in the small and relatively simple brains of insects is an interesting topic for research; first, because one can discover the underlying neuronal circuit-principles, which may apply also to higher animals and second, because these principles are useful for solving cross-modal tasks in technical equipment (e.g. Wessnitzer & Webb 2006).

I use the fruit fly to explore the interactions between olfactory and visual modalities as they pertain to associative learning. Associative learning paradigms in either modality are available for larval as well as for adult fruit flies: Larvae can learn odours as well as visual conditions (i.e. light or darkness) as predictors for sugar reward (Scherer et al. 2003; Gerber et al. 2004; Kaun et al. 2007). Adult fruit flies in turn can learn odours as predictors for electric shock punishment (Tully & Quinn 1985); in addition, they can associate illumination, colour and patterns with reinforcement (Heisenberg 1989; Wolf & Heisenberg 1991). Presently, I ask whether fruit flies do also learn about combinations of odours and visual cues, at either of these two developmental stages.

I use a so called 'biconditional discrimination' task. In Chapter I.1., fruit fly larvae are trained such that one odour is paired with sugar reward only in light, but not in darkness; the other odour in turn is paired with reward only in darkness, but not in light. Thus, neither the odours nor the visual conditions alone predict reward, only combinations of both do. After such training, larvae's learning can be probed in two different ways: In one experiment (Chapter I.1.Fig. 1), larvae are simultaneously offered all of the four odour-visual cue combinations that they have encountered during training. Had the larvae learned that two of these combinations predict reward, they would have preferred these against the other two combinations; this, however, is not the case. In another experiment (Chapter I.1.Fig. 4), larvae are allowed to choose, either in light, or in darkness, between the two odours that they have encountered during training. Had the larvae learned that one odour predicts reward only in light, whereas the other odour predicts reward only in darkness, they would have preferred the respective odour under the respective visual condition; this does not happen, either. Thus, there is no evidence that fruit fly larvae were to learn about combinations of olfactory and visual cues.

In Chapter I.2., I turn to adult fruit flies and ask whether they can solve such a task. The training-principle is the same as detailed above for larvae, except that an electric shock punishment is used instead of a sugar reward (Chapter I.2.Fig. 2): That is, during training, one odour is paired with shock only in light, but not in darkness; a second odour in turn is paired with shock only in darkness, but not in light. After such training, flies are offered, either in light or in darkness, the two odours that they have encountered during training. Had the flies learned which odour predicts shock under each visual condition, they would have avoided it; this does not happen. Thus, also in adult fruit flies, there is no evidence for learning about combinations of olfactory and visual cues.

With respect to both larval and adult fruit flies, additional experiments largely exclude the possibility that the observed lack of biconditional discrimination were an artefact of generally unfavourable training and testing parameters (Chapter I.1.Fig.s 2, 3 and 5; Chapter I.2.Fig.s 1 and 3).

These results speak against an interaction between olfactory and visual modalities in fruit flies, at the very least interactions which would enable biconditional discrimination. Notably, other studies in flies do suggest some cross-talk between these two sensory modalities (e.g. Guo & Götz 1997; Guo & Guo 2005). How can these results be reconciled? I suggest classifying different kinds of interaction between sensory modalities according to their site along the sensory-motor continuum: I consider an interaction 'truly' cross-modal if it is between the specific features of the stimuli. I call an interaction 'amodal' if it instead engages the behavioural tendencies or 'values' elicited by each stimulus.

As an example for an 'amodal' interaction, take the experiment of Guo and Götz (1997): Fruit flies are trained such that flying towards a visual cue results in the presentation of an unpleasant odour; subsequently, flies learn to avoid flying towards that particular visual cue. This association between the visual cue and the odour speaks for an interaction between the two modalities. However, at the site of convergence, only the specific features of the visual cue are preserved, those of the odour are not. Rather, the odour, in addition to evoking its specific representation, also induces a 'value' signal, carried by the dopaminergic neurons (Schwaerzel et al. 2003; Schroll et al. 2006); most likely these dopaminergic neurons can be activated by any other aversive stimulus as well. The aversive learning of the visual cue is thus due to an interaction of this 'amodal' value signal with olfactory processing.

As an example for a 'truly' cross-modal interaction, take sensory pre-conditioning (Guo & Guo 2005): In the first experimental phase, fruit flies are concomitantly presented with an odour and a visual cue. In the second experimental phase, flies are trained such that flying towards the particular odour results in unpleasant heat. After such training, flies not only avoid the heat-associated odour, but they also avoid the visual cue, which itself has never been associated with heat. Thus, the initial concomitant presentation of the two stimuli has endowed the visual cue with the ability to 'call up' a functional representation of the odour. This necessitates an interaction between olfactory processing and visual processing.

A yet different kind of 'truly' cross-modal interaction would be required to solve a 'biconditional discrimination' task (Chapters I.1. and I.2.): Fruit flies are trained such that a particular odour is paired with reinforcement only in light, but not in darkness; another odour in turn is paired with reinforcement only in darkness, but not in light. Thus, neither the odours nor the visual cues alone do faithfully predict reinforcement; only combinations of both do. Solving this task would require that the representation of the odour on the one hand and the representation of the visual cue on the other hand converge onto a combinatorial olfactory-visual representation (Rudy & Sutherland 1992). Thus, the interaction would take place downstream of both olfactory and visual sensory processing. Neither in larval (Chapter I.1.) nor in adult (Chapter I.2.) fruit flies is such interaction evident.

To conclude, it seems that different behavioural tasks require different kinds of interaction between sensory modalities; whether a given kind of interaction will be found depends on the neuronal infrastructure, which is a function of the species and the developmental stage.

Predictive learning of pain-relief in fruit flies

Choosing correctly what to do is difficult; having a prediction as to what will happen next is helpful in this respect. Animals can make such predictions, based on cues that they have learned by experience. But *what* is worth predicting? In the simplest terms, bad things, and good things. For example, fruit flies, when trained with sequential presentations of an odour and electric shock (odour-shock training) subsequently avoid the odour because it predicts something bad (punishment learning: Tully & Quinn

1985). Training with pairings of odour and sugar on the other hand teaches the flies to approach the odour as it predicts something good (reward learning: Tempel et al. 1983).

Bad or good, each event has a beginning and an end, calling for differential, maybe even opposite anticipatory action. Indeed, fruit flies can also learn to predict the end of something bad: If during training electric shock comes first and only then the odour is presented (shock-odour training), flies approach the odour during the subsequent test (relief learning: Tanimoto et al. 2004). Thus, in fruit flies, shock supports two opposite kinds of learning: Those stimuli that predict the beginning of shock are responded to aversively (punishment learning); whereas those stimuli that signal the end of shock induce an appetitive response (relief learning).

Such dual effects of painful stimuli apply to other animals as well (e.g. dog: Moscovitch & LoLordo 1968; rabbit: Plotkin & Oakley 1975; rat: Maier et al. 1976; snail: Britton & Farley 1999); indeed, Solomon and Corbit (1974) suggest that a painful stimulus, in addition to this primary effect, generally induces with its offset an 'opponent' state of relief; both the painful and the relieving states manifest themselves in physiology as well as in behaviour. Wagner (1981) builds his theory of associative learning on the principle that both the painful onset and the relieving offset of a stimulus can act as opposing reinforcers.

To understand the behavioural consequences of painful, traumatic experiences, it is therefore necessary to study how their beginning and their end are processed and learned about, and how these opposing kinds of learning are balanced. Fruit flies seem to be a suitable model to do so: Comparable behavioural assays are available for the learning of the onset and offset of shock (Tanimoto et al. 2004); these can be combined with genetic/ transgenic tools to analyse the underlying neurobiology. Thus using the fruit fly, I first investigate the parametric features and psychological mechanisms of relief learning; I then embark upon neurobiological analysis.

Parametric features of relief learning

The knowledge of the parametric features of relief learning, as provided in Chapter II.1., is threefold useful: First, it enables subsequent neurobiological analysis of relief learning; second, it will form the basis for a comprehensive mathematical model of relief learning; and third, it will guide other

researchers in uncovering relief learning in their own experimental systems.

Relief learning is a small but robust and reproducible behavioural effect, found in both genders (Chapter II.1.Fig.s 1 and 3). It reaches asymptotic levels after six training trials (Chapter II.1.Fig.s 4 and 8A). Out of five chosen odour-pairs, two support relief learning at all concentrations tested; for one odour-pair, optimal relief learning is observed at an intermediate odour concentration; for the remaining two odour-pairs, relief learning cannot be demonstrated (Chapter II.1.Fig. 5). Relief learning is maximal using relatively mild shocks (Chapter II.1.Fig. 6), supporting stable retention for the first 2 hours after training (Chapter II.1.Fig. 7).

In short, those researchers who aim at uncovering relief learning in their experimental systems are advised in the first place, to be patient, as they will probably be chasing a relatively subtle effect; further, they should use relatively many repetitions of training and a relatively low intensity of reinforcement. Indeed, this parametric study in fruit flies has already aided the uncovering of pain-relief learning in humans (personal communication: M. Andreatta, A. Mühlberger, P. Pauli, Universität Würzburg).

Psychological mechanisms of relief learning

Chapter II.1. also tackles two important questions concerning the psychological mechanisms of relief learning: The first one concerns the nature of the learned behaviour; the second one enquires into the mechanism of learning.

Regarding the nature of the learned behaviour, consider that for relief learning, flies usually are trained with two equally repellent odours (Chapter II.1.Fig.s 1B and C): A control odour is presented very long before shock; a to-be-learned odour closely follows shock. After such training, in a choice situation, flies show a relative preference for the learned odour. Actually, there are two alternative psychological explanations for this relative preference (Chapter II.1.Fig. 2A): Shock-odour training may indeed have established the learned odour as a predictor for relief, resulting in conditioned approach. Or, shock-odour training may have weakened the processing of the learned odour, decreasing its aversiveness. I experimentally distinguish between these two scenarios (Chapter II.1.Fig. 2B): Flies are trained with a single odour and shock (Chapter II.1.Fig. 2C). One group

receives the odour very long before or very long after the shock, such that the two stimuli cannot be associated with each other. I adjust the odour concentration such that after this kind of training, flies do approach the odour and I take this response as the baseline. I train another group of flies for relief learning, presenting the odour shortly after shock. The subsequent response of this group to the odour then is then compared to the baseline, mentioned above. It turns out that relief learning has indeed increased the appetitiveness of the odour, as compared to baseline; in other words, relief learning has established genuine conditioned approach behaviour (Chapter II.1.Fig. 2D).

Regarding the mechanism of relief learning, two alternative psychological mechanisms have been suggested: On the one hand, both the beginning and the end of shock may act as opposing reinforcers (Solomon & Corbit 1974; Wagner 1981); an odour that predicts the painful beginning of shock is avoided, whereas an odour that signals the relieving end of shock is approached. Alternatively, during training, the experimental context may become associated with the shock (Sutton & Barto 1990; Chang et al. 2003), such that within this context shock is predicted. Each time the shock stimulus ends, there follows a relatively long period of time, which is free of shocks; the mismatch in this period between the context-based prediction that the shock should be present and its actual absence (i.e. negative prediction error: Tobler et al. 2003) may act as a reinforcer for the odour. These two scenarios can be experimentally distinguished: Namely, had the second scenario been correct, pre-training for context-shock associations would have enhanced subsequent relief learning, this is not the case (Chapter II.1.Fig.s 8B-D).

Loss of white *function coherently affects punishment learning and relief learning;*
white *mutant flies form overall more 'negative' memories about a shock-episode.*

Chapter II.2. reports a remarkable phenomenon in the flies that are mutant for the so called *white* gene (Chapter II.2.Fig. 1B): Namely, the memories of an experience with shock are overall more 'negative' for the *white* mutants, as compared to the wild-type flies. That is, *white* mutants build stronger punishment memories for odours that precede shock and they form weaker relief memories for odours that follow shock. Importantly, these effects are restricted to the learning about shock; the reflexive responsiveness to shock itself remains unaltered (Chapter II.2.Fig. 1C). In an attempt to explain these

coherent effects upon punishment and relief learning, I probe the *white* mutants for abnormalities in biogenic amines. I am motivated to do so, because the White protein contributes to membrane transporters, which provide neurons with the precursor for serotonin as well as the precursor for a cofactor of serotonin- and dopamine-synthesis (Dreesen et al. 1988; Tearle et al. 1989; Koshimura et al. 2000). In line with this kind of function, *white* mutant flies reportedly have lower whole-head amounts of serotonin and dopamine as compared to wild-type flies (Sitaraman et al. 2008). However, having analysed brain homogenates by liquid chromatography, coupled to mass spectrometry, I find no difference between *white* mutants and wild-type flies with respect to the amounts of octopamine, tyramine, dopamine or serotonin (Chapter II.2.Fig. 3). Thus, the molecular mechanism underlying the effect of the *white* gene on learning remains unresolved.

Nevertheless, these results have twofold significance: First, the *white* mutation is a common tool in fruit fly genetics: Transgenic flies are typically *white* mutant and bear copies of a truncated *white*-cDNA coupled to the transgenes as marker. The behavioural consequences of the loss of *white* function may confound those behavioural experiments that use such transgenic flies. Second, the *coherent* effects of the loss of *white* function on the two opposing kinds of learning induced by shock may reflect a basic principle as to how a painful, traumatic experience moulds behaviour: Namely, the aversive memory about the beginning of such experience on the one hand and the appetitive memory about its end on the other hand, seem to have some common determinants to keep them in a balance. Indeed, Solomon & Corbit (1974) suggest that a primarily painful stimulus induces by its offset an additional 'opponent' state of relief and that animal behaviour is governed by the balance between these two opponent states. Such balance might indeed be broken under pathological conditions (e.g. anxiety: Vincent & Kukstas 1998; schizophrenia: Grossberg 2000; the corresponding balance with respect to rewarding stimuli is important for addiction as reviewed by Koob [2008])

Thus, it is important to delineate the molecular and neuronal pivots of the balance between pain and relief, and fruit flies may be a suitable model to do so, specifically with regard to the key molecular players; indeed, the human homologues of the *white* gene are implicated in mood and panic disorders (Straub et al. 1994; Croop et al. 1997; Nakamura et al. 1999).

No evidence for a role for the common biogenic amines in relief learning

Chapter II.3. compares relief learning to the learning of punishment and of reward. Punishment and reward learning are doubly dissociated in terms of the biogenic amines which carry the underlying internal reinforcement signals: Shock, and probably other aversive stimuli as well, activate dopaminergic neurons (Riemensperger et al. 2005). Output from these neurons is necessary for punishment learning, but not for reward learning (Schwaerzel et al. 2003). Reportedly, activation of the dopaminergic neurons in turn can act as aversive reinforcement (Schroll et al. 2006). A corresponding appetitive reinforcement signal is carried by the octopaminergic neurons: Octopamine is necessary for reward but not for punishment learning (Schwaerzel et al. 2003); activation of octopaminergic/ tyraminergic neurons in turn can reportedly act as appetitive reinforcement (Schroll et al. 2006; but see Schipanski 2007). This double dissociation between the biogenic amines that signal appetitive and aversive reinforcement applies also to other insects (honeybee: reviewed by Giurfa [2007]; cricket: Unoki et al. 2005).

Might relief learning rely on the same internal reinforcement signal as reward or punishment learning? The answer is no. Using mutant flies that cannot synthesize octopamine, I show that octopamine is dispensible for relief learning (Chapter II.3.Fig. 3B); importantly, I verify the requirement for octopamine in reward learning (Chapter II.3.Fig. 3A). In an independent experiment, blocking output from a subset of octopaminergic/ tyraminergic neurons also leaves relief learning intact (Chapter II.3.Fig. 5B).

When I block output from a subset of dopaminergic neurons, I find relief learning unaffected (Chapter II.3.Fig. 6B); on the other hand, such output is required for punishment learning (Chapter II.3.Fig. 6A). In a follow-up experiment, blocking output from another, independent subset of dopaminergic neurons also leaves relief learning intact (Chapter II.3.Fig. 7B).

Finally, I block output from two independent subsets of serotonergic neurons and find relief learning to be unaffected also in these cases (Chapter II.3.Fig.s 7B and 8B).

Thus, I conclude that relief learning is distinct from both punishment and reward learning in terms of the requirement for biogenic amine signaling. With respect to none of the common biogenic amines in the fruit fly brain, do we find evidence for a role in relief learning.

Outlook

To summarize, I provide a detailed parametric analysis of relief learning and answers to two important questions about the underlying psychological mechanisms (Chapter II.1.): First, relief learning establishes genuine conditioned approach behaviour; and second, it is most likely not mediated by context associations. I find indications that punishment learning on the one hand and relief learning on the other hand have common genetic determinats; the *white* gene is critical for the balance between the two (Chapter II.2.). On a cellular level, in terms of biogenic amine signalling, however, I find that relief learning is distinct from both punishment learning and reward learning: While punishment and reward are respectively signalled by dopaminergic and octopaminergic neurons, relief learning requires neither of the two systems (Chapter II.3.). I see three lines of enquiry as most urgent for future research.

First, the neuronal circuit-principle underlying relief learning might be the same as that underlying the learning of punishment and reward. Namely, during punishment or reward learning, the odour-evoked neural signal is thought to coincide with the respective internal reinforcement signal. This coincidence triggers the decisive synaptic change, which subsequently enables the appropriate conditioned behaviour. Similarly, relief learning may rely on the coincidence between an odour-signal and an internal reinforcement signal, induced by the offset of shock (Solomon & Corbit 1974; Wagner 1981). However, which cells do actually carry such an internal reinforcement signal for relief? As my results exclude the roles of the biogenic amines at least to some extent (Chapter II.3.), I might next consider *neuropeptides*, which also function as neuromodulators, act on the same kind of receptors, linked to the same intracellular signalling cascades, as the biogenic amines (reviewed by Nässel [2002]).

The second question that arises concerns the nature and the cellular site of the synaptic plasticity that underlies relief learning. With respect to punishment learning, current evidence points to the mushroom body Kenyon cells as the harbour of the key synaptic change that is, the site of the memory trace (reviewed by Heisenberg [2003]; Gerber et al. [2004]): Kenyon cells are activated by odours (Wang et al. 2004; Turner et al. 2008), and they also receive the shock-induced dopaminergic

reinforcement signal. The coincidence of these two signals is thought to induce the cAMP signalling cascade, leading to the strengthening of the output synapses. This strengthened output is then thought to enable the odour to induce conditioned avoidance, when it is encountered again. For reward learning on the other hand, two such cAMP signalling-dependent memory traces seem to be established; one at the Kenyon cells and another independent one at the upstream olfactory projection neurons (Thum et al. 2007). Might relief learning induce the same kind of memory trace(s) at the same cellular site(s) as punishment or reward learning? This can be tackled using the experimental strategy that has been successfully employed with respect to punishment and reward learning (reviewed by Gerber et al. [2004]).

Third, I suggest considering event timing-dependent bi-directional synaptic plasticity as a mechanism for punishment *versus* relief learning. As detailed above, odour-shock training is thought to strengthen the Kenyon cell output to those neurons that mediate conditioned avoidance. Shock-odour training in turn might weaken this very same output, rendering the avoidance of the odour less likely than the baseline situation, thus resulting in relative approach. Such bi-directional changes in synaptic strength often depend on the relative timing of pre and post-synaptic activity (reviewed by Caporale & Dan [2008]): When the pre-synaptic action potentials happen within a ~ 10 ms interval before the post-synaptic ones, the synapses are potentiated; a 'reversed' sequence of action potentials depresses the synapses. Such *spike timing-dependent plasticity* can indeed be experimentally induced at the Kenyon cell output synapses of the locust (Cassenaer & Laurent 2007). Drew & Abbott (2006) explicate the necessary assumptions for accommodating such spike timing-dependent plasticity at the Kenyon cell output as a mechanism for punishment *versus* relief learning: First, the neurons, which are post-synaptic to Kenyon cells and mediate conditioned avoidance, should be responsive to shock. If that were the case, odour-shock training would result in pre-then-post synaptic action potentials, and thus potentiate the synapses; shock-odour training would in turn induce a 'reversed' sequence of action potentials and thus depress the synapses. Spike timing-dependent plasticity operates at the scale of tens of *milliseconds*; during behavioural training on the other hand, odour and shock are separated by tens of *seconds*. To bridge this long temporal gap, Drew and Abbott (2006) suggest assuming that both the odour response of the Kenyon cells and the shock-response of the postsynaptic neurons

persist for a few seconds, even once the respective stimuli are turned off. These assumptions must be tested to put any reasonable link between spike timing dependent plasticity on one hand and punishment *versus* relief learning, on the other hand. Along these lines I note that the coherent effects of the *white* mutation on punishment and relief learning (Chapter II.2.) can easily be incorporated into such a scenario.

To conclude, the efforts reported in this thesis, seem to have yielded many open ends, which will in the future hopefully be weaved into a comprehensive picture of relief learning.

References

Britton, G. & Farley, J. 1999. Behavioral and neural bases of noncoincidence learning in Hermissenda. *J Neurosci*, **19,** 9126-32.

Caporale, N. & Dan, Y. 2008. Spike Timing-Dependent Plasticity: A Hebbian Learning Rule. *Annu Rev Neurosci*.

Cassenaer, S. & Laurent, G. 2007. Hebbian STDP in mushroom bodies facilitates the synchronous flow of olfactory information in locusts. *Nature*, **448,** 709-13.

Chang, R. C., Blaisdell, A. P. & Miller, R. R. 2003. Backward conditioning: mediation by the context. *J Exp Psychol Anim Behav Process*, **29,** 171-83.

Croop, J. M., Tiller, G. E., Fletcher, J. A., Lux, M. L., Raab, E., Goldenson, D., Son, D., Arciniegas, S. & Wu, R. L. 1997. Isolation and characterization of a mammalian homolog of the Drosophila white gene. *Gene*, **185,** 77-85.

Dreesen, T. D., Johnson, D. H. & Henikoff, S. 1988. The brown protein of Drosophila melanogaster is similar to the white protein and to components of active transport complexes. *Mol Cell Biol*, **8,** 5206-15.

Drew, P. J. & Abbott, L. F. 2006. Extending the effects of spike-timing-dependent plasticity to behavioral timescales. *Proc Natl Acad Sci U S A*, **103,** 8876-81.

Gerber, B., Scherer, S., Neuser, K., Michels, B., Hendel, T., Stocker, R. F. & Heisenberg, M. 2004. Visual learning in individually assayed Drosophila larvae. *J Exp Biol*, **207,** 179-88.

Gerber, B., Tanimoto, H. & Heisenberg, M. 2004. An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol*, **14,** 737-44.

Giurfa, M. 2007. Behavioral and neural analysis of associative learning in the honeybee: a taste from the magic well. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*, **193,** 801-24.

Grossberg, S. 2000. The imbalanced brain: from normal behavior to schizophrenia. *Biol Psychiatry*, **48,** 81-98.

Guo, A. & Gotz, K. G. 1997. Association of visual objects and olfactory cues in Drosophila. *Learn Mem*, **4,** 192-204.

Guo, J. & Guo, A. 2005. Crossmodal interactions between olfactory and visual learning in Drosophila. *Science*, **309,** 307-10.

Heisenberg, M. 1989. Genetic approach to learning and memory (mnemogenetics) in Drosophila melanogaster. In: *Fortschritte der Zoologie. Fundamentals of memory formation: neuronal plasticity and brain function.* (Ed. by M. Lindauer), pp. 3-45. Stuttgart, Germany: G. Fischer.

Heisenberg, M. 2003. Mushroom body memoir: from maps to models. *Nat Rev Neurosci*, **4,** 266-75.

Kaun, K. R., Hendel, T., Gerber, B. & Sokolowski, M. B. 2007. Natural variation in Drosophila larval reward learning and memory due to a cGMP-dependent protein kinase. *Learn Mem*, **14,** 342-9.

Koob, G. F. & Le Moal, M. 2008. Addiction and the brain antireward system. *Annu Rev Psychol*, **59,** 29-53.

Koshimura, K., Murakami, Y., Tanaka, J. & Kato, Y. 2000. The role of 6R-tetrahydrobiopterin in the nervous system. *Prog Neurobiol*, **61,** 415-38.

Maier, S. F., Rapaport, P. & Wheatley, K. L. 1976. Conditioned inhibition and the UCS-CS interval. *Anim Learn Behav*, **4**, 217-20.

Moskovitch, A. & LoLordo, V. M. 1968. Role of safety in the Pavlovian backward fear conditioning procedure. *J Comp Physiol Psychol*, **66**, 673-78.

Nakamura, M., Ueno, S., Sano, A. & Tanabe, H. 1999. Polymorphisms of the human homologue of the Drosophila white gene are associated with mood and panic disorders. *Mol Psychiatry*, **4,** 155-62.

Nassel, D. R. 2002. Neuropeptides in the nervous system of Drosophila and other insects: multiple roles as neuromodulators and neurohormones. *Prog Neurobiol*, **68,** 1-84.

Plotkin, H. C. & Oakley, D. A. 1975. Backward conditioning in the rabbit (Oryctolagus cuniculus). *J Comp Physiol Psychol*, **88**, 586-90.

Rein, K., Zockler, M., Mader, M. T., Grubel, C. & Heisenberg, M. 2002. The Drosophila standard brain. *Curr Biol*, **12,** 227-31.

Riemensperger, T., Voller, T., Stock, P., Buchner, E. & Fiala, A. 2005. Punishment prediction by dopaminergic neurons in Drosophila. *Curr Biol*, **15,** 1953-60.

Rudy, J. W. & Sutherland, R. J. 1992. Configural and elemental associations and the memory coherence problem. *J Cogn Neurosci*, **4,** 208-16

Scherer, S., Stocker, R. F. & Gerber, B. 2003. Olfactory learning in individually assayed Drosophila larvae. *Learn Mem*, **10,** 217-25.

Scherer, S., Stocker, R. F. & Gerber, B. 2003. Olfactory learning in individually assayed Drosophila larvae. *Learn Mem*, **10,** 217-25.

Schipanski A. 2007. Reinforcement processing in fruit fly larvae. Diploma thesis. Universität Würzburg.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Voller, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. & Fiala, A. 2006. Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae. *Curr Biol*, **16,** 1741-7.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. & Heisenberg, M. 2003. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. *J Neurosci*, **23,** 10495-502.

Seol, G. H., Ziburkus, J., Huang, S., Song, L., Kim, I. T., Takamiya, K., Huganir, R. L., Lee, H. K. & Kirkwood, A. 2007. Neuromodulators control the polarity of spike-timing-dependent synaptic plasticity. *Neuron*, **55,** 919-29.

Sitaraman, D., Zars, M., Laferriere, H., Chen, Y. C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G. E. & Zars, T. 2008. Serotonin is necessary for place memory in Drosophila. *Proc Natl Acad Sci U S A*, **105,** 5579-84.

Sokolowski, M. B. 2001. Drosophila: genetics meets behaviour. *Nat Rev Genet*, **2,** 879-90.

Solomon, R. L., & Corbit, J. D. 1974 An opponent-process theory of acquired motivation. I. Temporal dynamics of affect. *Psychol Rev,* **81(2)**, 119-45.

Straub, R. E., Lehner, T., Luo, Y., Loth, J. E., Shao, W., Sharpe, L., Alexander, J. R., Das, K., Simon, R., Fieve, R. R. & et al. 1994. A possible vulnerability locus for bipolar affective disorder on chromosome 21q22.3. *Nat Genet*, **8,** 291-6.

Sutton, R. S. & Barto, A. G. 1990. Time derivative models of Pavlovian reinforcement. In: *Learning and Computational Neuroscience: Foundations of Adaptive– Networks* (Ed. by M. R. Gabriel & J. W. Moore), pp. 497-537. Cambridge, MA: MIT Press.

Tanimoto, H., Heisenberg, M. & Gerber, B. 2004. Experimental psychology: event timing turns punishment to reward. *Nature*, **430,** 983.

Tearle, R. G., Belote, J. M., McKeown, M., Baker, B. S. & Howells, A. J. 1989. Cloning and characterization of the scarlet gene of Drosophila melanogaster. *Genetics*, **122,** 595-606.

Tempel, B. L., Bonini, N., Dawson, D. R. & Quinn, W. G. 1983. Reward learning in normal and mutant Drosophila. *Proc Natl Acad Sci U S A*, **80,** 1482-6.

Thum, A. S., Jenett, A., Ito, K., Heisenberg, M. & Tanimoto, H. 2007. Multiple memory traces for olfactory reward learning in Drosophila. *J Neurosci*, **27,** 11132-8.

Tully, T. & Quinn, W. G. 1985. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J Comp Physiol [A]*, **157,** 263-77.

Turner, G. C., Bazhenov, M. & Laurent, G. 2008. Olfactory representations by Drosophila mushroom body neurons. *J Neurophysiol*, **99,** 734-46.

Unoki, S., Matsumoto, Y. & Mizunami, M. 2005. Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *Eur J Neurosci*, **22,** 1409-16.

Vincent, J. D. & Kukstas, L. A. 1998. Opponent processes and anxiety: toward a neurophysiological formulation. *Acta Psychiatr Scand Suppl*, **393,** 50-5.

Wagner, A. R. 1981. SOP: A Model of Automatic Memory Processing in Animal Behavior. In: *Information Processing in Animals: Memory Mechanisms* (Ed. by N. E. Spear & R. R. Miller), pp. 5−47. Hillsdale, New Jersey: Erlbaum.

Wang, Y., Guo, H. F., Pologruto, T. A., Hannan, F., Hakker, I., Svoboda, K. & Zhong, Y. 2004. Stereotyped odor-evoked activity in the mushroom body of Drosophila revealed by green fluorescent protein-based Ca2+ imaging. *J Neurosci*, **24,** 6507-14.

Wessnitzer, J. & Webb, B. 2006. Multimodal sensory integration in insects--towards insect brain control architectures. *Bioinspir Biomim*, **1,** 63-75.

Wolf, R. & Heisenberg, M. 1991. Basic organization of operant behavior as revealed in Drosophila flight orientation. *J Comp Physiol [A]*, **169,** 699-705.

Yu, W. & Hardin, P. E. 2006. Circadian oscillators of Drosophila and mammals. *J Cell Sci*, **119,** 4793-5.

Chapter I.

Do fruit flies learn about combinations of olfactory and visual cues?

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ORIGINAL PAPER

Olfactory learning and behaviour are 'insulated' against visual processing in larval *Drosophila*

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Abstract We investigate the organization of behaviour across sensory modalities, using larval *Drosophila melanogaster*. We ask whether olfactory learning and behaviour are affected by visual processing. We find that: (1) Visual choice does not affect concomitant odour choice. (2) Visual context does not influence odour learning, nor do changes of visual context between training and test affect retrieval of odour memory. (3) Larvae cannot solve a biconditional discrimination task, despite generally permissive conditions. In this task, larvae are required to establish conditional associations: in light, one odour is rewarded and the other one is not, whereas in dark the opposite contingency is established. After such training, choice between the two odours is equal under light and dark testing conditions, suggesting that larvae do not establish odour memories specifically for one visual context only. Together, these data suggest that, in larval *Drosophila*, olfactory learning and behaviour are 'insulated' against visual processing.

Keywords Olfaction · Vision · Gustation · *Drosophila* larva · Learning

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Abbreviations

AM	Amylacetate
FRU	Fructose
LI	Learning index
OCT	1-octanol
PREF	Preference
QUI	Quinine hemisulfate
+	Positive reinforcement
–	Negative reinforcement

Introduction

The simultaneous occurrence of stimuli from many different sensory modalities poses a problem: although many things happen at a time which may deserve attention and possibly action, one can typically do only one thing at a time. Thus, animals need to allocate attention, extract predictive relations, and organize behaviour across stimuli and across sensory modalities. Similar problems need to be solved for the design of software for robots and navigational devices, and maybe a better understanding of the biological solutions to these kinds of problem can be helpful in this respect.

So far, less is known about the cross-modality as compared to the within-modality organization of behaviour. Concerning cross-modality effects in insect associative learning, rather few behavioural studies have been reported (Bitterman 1996 for review of the bee literature; Couvillon et al. 2001; Gerber and Smith 1998; Gerber and Menzel 2000; Guo and Guo 2005; Matsumoto and Mizunami 2004). Here, we use

the *Drosophila* larva to investigate the organization of olfactory learning and behaviour and its possible interaction with visual processing. The *Drosophila* larva is a useful system for such an approach because it combines learning ability (Gerber et al. 2004; Hendel et al. 2005; Neuser et al. 2005; Scherer et al. 2003), simplicity in terms of cell number (e.g. Ramaekers et al. 2005) and accessibility by neurogenetic methods (Michels et al. 2005; for reviews concerning *Drosophila* in general, see Heisenberg 2003; Sokolowski 2001). With respect to learning ability, two about equally potent learning paradigms have been established, one for the association between sugar reward and odours (Hendel et al. 2005; Neuser et al. 2005; Michels et al. 2005; Scherer et al. 2003), and one for the association between sugar reward and visual stimuli (Gerber et al. 2004). Both paradigms use a reciprocal training regime, such that one group is rewarded when stimulus A is presented but not when stimulus B is presented (A+/B), whereas for the other group, A is presented without, but B with reward (A/B+). Then, animals from both groups are tested for their relative preference in an A versus B binary choice situation. Critically, one can conclude that associative learning has taken place if the animals trained A+/B have a preference for A which is relatively higher than the ones trained A/B+. Logically, to reach this conclusion it is not required that naïve larvae are indifferent between the two stimuli—this is because such baseline preference would merely lead to an offset in preference values for both groups, but cannot cause differences in preference between them. Concerning light and darkness as visual stimuli, this kind of procedure can be used to associatively up- and downregulate the typically moderate naive preference of *Drosophila* larvae for darkness (Gerber et al. 2004). Using two odours, e.g. amylacetate and 1-octanol which are attractive to larvae just as most other odours are as well, one can correspondingly modulate the relative preference between these two odours in an associative way (Hendel et al. 2005; Michels et al. 2005; Neuser et al. 2005; Scherer et al. 2003).

Here, we want to investigate whether there is any interaction between visual and olfactory processing either in terms of learning (Experiments 1, 4, 5), or in terms of naïve behaviour (Experiments 2, 3). We start from a biconditional discrimination experiment (Experiment 1), in which larvae are trained to establish odour memories specifically for one but not the other visual context. We find no evidence for such discrimination ability. As the test situation in these experiments involves concomitant visual and olfactory choice, we test whether this lack of biconditional discrimination ability may be due to visual stimuli overriding any olfactory behaviour (or vice versa). We therefore test, in experimentally naïve animals, whether concomitant visual and olfactory choice behaviour influence each other (Experiments 2, 3). We find that although visual choice leaves odour choice unaffected, odour choice does impair visual choice; we therefore resume to a modified biconditional discrimination experiment which does not require concomitant visual and odour choice (Experiment 4), but still do not find evidence for biconditional discrimination. Given that both biconditional discrimination procedures require odour learning both in darkness and in light, we further ask whether odour learning indeed is possible under either visual condition (Experiment 5) and find that this is the case. Finally, within Experiment 5 we also test whether changes in visual context between training and test may influence the retrieval of odour memory, which is not the case, either.

General materials and methods

We first describe only those methods which are used across almost all experiments; methods specific for single experiments, in particular the design of the behavioural experiments, are mentioned along with the results.

Larvae

Flies of the Canton-S wild-type strain are kept in mass culture maintained at 24°C temperature, 60–70% relative humidity and subject to a 14:10 h light: dark cycle. Daily at around noon, adult flies are transferred from their current vial into a fresh food vial where they are allowed to lay eggs for 24 h. Five days later, experiments are performed, such that larvae are aged 96–120 h after egg-laying.

On experimental days, a spoon-full of food substrate containing larvae is taken and transferred to a small glass vial. From there, individual animals are removed upon demand using a paintbrush, briefly washed in tap water, and placed in the experimental arena.

Petri dishes, reinforcers, odours, light source

Petri dishes for experiments are prepared fresh daily. Agarose solution (1%; electrophoresis grade, Roth, Karlsruhe, Germany) is boiled in a microwave oven

and allowed to cool down for 30 min, with constant gentle stirring. Petri dishes (9 cm diameter; Sarstedt, Nümbrecht, Germany) are then filled with a thin layer of agarose, allowed to solidify for 20 min, covered with their lids and stored at room temperature until the following day.

Depending on experimental design (see below), quinine hemisulfate (QUI; purity 92%; Sigma, Steinheim, Germany) and fructose (FRU; purity 99%; Sigma) are used as putative negative and positive reinforcers, respectively (these stimuli had been used in our earlier work: Gerber et al. 2004; Hendel et al. 2005; Neuser et al. 2005; Scherer et al. 2003). These tastants are added to the agarose 10 min after boiling to reach final concentrations of 0.2% QUI and 1 M FRU. At these concentrations, the degree of preference for FRU in a FRU versus agarose test is equal to the degree of avoidance of QUI in a QUI versus agarose test (Hendel et al. 2005).

As olfactory stimuli, 1-octanol (OCT; purity 99.5%; Fluka) and amylacetate (AM; purity 99%; Aldrich) are used; these odours had been used in our earlier work (see references in the previous paragraph). AM is diluted in paraffin oil (1:100 for Experiment 2; 1:50 for Experiments 1, 4 and 5; 1:10 for Experiment 3; for the rationale of choosing these concentrations, see Results of Experiment 3). Odours are applied by adding 10 µl of odour substance into custom-made Teflon containers (inner diameter 5 mm) that are closed by a perforated lid (seven holes, 0.5 mm diameter).

The cold light source used in some experiments (Intralux 6000 in combination with the 5″ light table; VOLPI AG, Schlieren, Switzerland) has a homogeneous emission spectrum in the human visual domain, but no UV or IR emission. A Perspex tray is placed between the light source and the petri dish such that the bottom of the petri dish is elevated 5 mm above the surface of the light table. To implement an X-plate photo-behaviour assay, parts of the petri dish are shielded from light with a black cardboard between light source and tray, creating an X-plate with two dark and two light quadrants. The cardboard is positioned 3 mm above the light source and 2 mm below the petri dish. Between the light source and the cardboard, a 1 mm thick aluminium shield is inserted to prevent heating of the cardboard by light absorption. In cases where the complete petri dish is to be dark, all four quadrants are covered in this way.

All experiments are performed under a fume hood. Room temperature ranges between 20 and 25°C. Immediately before experiments involving odours, we replace the regular lids of petri dishes with lids perforated in the centre by 61, 1-mm holes.

Results

Experiment 1: Can larvae solve a biconditional discrimination task?

This experiment tests whether larvae can solve a biconditional discrimination task across sensory modalities (Table 1): one odour is rewarded in *LIGHT* but not in **DARK**, whereas the second odour is rewarded in **DARK** but not in *LIGHT*. After such training, larvae are given a choice between all four stimulus combinations. If biconditional discrimination is possible, larvae should prefer those two combinations which were rewarded in training ('target quadrants'). Importantly, if either the odours (Hendel et al. 2005; Neuser et al. 2005; Michels et al. 2005; Scherer et al. 2003) or the visual stimuli (Gerber et al. 2004) are trained in isolation, simple discrimination learning is possible.

Specifically, two groups are trained in a reciprocal way: one receives reward when OCT is presented in **DARK** (**OCT+**) but not when OCT is presented in *LIGHT* (*OCT*); concerning AM, they receive reward when AM is presented in *LIGHT* (*AM+*) but not when presented in **DARK** (**AM**). Thus, for this group, training is *OCT* **OCT+** **AM** *AM+*. Consequently, target quadrants for this group are **OCT** and *AM*. The second group is trained reciprocally: *OCT+* **OCT** **AM+** *AM*. Thus, for this group target quadrants are *OCT* and **AM.** For both groups, successful biconditional discrimination is indicated if the target quadrants are preferred over the non-target quadrants.

Groups of 10–12 larvae receive five training cycles. Each cycle includes four trials, one of each of the four trial types (i.e. *OCT*, **OCT+**, **AM**, and *AM+* for one group; *OCT+*, **OCT**, **AM+** and *AM* for the other group). Each trial lasts 1 min and is followed by the next trial without any break. Across repetitions of the experiment, the sequence of trial types within the cycles is pseudorandomly altered. That is, for a given group of 10–12 larvae, the sequence of trial types is

Table 1 Experiment 1

Group	Training	Test	Target
Group 1	*OCT* **OCT+** **AM** *AM+*	*OCT* **OCT** **AM** *AM*	**OCT** *AM*
Group 2	*OCT+* **OCT** **AM+** *AM*	*OCT* **OCT** **AM** *AM*	*OCT* **AM**

AM, amylacetate; OCT, 1-octanol; + positive reinforcement

AM and OCT in non-bold, italic font refers to presentation of these stimuli in *LIGHT*, whereas bold, non-italic font indicates presentation in **DARK**

always the same, in all cycles. The next group of larvae is then trained with another sequence of trial types throughout. All trial types serve equally often as first trials. The sequences are arranged such that no two trials in a row are rewarded.

After training, larvae are placed to the centre of a test plate with two **DARK** and two *LIGHT* quadrants. On opposite sides of the plate, we place the odour containers, one with AM, and one with OCT. Thus, the test plate offers four types of quadrant: *OCT*, **OCT**, **AM** and *AM*. The number of larvae in each of the four quadrants is recorded after allowing dispersal for 1 min. For each test plate, the preference towards quadrants **OCT** and *AM* ($\mathrm{PREF}_{\mathbf{OCT}/AM}$) is calculated as:

$$\mathrm{PREF}_{\mathbf{OCT}/AM} = ((\#_{\mathbf{OCT}} + \#_{AM}) - (\#_{OCT} + \#_{\mathbf{AM}}))/\#_{\mathrm{Total}}. \quad (1)$$

We use '#' to indicate the number of larvae observed in the respective quadrant (here as in all following experiments, the criterion for scoring was the position of the larva's mouthhook.). Thus, if the animals can solve the biconditional discrimination task, the group trained *OCT* **OCT+ AM** *AM*+ should show a higher $\mathrm{PREF}_{\mathbf{OCT}/AM}$ value than the one trained *OCT*+ **OCT AM+** *AM*. We use a Mann–Whitney *U* test to compare the $\mathrm{PREF}_{\mathbf{OCT}/AM}$ values between the two groups.

Obviously, larvae from both groups display statistically indistinguishable preference values (Fig. 1; $P = 0.97$, $Z = 0.038$; $N = 16, 16$). Thus, this experiment does not provide evidence for an ability to solve a biconditional discrimination paradigm across sensory modalities.

Experiment 2: Interference between olfaction and vision in untrained larvae?

The lack of biconditional learning in Experiment 1 might have resulted from an inability to make two choices simultaneously (i.e. *LIGHT* versus **DARK** as well as OCT versus AM). Experiment 2 therefore tests whether, in untrained larvae, concomitant visual and olfactory choices interfere with each other. The experiment uses three experimental groups. The first group of larvae is tested for only olfactory choice (AM versus OCT; ODOURS group). The second group is tested for only visual choice in an X-plate **DARK**–*LIGHT* assay (VISUAL group). For the third group, odour containers are placed on opposite sides of the X-plate, on the border of **DARK** and *LIGHT* quadrants. Thus, larvae are concomitantly confronted with both an olfactory and a visual choice (VISUAL AND ODOURS). The data from this third group are analysed either by ignoring the visual condition to describe odour choice, or by ignoring the odour condition to describe visual choice. Thus, we can compare the behaviour of larvae from the ODOURS group with the odour choice behaviour of the larvae from the VISUAL AND ODOURS group. Any difference in such a comparison would point to an interaction between vision and olfaction. Obviously, the same is possible with respect to visual behaviour.

Larvae (9–11 animals) are placed in the middle of the test plate. After 5 min, the numbers of larvae are scored. For the ODOURS group this involves the number on either the OCT or AM side; for the VISUAL group the number of larvae in either of the two dark and either of the two light quadrants; for the VISUAL AND ODOURS group, we scored each of the four quadrants (*AM*, **AM**, **OCT**, *OCT*) separately. We allow 5 min instead of only 1 min as in Experiment 1 because we reason that maybe interactions show up only after animals had the opportunity to extensively sample all four quadrants in the VISUAL AND ODOURS group. Odour preferences ($\mathrm{PREF}_{\mathrm{Odour}}$) for the ODOURS and the VISUAL AND ODOURS groups are then calculated as follows:

$$\mathrm{PREF}_{\mathrm{Odour}} = (\#_{\mathrm{AM}} - \#_{\mathrm{OCT}})/\#_{\mathrm{Total}}. \quad (2)$$

We use '$\#_{\mathrm{AM}}$' and '$\#_{\mathrm{OCT}}$' to indicate the number of larvae found on the AM or OCT sides, respectively; '$\#_{\mathrm{Total}}$' indicates the total number larvae. Similarly, visual preferences ($\mathrm{PREF}_{\mathrm{Visual}}$) for the VISUAL and the VISUAL AND ODOURS groups are calculated as:

$$\mathrm{PREF}_{\mathrm{Visual}} = (\#_{\mathrm{Dark}} - \#_{\mathrm{Light}})/\#_{\mathrm{Total}}. \quad (3)$$

We compare PREF values between groups using the Mann–Whitney *U* test. We use one-sample sign tests to determine whether these PREF values are significantly different from zero; in cases where multiple groups are compared to zero, we adjust significance levels using a Bonferroni correction to maintain an experiment-wide error rate of $P < 0.05$; this is done by dividing 0.05 by the number of single comparisons.

Concerning odour choice, $\mathrm{PREF}_{\mathrm{Odour}}$ values of larvae from the ODOURS and the VISUAL AND ODOURS groups are presented in Fig. 2a. Odour choice is statistically indistinguishable in both conditions (Fig. 2a; $P = 0.56$, $Z = -0.58$; $N = 44, 44$); after pooling of the data from groups ODOURS and VISUAL AND ODOURS, we find a median $\mathrm{PREF}_{\mathrm{Odour}}$ value of 0.00, indicating no preference between the two odours at these concentrations.

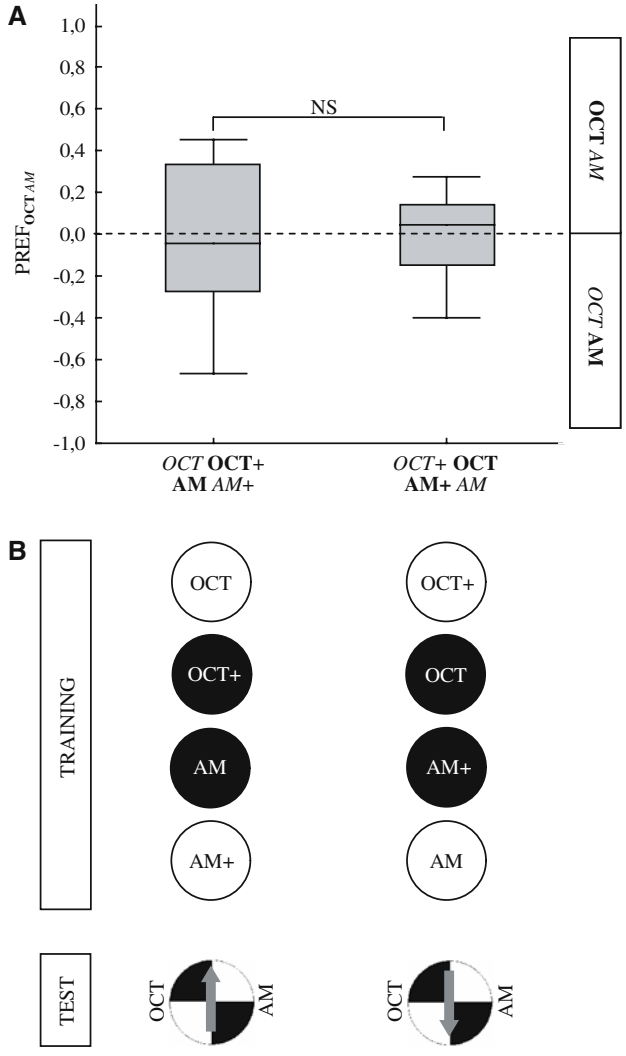

Fig. 1 Experiment 1, testing whether larvae can solve a biconditional discrimination task across sensory modalities (see Table 1 and sketch in (**b**) for the experimental design). **a**. Larvae trained *OCT* **OCT+ AM** *AM+* should have a higher preference for their target quadrants (**OCT** and *AM*) than the ones trained *OCT+* **OCT AM+** *AM*. This is not the case. The *arrows* point to the target quadrants. Sample sizes are $N = 16$ for each group. *NS*: $P > 0.05$. The *box plots* represent the median as the middle line and 10 and 90 and 25 and 75% quantiles as whiskers and box boundaries, respectively. AM and OCT in non-bold, italic font refers to presentation of these stimuli in *LIGHT*, whereas bold, non-italic font indicates presentation in **DARK**

Concerning visual choice, the dark preference in the VISUAL AND ODOURS group is diminished as compared to the VISUAL group (Fig. 2b; $P < 0.05$, $Z = 3.54$; N =44, 44). Actually, in the VISUAL AND ODOURS group, the dark preference falls short of significance (Fig. 2b; $P = 0.08$; $N = 44$), whereas the VISUAL group shows a clear dark preference (Fig. 2b; $P < 0.025$; $N = 44$). Thus, we find that concomitantly ongoing olfactory choice interferes with visual choice. Concerning olfactory behaviour, however, we have no evidence for an effect of visual choice on olfactory choice. In Experiment 3, we further scrutinize this asymmetric result.

Experiment 3: Interference between olfaction and vision revisited

The lack of effect of visual choice upon concomitant odour choice in Experiment 2 (Fig. 2a) may be due to either a true absence of effect, or may be an artefact of the equal distribution of the animals between the two odours. That is, because the preference between OCT and AM is zero, we are obviously unable to see whether concomitant visual choice may disrupt odour choice. Therefore, we replicate Experiment 2, but use a higher AM concentration (1:10 dilution) than in Experiment 2 (1:100 dilution), such that we can expect a moderate preference for AM. As in Experiment 2, experimentally naïve larvae are tested.

Odour choice is statistically indistinguishable between the ODOURS group and the VISUAL AND ODOURS group (Fig. 3a; $P = 0.62$, $Z = -0.49$; $N = 59$, 59); after pooling of the data from both groups, we find a median $PREF_{Odour}$ value of 0.4, which is significantly different from zero ($P < 0.05$; $N = 118$), indicating a preference towards AM.

Concerning visual choice, we find that dark preference ($PREF_{Visual}$) is slightly, yet significantly lower in the presence of concomitantly ongoing odour choice (Fig. 3b; $P < 0.05$, $Z = 2.50$; $N = 59$, 59). In both groups, the dark preference is significantly different from zero ($P < 0.025$; $N = 59$ in both cases).

These data confirm the results of Experiment 2, in that odour choice hinders concomitant visual choice, whereas odour choice is not affected by concomitant visual choice.

It should be noted that Experiment 2 use a lower, and Experiment 3 a higher concentration of AM than Experiment 1, which uses a 1:50 dilution. This is because we reasoned that for relatively low concentrations of odour, odour choice may be even more susceptible to visual interference, whereas under conditions of high odour concentration an effect of odour choice on visual behaviour may be stronger.

In any event, the fact that when odour choice is ongoing, visual choice is impaired may provide an explanation for the lack of biconditional discrimination ability as seen in Experiment 1. This is because the setup of the test situation in Experiment 1 involves concomitant odour and visual choice. In Experiment 4, we

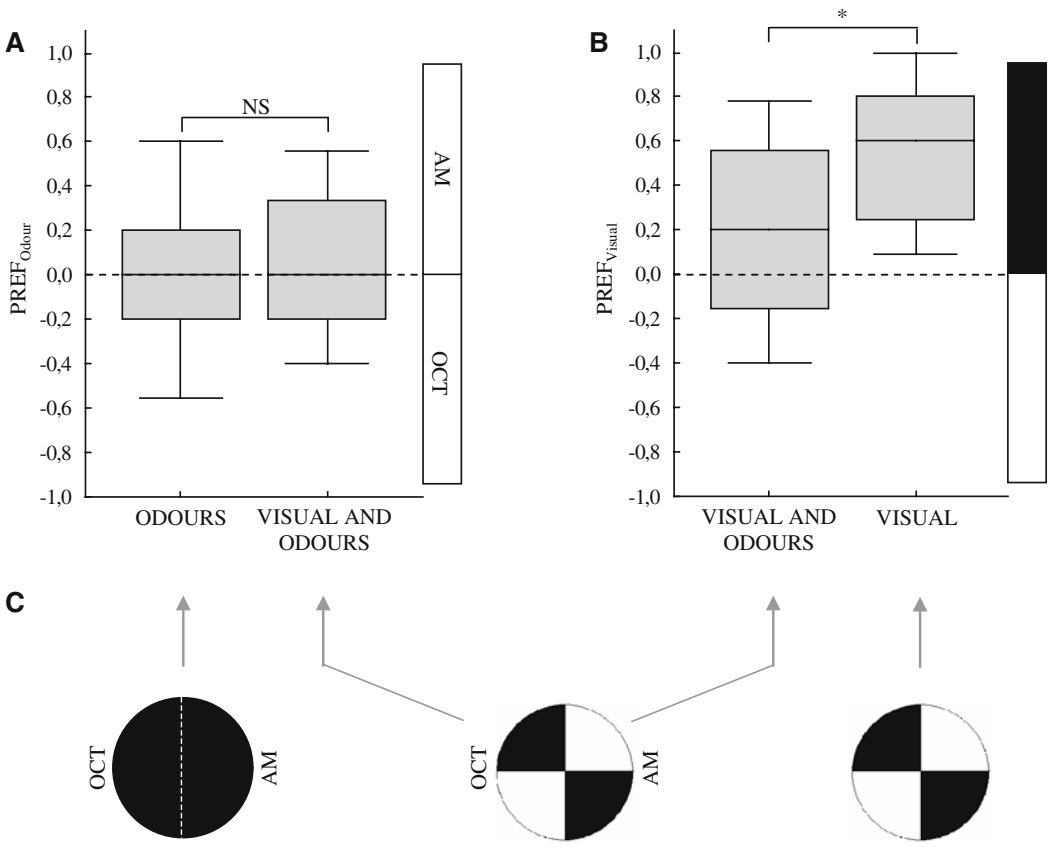

Fig. 2 Experiment 2, testing whether concomitant olfactory and visual choices interfere with each other (see sketch in (**c**) for the experimental design). Larvae are tested either for olfactory choice only (ODOURS), for visual choice only (VISUAL), or for both kinds of choice concomitantly (VISUAL AND ODOURS). **a** Odour choice is unaltered by concomitant visual choice. **b** Preference towards dark is diminished by concomitant olfactory choice. Sample sizes are $N = 44$ for each group. *NS*: $P > 0.05$, $*P < 0.05$. The *box plots* represent the median as the middle line and 10 and 90 and 25 and 75% quantiles as whiskers and box boundaries, respectively

therefore use a test situation for biconditional discrimination which does not involve such concomitant choice.

Experiment 4: Biconditional discrimination revisited

This experiment is similar to Experiment 1 in that it is a biconditional discrimination task. In Experiment 4, however, the test situation does not involve concomitant olfactory and visual choice (see Table 2). In an attempt to further increase the likelihood of observing biconditional discrimination, we use QUI as a potential punishment instead of merely presenting odours without any reinforcer. Using QUI seems reasonable although Hendel et al. (2005) and Gerber et al. (2004) showed that QUI is without any apparent reinforcing effect; this is because such reinforcing effects may not be detectable in simple, elementary conditioning, but may show in more demanding tasks like biconditional discrimination. In any event, larvae are trained either *OCT*– **OCT+ AM–** *AM*+ or *OCT*+ **OCT– AM+** *AM*–. Furthermore, we test the larvae individually, which allows for some temporal resolution of behaviour during test (see below). Finally, Experiment 4 uses four instead of five training cycles.

The main difference between Experiment 1 and 4, however, is that in Experiment 4 larvae are tested for their odour choice, either in a completely **DARK** or in a completely *LIGHT* condition, rather than in the four-quadrant X-plate assay. Each larva is individually placed in the middle of a pure agarose assay plate with a container of AM on one side and one of OCT on the other side. The position of each larva is then noted every 20 s for 5 min as 'AM', or 'OCT'. A preference value (PREF) is calculated for each larva as:

$$\mathrm{PREF} = (\#_{\mathrm{AM}} - \#_{\mathrm{OCT}})/\#_{\mathrm{Total}}. \quad (4)$$

We use '$\#_{\mathrm{AM}}$' and '$\#_{\mathrm{OCT}}$' to indicate the number of times that the larva was found on the AM or OCT sides, respectively; '$\#_{\mathrm{Total}}$' indicates the total number of observations for this larva.

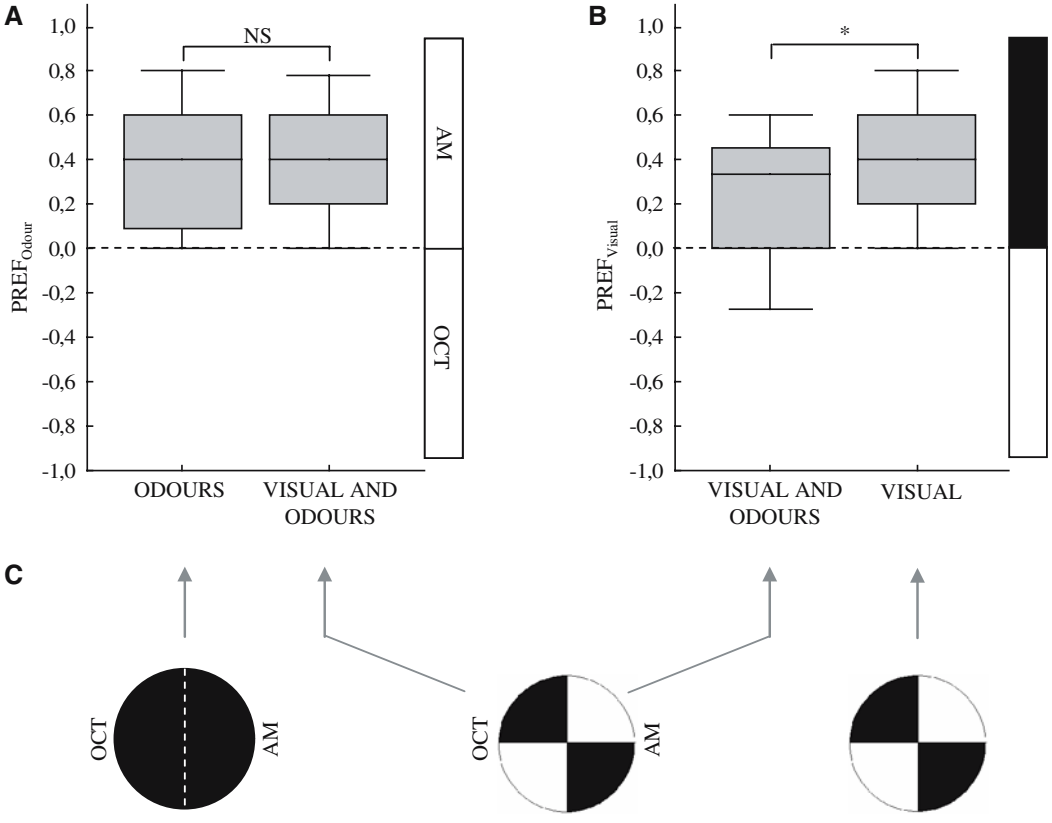

Fig. 3 Experiment 3, which is a repetition of Experiment 2 but uses olfactory conditions supporting unequal distribution of larvae between the two odours; this is achieved by using a higher concentration of AM (diluted 1:10) than in Experiment 2 (diluted 1:100) (see sketch in (**c**) for the experimental design). **a** Odour choice is unaltered by concomitant visual choice even under conditions supporting AM preference. **b** As in Experiment 2, preference towards dark is diminished by concomitant olfactory choice. Sample sizes are $N = 59$ for each group. *NS*: $P > 0.05$, $*P < 0.05$. The *box plots* represent the median as the middle line and 10 and 90 and 25 and 75% quantiles as whiskers and box boundaries, respectively

When tested in a completely *LIGHT* condition, the target side for larvae trained *OCT*– **OCT+ AM–** *AM*+ is AM. Therefore, under this testing condition, these animals should show a higher AM preference than the ones trained OCT+ **OCT– AM+** *AM*–. We find, however, no difference in PREF values between these groups (Fig. 4a; $P = 1.00$, $Z = 0.00$; $N = 16, 16$). The same is true for those two groups which are tested in **DARK**; that is, for the group trained *OCT*– **OCT+ AM–** *AM*+ the target side is OCT, whereas for the one trained *OCT*+ **OCT– AM+** *AM*– the target side is AM. However, both groups show indistinguishable PREF values when tested in **DARK** (Fig. 4b; $P = 0.22$, $Z = 1.22$; $N = 16, 16$). Under neither condition does an analysis of the time-resolved individual-animal data suggest any different conclusion (not shown). Thus, this experiment does not provide evidence for biconditional associative learning, even under testing conditions which should be generally permissive.

Experiment 5: Effects of visual context on olfactory learning or memory?

The design of Experiment 4 demands that larvae use their odour memories in a conditional way. However, an inability to do so may be due to a more general inability to establish or express odour memories under

Table 2 Experiment 4

Group	Training	Test	Target
Group 1	*OCT*– **OCT+ AM–** *AM*+	*OCT AM*	*AM*
Group 2	*OCT*+ **OCT– AM+** *AM*–	*OCT AM*	*OCT*
Group 3	*OCT*– **OCT+ AM–** *AM*+	**OCT AM**	**OCT**
Group 4	*OCT*+ **OCT– AM+** *AM*–	**OCT AM**	**AM**

AM amylacetate, OCT 1-octanol; + positive reinforcement; – negative reinforcement

AM and OCT in non-bold, italic font refers to presentation of these stimuli in *LIGHT*, whereas bold, non-italic font indicates presentation in **DARK**

LIGHT or **DARK** conditions. Experiment 5 therefore is designed to test whether visual context (presence or absence of light) influences olfactory learning, and whether changes in visual context between training and test affect memory retention.

One group of larvae is rewarded in the presence of AM but not in the presence of OCT (AM+/OCT), whereas the other group is trained reciprocally (AM/OCT+). Then, both groups are tested for their preference concerning AM versus OCT. Associative learning is indicated by a relatively stronger preference for AM after AM+/OCT training as compared to AM/OCT+ training. This difference is quantified by the learning index (LI; see below).

Larvae are trained in groups of eight. They receive three training cycles, each involving one rewarded and one unrewarded trial. Each trial lasts 1 min; between each trial, larvae are given a 1 min break. After such

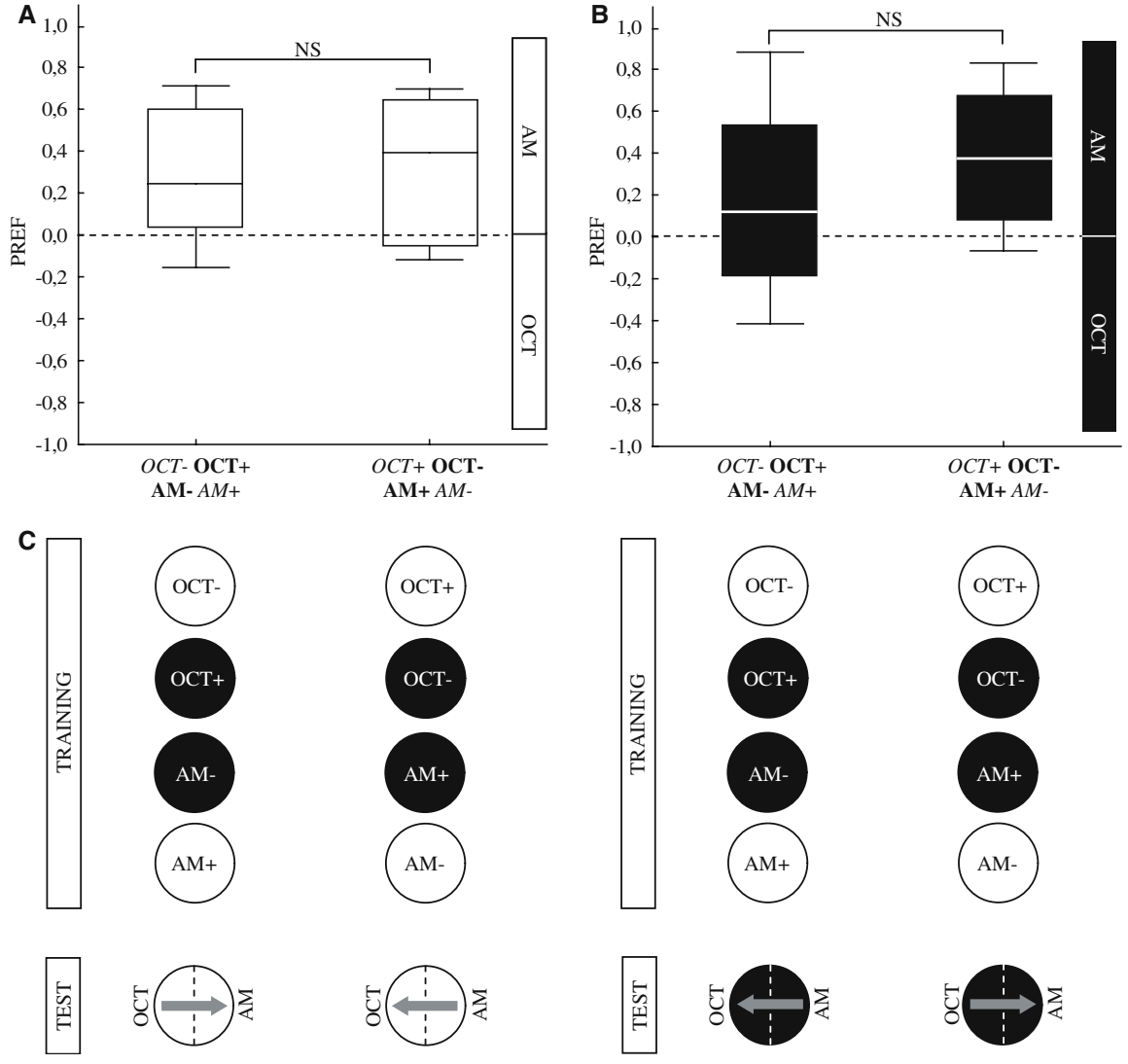

Fig. 4 Experiment 4, which like Experiment 1 is a biconditional discrimination paradigm, but using a modified testing situation (see Table 2 and sketch in (**c**) for the experimental design). **a** If tested in *LIGHT*, the target side for larvae trained *OCT–* **OCT+ AM–** *AM+* is *AM*; thus, in *LIGHT*, they should have a higher preference for AM than the ones trained *OCT+* **OCT– AM+** *AM–*. This is not the case. **b** If tested in **DARK,** the target side for larvae trained *OCT–* **OCT+ AM–** *AM+* is **OCT**. They should therefore have a lower preference for AM than the ones trained *OCT+* **OCT– AM+** *AM–*. This is not the case. The *arrows* in (**c**) point to the target sides. Sample sizes are $N = 16$ for each group. *NS*: $P > 0.05$. The *box plots* represent the median as the middle line and 10 and 90 and 25 and 75% quantiles as whiskers and box boundaries, respectively. AM and OCT in non-bold, italic font refers to presentation of these stimuli in *LIGHT*, whereas bold, non-italic font indicates presentation in **DARK**

training, larvae are tested individually, as detailed in Experiment 4. A PREF value for each larva is also calculated as in Eq. 4. On the basis of these PREF values, we calculate the learning index for pairs of larvae as:

$$\mathrm{LI} = (\mathrm{PREF}_{\mathrm{AM+/OCT}} - \mathrm{PREF}_{\mathrm{AM/OCT+}})/2. \quad (5)$$

$\mathrm{PREF}_{\mathrm{AM+/OCT}}$ is the preference value of a larva from the AM+/OCT group and $\mathrm{PREF}_{\mathrm{AM/OCT+}}$ is the preference value of the concurrently trained larva from the AM/OCT+ group (for a discussion see Hendel et al. 2005). As the LI measures the relative difference in preference between reciprocally trained groups, it gives a pure measure of associative learning; this is because all other parameters (handling, odour exposure, reward exposure, passage of time) are equal between groups.

On the basis of this design, Experiment 5 runs four groups (see Table 3): one is trained and tested in **DARK** (DD), one is trained and tested in *LIGHT* (LL). A third group is trained in **DARK** but tested in *LIGHT* (DL), and the fourth group is trained in *LIGHT* but tested in **DARK** (LD). For the **DARK** condition, illumination with red light which is invisible to flies and fly larvae, is used; for the *LIGHT* condition, white light from standard fluorescent bulbs is used. A Kruskal–Wallis test is used to test whether LI values for the four conditions are significantly different from each other.

For all four groups, the learning indices are significantly above chance level (Fig. 5; $P < 0.0125$ in all cases; $N = 251, 257, 178, 182$). Importantly, learning indices do not differ between groups (Fig. 5; $P = 0.61$, $H = 1.82$, $df = 3$). These results suggest that visual context does not have an apparent influence on odour learning, nor do changes in visual context between training and test have an impact on the retrieval of odour memory.

Discussion

We report that olfactory processing and learning are 'insulated' against visual modulation in *Drosophila* larvae: first, visual choice does not affect concomitant odour choice (Fig. 3a). Second, visual context does not affect odour learning (Fig. 5), nor does changing visual context between training and test alter retrieval of odour memory (Fig. 5). Third, larvae do not form visual-context dependent olfactory memories, despite explicit training and generally permissive conditions (Figs. 1, 4).

Table 3 Experiment 5

Group	Training	Test	Target
DD	**AM+ OCT**	**AM OCT**	**AM**
DD	**AM OCT+**	**AM OCT**	**OCT**
LL	*AM+ OCT*	*AM OCT*	*AM*
LL	*AM OCT+*	*AM OCT*	*OCT*
DL	**AM+ OCT**	*AM OCT*	*AM*
DL	**AM OCT+**	*AM OCT*	*OCT*
LD	*AM+ OCT*	**AM OCT**	**AM**
LD	*AM OCT+*	**AM OCT**	**OCT**

AM amylacetate; D dark; L light; OCT 1-octanol; + positive reinforcement

AM and OCT in non-bold, italic font refers to presentation of these stimuli in *LIGHT*, whereas bold, non-italic font indicates presentation in **DARK**

Naïve odour choice is 'insulated' against visual influence

To test for an influence of visual choice on odour choice, we confront larvae with (1) a choice between two odours (ODOURS group); or (2) a choice between light and dark (VISUAL group); or (3) both kinds of choice simultaneously (VISUAL AND ODOURS group). We find that visual choice does not alter concomitant odour choice (Fig. 2a). A more rigidly designed follow-up experiment confirms this conclusion (Fig. 3a).

Concerning interactions between olfactory and visual behaviour, in adult, freely flying flies, localizing an odour source on the horizontal plane requires visual feedback (Frye et al. 2003). In this paradigm, an odour source is put eccentrically in a vertically-striped, cylindrical arena. Localizing the odour source involves modulation of two flight parameters: First, close to the odour source, flies increase the frequency of body saccades, a behaviour which will eventually trap them around the odour source; this is a purely olfactory effect. Second, when heading directly towards the odour source, flies allow a shorter collision distance to the wall before making a saccade. Flies measure the distance to the wall visually, by the vertical edges on the wall. Thus, one can argue (Frye et al. 2003) that a visually-guided behaviour is modulated by odour.

In another study, the interaction of olfactory and visual stimuli was investigated in tethered flying flies (Frye and Dickinson 2004). Tethered flies respond to optomotor stimuli by trying to stabilize the visual field by compensatory movements. One kind of behaviour to do so is to modulate left versus right wing beat amplitude. If during such a task an attractive odour is presented from the front, the very same difference in

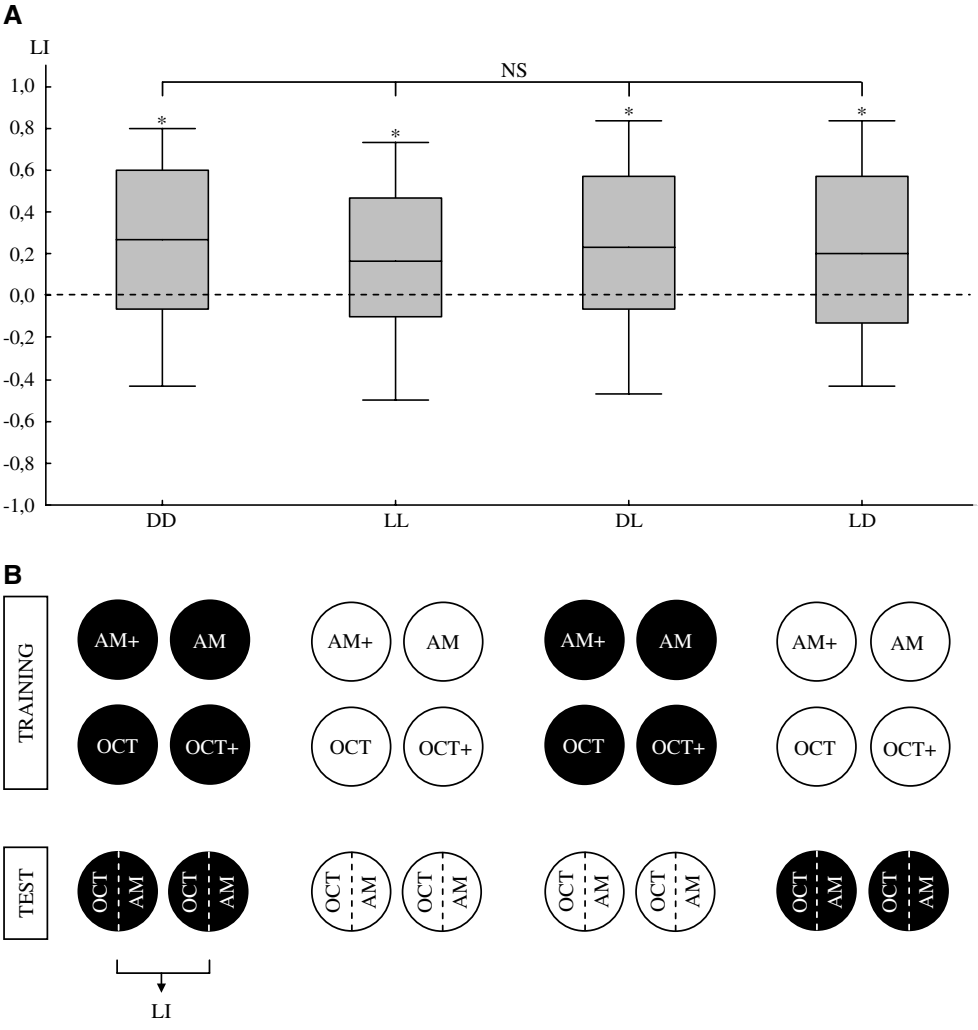

Fig. 5 Experiment 5, testing whether visual context influences olfactory learning, and whether changes in visual context between training and test affect memory retention (see Table 3 and sketch in **(b)** for the experimental design). **a** Larvae show equal learning performance (as measured by the learning index; see text), for all four experimental regimes: for train and test in dark (DD); train and test in light (LL); training in dark but test in light (DL); training in light but test in dark (LD). Sample sizes are from left to right $N = 251$, 257, 178 and 182. *NS*: $P > 0.05$; *$P < 0.0125$. The *box plots* represent the median as the middle line and 10 and 90 and 25 and 75% quantiles as whiskers and box boundaries, respectively

left versus right wing beat amplitude is observed—thus, the optomotor reflex is not altered by odour. Interestingly, under these conditions this side-asymmetry is seen on a higher over-all level of wing beat amplitudes. This over-all increase in amplitude is due to the odour, because the response to such an odour stimulus alone is a bilateral increase in wing beat amplitude (and frequency). Thus, wing beat amplitude is determined by the linear superposition of responses to olfactory and visual stimuli. This does not require the two modalities interacting with each other; indeed, the visual and the olfactory pathways supporting over-all versus side-specific modulations of wing beat amplitude may be dissociated until the muscular level (Frye and Dickinson 2004).

Taken together, it seems that across-modality interactions are specific for certain parameters (collision distance versus wing beat amplitude), behavioural routines (landing versus turning) and tasks (tracking down a goal versus choosing flight direction).

Naïve visual choice is susceptible to olfactory influence

Interestingly, although we find that visual choice does not alter concomitant olfactory choice, the reverse is not true: in two experiments we find that olfactory choice does interfere with concomitant visual choice (Figs. 2b, 3b). This asymmetry may be interpreted along two lines:

1. Maybe these two modalities are weighted unequally during orientation behaviour. The conjoint visual–olfactory response may be a weighted sum of the purely visual and the purely olfactory responses, the latter being weighted heavier. Taken the settings of Experiments 2 and 3, this would result in a diminished visual preference but a hardly altered olfactory preference in the VISUAL AND ODOURS group. Indeed such weighted summation is observed in flight control by visual

and mechanosensory stimuli in adult flies (Sherman and Dickinson 2004).

2. On the other hand, given the likely lack of visual far-distance orientation in the larva in our X-plate assay, visual stimuli may induce only undirected 'STAY-GO' responses whereas odour gradients may induce both such undirected STAY-GO responses and directed 'WHERE-to-go' responses. That is, in the VISUAL group, the only 'decision' is STAY-GO. In the VISUAL AND ODOURS group, the larvae may make two 'decisions': STAY-GO based on both visual and odour stimuli, and only then, based on the odours alone, 'decide' WHERE to go. Therefore, in the VISUAL AND ODOURS group, visual stimuli may over-all have less impact than in the VISUAL group. In the VISUAL AND ODOURS group, olfactory stimuli have access to both kinds of 'decision'; hence when compared to the ODOURS group any impact of visual stimuli on olfactory behaviour may remain undetectable. Interestingly, the aggregation behaviour of the haematophagous bug *Triatoma infestans* is similarly organized (Reisenman et al. 2000): light induces an undirected escape response and chemical cues then determine direction.

For larval *Drosophila*, we cannot decide which of these two scenarios is more adequate. In any event, concerning olfaction we can conclude that naïve olfactory behaviour is effectively 'insulated' against visual modulation in larval *Drosophila*. This brings us to the question whether olfactory *learning* is prone to visual modulation.

Visual context affects neither olfactory learning nor retrieval

We find that under four different conditions larvae show the same level of olfactory learning (Fig. 5): (1) training and test in dark; (2) training and test in light; (3) training in dark, test in light; and (4) training in light, test in dark. Thus, neither does visual context (dark or light) affect olfactory learning, nor does changing visual context between training and test alter olfactory memory retrieval.

This lack of visual modulation of olfactory learning and retrieval is striking when compared to adult *Drosophila*: odour discrimination learning in adults reportedly is not possible in a light context (M. Heisenberg, personal communication) and is therefore performed under red light. Honeybee olfactory conditioning using the proboscis extension reflex as an assay, however, is well possible in light. Interestingly, the reverse it not true, as there is a detrimental effect of olfaction on visual processing. That is, under conditions of intact olfaction, honeybees do not seem to establish associations between visual stimuli and sugar reward (Gerber and Smith 1998), at least one does not observe any conditioned extension of the proboscis after such training. If, however, the antennae are cut before training, visual learning can be observed in terms of conditioned proboscis extension towards visual stimuli (Hori et al. 2006; Kuwabara 1957). In adult flies, visual stimuli can 'potentiate' olfactory learning: Guo and Guo (2005) reported that odour learning in the so called flight simulator proceeds at a higher rate when odour-visual compound stimuli are used as compared to using odour stimuli alone. This 'potentiation' is opposite to the overshadowing effect found by Brembs and Heisenberg (2001) within the visual modality in flies and, in bees, between odours (Smith 1998) as well as between odours and antennal mechanosensory stimuli (Pelz et al. 1997). Different from our Experiment 5, however, all these studies do not investigate across-modality effects of a tonically present context on learning discrete stimuli from another sensory modality, but all look at interactions between such discrete stimuli which can directly trigger conditioned behaviour.

As mentioned before, we find that in addition to equal odour learning in light versus dark, changing visual context between training and test has no effect on olfactory memory retrieval. This context-independence of once established odour memories is in line with findings in bees showing that odour memories seem rather stable with respect to massive (free flight in natural habitat versus harnessed proboscis extension learning in the laboratory) changes in context (Gerber et al. 1996; Sandoz et al. 2000).

Does this mean that there are simply no across modality interactions in larvae? Two kinds of approach may still yield evidence for such interaction. First, pre-training manipulations reveal that odour learning in bees proceeds at a higher rate if context-reward associations are established before odour learning (Gerber and Menzel 2000), or if a visual stimulus, which had previously been associated with reward, is trained in conjunction with the odour (Gerber and Smith 1998). Such potentiating effects are seen neither between two odours in bees (Gerber and Ullrich 1999; Guerrieri et al. 2005; but see Hosler and Smith 2000) nor between colours and patterns in flies (Brembs and Heisenberg 2001). Second, if odours and visual stimuli are jointly presented without reinforcement, flies form an association between the two; this association can then be the basis for the so called sensory precondi-

tioning effect. That is, if afterwards either the odour or the visual stimulus alone is used for reinforcement learning, Guo and Guo (2005) found that responses develop also to the not-trained companion. Similar effects were previously found within the visual domain in flies (Brembs and Heisenberg 2001) and between odours in bees (Müller et al. 2000). Given that these approaches have not yet been tried in larvae, they may eventually reveal cross-modality interactions.

Larvae cannot form visual-context dependent olfactory memories despite explicit training

In an attempt to demonstrate biconditional discrimination, we train larvae such that one odour is rewarded in light, but not in dark; whereas another odour is rewarded in dark, but not in light (Table 1). Following such training, larvae are presented with all four stimulus combinations simultaneously. If biconditional learning were possible, larvae should choose the previously rewarded combinations over the non-rewarded ones. This is not the case (Fig. 1). However, successful performance in this task would have required the ability to make concomitant visual and odour choices; while these requirements are met concerning odour choice, visual choice is impaired along with concomitant odour choice (Figs. 2b, 3b). The test situation in a follow-up experiment is therefore designed such that it does not demand simultaneous odour and visual choice: larvae are trained essentially the same as in the previous experiment, but are tested for odour preference either in a uniformly dark or in a uniformly light situation (Table 2). If biconditional learning were possible, larvae should choose the odour previously rewarded in light only when tested in light; when tested in dark, in turn, they should choose the odour which was previously rewarded in dark. This, again, is not the case (Fig. 4), suggesting that the lack of evidence for biconditional discrimination learning is not limited to the particulars of the testing situation.

An explanation as to why this task is not manageable to the larvae could be that, when trained in light, no odour memories can form and/or that changes in visual context between training and test (as entailed in the design of Experiment 5; see Table 3) may be prohibitive for memory retrieval. This seems unlikely, given the observation that neither visual context affects odour learning, nor does a change in context affect odour memory retrieval (Fig. 5). We are thus inclined to conclude that, despite otherwise permissive conditions, larvae are unable to solve a biconditional discrimination task across sensory modalities.

In contrast, Matsumoto and Mizunami (2004) have shown that crickets can solve such a task in a paradigm similar to our Experiment 4. In light, one odour was rewarded with water whereas another odour was punished with saline. In dark, the contingencies were reversed. After such training, crickets preferred the odour that was rewarded in light when tested in light. In turn, when tested in dark, they preferred the odour that was rewarded in dark. Thus, it seems that in principle biconditional discrimination across sensory modalities is possible in insects (for within-modality versions see Hellstern et al. 1995; Chandra and Smith 1998; Schubert et al. 2002). Speculations as to whether a specific part of the circuitry to solve such a task is missing or still immature in larvae must be postponed until the neuronal substrate for biconditional discrimination in adult insects is better understood.

Outlook

Taken together, there does not seem to be a general rule describing whether and in which way two stimuli interact. Instead, interactions seem to depend on multiple factors and their interaction, including experimental design, species, life stage, nature of stimuli as context or phasic stimulus, behaviour examined as well as on status of the stimuli as within- or across sensory modality. This complexity, we believe, should not be dispiriting researchers to further investigate the topic, but should underscore the need to study stimulus interaction strictly on a case-by-case basis. At the very least, it should prevent too broad generalizations.

In larval *Drosophila*, olfactory learning as well as naive olfactory behaviour seems 'insulated' against visual modulation. It will be interesting to investigate whether this insulation results from an absence of convergence of visual and olfactory sensory–motor pathways or whether it is actively maintained, e.g. by virtue of the mushroom bodies (Liu et al. 1999). If the latter were true, disruption of mushroom body function may lead to a breakdown of this 'insulation'.

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References

Bitterman ME (1996) Comparative analysis of learning in honeybees. Anim Learn Behav 24:123–141

Brembs B, Heisenberg M (2001) Conditioning with compound stimuli in *Drosophila* at the flight simulator. J Exp Biol 204:2849–2859

Chandra S, Smith BH (1998) An analysis of synthetic processing of odor mixtures in the honeybee (*Apis mellifera*). J Exp Biol 201:3113–3121

Couvillon PA, Campos AC, Bass TD, Bitterman ME (2001) Intermodal blocking in honeybees. Q J Exp Psychol B 54:369–381

Frye MA, Dickinson MH (2004) Motor output reflects the linear superposition of visual and olfactory inputs in *Drosophila*. J Exp Biol 207:123–131

Frye MA, Tarsitano M, Dickinson MH (2003) Odor localization requires visual feedback during free flight in *Drosophila melanogaster*. J Exp Biol 206:843–855

Gerber B, Geberzahn N, Hellstern F, Klein J, Kowalsky O, Wüstenberg D, Menzel R (1996) Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. Anim Behav 52:1079–1085

Gerber B, Menzel R (2000) Contextual modulation of memory consolidation. Learn Mem 7:151–158

Gerber B, Scherer S, Neuser K, Michels B, Hendel T, Stocker RF, Heisenberg M (2004) Visual learning in individually assayed *Drosophila* larvae. J Exp Biol 207:179–188

Gerber B, Smith BH (1998) Visual modulation of olfactory learning in honeybees. J Exp Biol 201:2213–2217

Gerber B, Ullrich J (1999) No evidence for olfactory blocking in honeybee classical conditioning. J Exp Biol 202:1839–1854

Guerrieri F, Lachnit H, Gerber B, Giurfa M (2005) Olfactory blocking and odorant similarity in the honeybee. Learn Mem 12:86–95

Guo J, Guo A (2005) Crossmodal interactions between olfactory and visual learning in *Drosophila*. Science 309:307–310

Hellstern F, Wüstenberg D, Hammer M (1995) Contextual learning in honeybees under laboratory conditions. In: Elsner N, Menzel R (eds) Learning and memory: proceedings of the 23rd Göttingen neurobiology conference, vol. 1, pp 30. Stuttgart: Georg Thieme Verlag

Heisenberg M (2003) Mushroom body memoir: from maps to models. Nat Rev Neurosci 4:266–275

Hendel T, Michels B, Neuser K, Schipanski A, Kaun K, Sokolowski MB, Marohn F, Michel R, Heisenberg M, Gerber B (2005) The carrot, not the stick: appetitive rather than aversive gustatory stimuli support associative olfactory learning in individually assayed *Drosophila* larvae. J Comp Physiol A 191:265–279

Hori S, Takeuchi H, Arikawa K, Kinoshita M, Ichikawa N, Sasaki M, Kubo T (2006) Associative visual learning, color discrimination, and chromatic adaptation in the harnessed honeybee *Apis mellifera L*. J Comp Physiol A (in press)

Hosler JS, Smith BH (2000) Blocking and the detection of odor components in blends. J Exp Biol 203:2797–2806

Kuwabara M (1957) Bildung des bedingten Reflexes von Pavlovs Typus bei der Honigbiene, *Apis mellifica*. J Faculty Sci, Hokkaido University, Series VI, Zoology 13:458–464

Liu L, Wolf R, Ernst R, Heisenberg M (1999) Context generalization in *Drosophila* visual learning requires the mushroom bodies. Nature 400:753–756

Matsumoto Y, Mizunami M (2004) Context-dependent olfactory learning in an insect. Learn Mem 11:288–293

Michels B, Diegelmann S, Tanimoto H, Schwenkert I, Buchner E, Gerber B (2005) A role of synapsin for associative learning: the *Drosophila* larva as a study case. Learn Mem 12:224–231

Müller D, Gerber B, Hellstern F, Hammer M, Menzel R (2000) Sensory preconditioning in honeybees. J Exp Biol 203:1351–1364

Neuser K, Husse J, Stock P, Gerber B (2005) Appetitive olfactory learning in *Drosophila* larvae: effects of repetition, reward strength, age, gender, assay type and memory span. Anim Behav 69:891–898

Pelz C, Gerber B, Menzel R (1997) Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. J Exp Biol 200:837–847

Ramaekers A, Magnenat E, Marin E, Gendre N, Jefferis G, Luo L, Stocker R (2005) Glomerular maps without cellular redundancy at successive levels of the *Drosophila* larval olfactory circuit. Curr Biol 15:1–11

Reisenman CE, Lorenzo Figueiras AN, Giurfa M, Lazzari CR (2000) Interaction of visual and olfactory cues in the aggregation behaviour of the haematophagous bug *Triatoma infestans*. J Comp Physiol [A] 186:961–968

Sandoz JC, Laloi D, Odoux JF, Pham-Delegue MH (2000) Olfactory information transfer in the honeybee: compared efficiency of classical conditioning and early exposure. Anim Behav 59:1025–1034

Scherer S, Stocker RF, Gerber B (2003) Olfactory learning in individually assayed *Drosophila* larvae. Learn Mem 10:217–225

Schubert M, Lachnit H, Francucci S, Giurfa M (2002) Nonelemental visual learning in honeybees. Anim Behav 64:175–184

Sherman A, Dickinson MH (2004) Summation of visual and mechanosensory feedback in *Drosophila* flight control. J Exp Biol 207:133–142

Smith BH (1998) Analysis of interaction in binary odorant mixtures. Physiol Behav 65:397–407

Sokolowski MB (2001) *Drosophila*: genetics meets behaviour. Nat Rev Genet 2:879–890

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SHORT COMMUNICATION

No evidence for visual context-dependency of olfactory learning in *Drosophila*

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Abstract How is behaviour organised across sensory modalities? Specifically, we ask concerning the fruit fly *Drosophila melanogaster* how visual context affects olfactory learning and recall and whether information about visual context is getting integrated into olfactory memory. We find that changing visual context between training and test does not deteriorate olfactory memory scores, suggesting that these olfactory memories can drive behaviour despite a mismatch of visual context between training and test. Rather, both the establishment and the recall of olfactory memory are generally facilitated by light. In a follow-up experiment, we find no evidence for learning about combinations of odours and visual context as predictors for reinforcement even after explicit training in a so-called biconditional discrimination task. Thus, a 'true' interaction between visual and olfactory modalities is not evident; instead, light seems to influence olfactory learning and recall unspecifically, for example by altering motor activity, alertness or olfactory acuity.

Keywords Olfaction · Vision · Learning · Context · Biconditional discrimination

Introduction

Animals need to simultaneously deal with stimuli from different sensory modalities. Choosing which to ignore, respond to or learn about is thus a biologically important task, potentially requiring a cross-talk between sensory modalities and an integration with the particular behavioural demands. Whether such cross-talk can be demonstrated in insect behaviour and how the relatively simple brains of insects may accomplish such tasks are thus interesting questions for basic research (examples of such analyses of cross-talk between sensory modalities come from various species: fruit fly: Guo and Guo 2005; honeybee: Gerber and Smith 1998; cricket: Matsumoto and Mizunami 2004; cockroach: Sato et al. 2006; bumblebee: Fauria et al. 2002; for examples concerning non-insect invertebrates: Hvorecny et al. 2007; see "Discussion" for details).

We use the fruit fly *Drosophila melanogaster* to explore interactions between olfactory and visual modalities. Fruit flies readily learn odours as predictors for an aversive electric shock (Tully and Quinn 1985). In addition, flies can associate illumination, color or patterns with reinforcement (Heisenberg 1989; Wolf and Heisenberg 1991). Olfactory (reviewed in Gerber et al. 2004), and to some extent also visual learning (Liu et al. 2006), in fruit flies are fairly well studied at the cellular and molecular level, but the interaction between them has so far not been investigated. The present study asks how visual context affects olfactory learning and recall and whether fruit flies integrate the information about the visual context into their olfactory memory.

Materials and methods

Flies and experimental setup

D. melanogaster of the Canton-Special wild-type strain are maintained as mass culture at 25°C, 60–70% relative humidity and under a 14:10-h light/dark cycle. On the day before experiments, 1- to 4-day-old flies are collected in

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fresh food vials and kept overnight at 18°C and 60–70% relative humidity. Experiments are performed at 22–25°C and 75–85% relative humidity, either under dim red light which does not allow flies to see (dark) or with illumination from a 50-W light bulb, placed ~50 cm above the experimental setup (light). Flies are trained and tested in groups of ~100. As odourants, 90 µl benzaldehyde (BA) or 340 µl 3-octanol (OCT; both from Fluka, Steinheim, Germany) are applied undiluted in 1-cm-deep Teflon containers of 5- and 14-mm diameters, respectively. Otherwise, the setup is as described by Schwaerzel et al. (2003).

Effect of visual context on olfactory learning and recall

We compare levels of olfactory learning and recall under four conditions (Fig. 1b): training and test in dark (DD); training and test in light (LL); training in light, test in dark (LD); training in dark, test in light (DL).

Training starts by loading the flies into the setup (0:00). The control odour is presented from 4:00 min to 5:00 min. The to-be-learned odour follows from 6:00 min to 7:00 min. At 6:15 min, electric shock is given as 12 pulses of 100 V, each 1.2 s long, with 5 s inter-pulse interval. At 13:00 min, flies are transferred for testing to the choice point of a T-maze where they can distribute between the two odours for 2 min. These parameters follow Schwaerzel et al. (2003). In half of the cases, we use BA as the to-be-learned and OCT as the control odour (OCT/ BA-Shock), whereas in the other half of the cases, training is reciprocal (BA/ OCT-Shock; Fig. 1a). In half of the cases, training starts with the control odour, whereas in the other half, the to-be-learned odour has precedence. We determine the number of flies in each arm of the maze and calculate an odour preference (PREF) as:

$$\mathrm{PREF} = [(\#_{\mathrm{BA}} - \#_{\mathrm{OCT}})/\#_{\mathrm{Total}}] \times 100 \qquad (1)$$

A learning index (LI) is then calculated as the difference in preference between the reciprocally trained groups:

$$\mathrm{LI} = \left(\mathrm{PREF}_{\mathrm{OCT/\ BA\text{-}Shock}} - \mathrm{PREF}_{\mathrm{BA/\ OCT\text{-}Shock}}\right)/2 \qquad (2)$$

$\mathrm{PREF}_{\mathrm{OCT/\ BA\text{-}Shock}}$ and $\mathrm{PREF}_{\mathrm{BA/\ OCT\text{-}Shock}}$ denote the preferences of the respectively trained groups. Negative LIs indicate avoidance of the learned odour.

We use one-sample sign tests to compare the LIs of each group to zero. A Bonferroni correction keeps the experiment-wide error rate at 5% by dividing the significance level α by the number of comparisons (e.g. in the case with four comparisons α=0.05/4). For comparing LIs between groups, we use a 2×2 factorial analysis of variance (ANOVA) after having probed for normality by the Lilliefors test. We present the data as box plots; in these plots, the midline represents the median, whereas box-boundaries and whiskers represent the 25% and 75% as well as 10% and 90% quartiles, respectively.

Biconditional discrimination

To test whether with explicit training *Drosophila* can establish visual context-dependent olfactory memories, we use a so-called 'biconditional discrimination' design. For a given group of flies, one odour is paired with shock in light, but not in darkness; another odour, in turn, is paired with shock in darkness, but not in light. Thus, neither the odours nor the visual situation alone can unambiguously predict shock—only the combinations of both can.

We use four groups (Fig. 2a,b): one group, in light, receives shock with BA, but not with OCT, whereas in darkness, contingencies are reversed. A second group is trained reciprocally. Both groups are then tested in dark for their preference between BA and OCT. The two further groups are trained the same as the ones already mentioned, but are tested in light.

Reasoning that biconditional discrimination is a more difficult task for the flies to master and that it may require some repetition, we use more but 'weaker' training trials than in the first experiment: Training consists of six blocks, each with the four respective kinds of training trial. Across repetitions of the experiment, we pseudo-randomise the order of trials, avoiding two 'shocked-trials' in a row. Each trial lasts 2 min and is immediately followed by the next. At 0:00 min, visual context is set. Odour is presented at 0:45 min for 15 s. In 'shocked-trials', shock is presented at 1:00 min as four pulses of 100 V, each 1.2 s long and with 5 s inter-pulse interval. Thus, odour precedes shock with an onset-to-onset interval of 15 s; the visual context (either light or dark), on the other hand, spans the entire training trial. At 5 min after the end of the last training trial, flies are transferred to the choice point of a T-maze and are allowed 2 min to distribute between the two odours. The visual context during this 2-min choice period then can be light or dark. The PREF values are calculated according to Eq. 1. We compare the PREF values between reciprocally trained groups with a Mann–Whitney *U* test.

In a follow-up experiment, we test for 'usual' odour-shock learning and recall using the same training and test parameters as in the biconditional discrimination experiment; 'usual' here means that the complete experiment is run in darkness and by reliably pairing one odour with shock (Fig. 2c). Specifically, we use two reciprocally trained groups (i.e. OCT/ BA-Shock and BA/ OCT-Shock), whose PREF values are calculated according to Eq. 1 for comparison to each other with a Mann–Whitney *U* test. Based on the difference in preference between reciprocally trained groups, we additionally calculate LIs as in Eq. 2. LIs are compared to zero using a one-sample sign test.

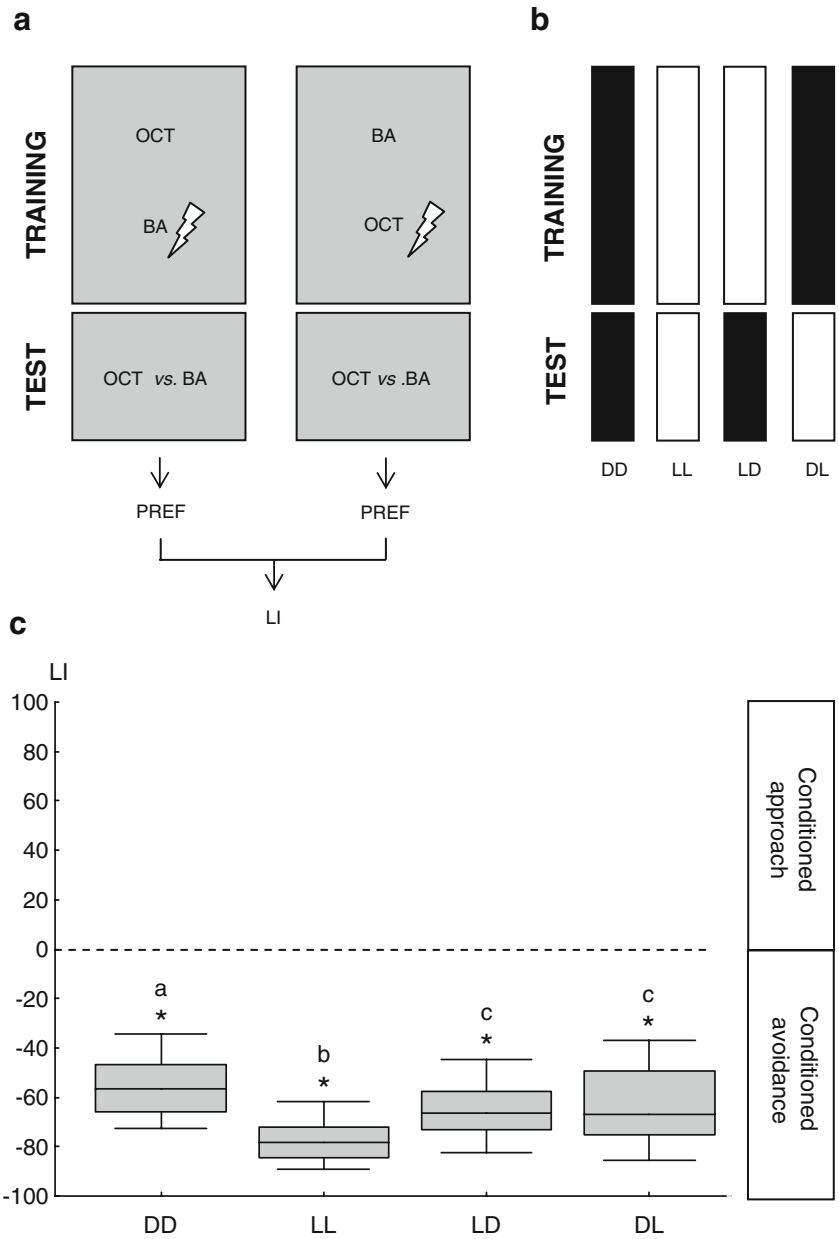

Fig. 1 Light facilitates both establishment and recall of olfactory memory. **a** For each visual condition, we train two groups: one receives 3-octanol (*OCT*) as the control odour, while benzaldehyde (*BA*) is paired with electric shock, whereas the second group is trained reciprocally. Each group is then given the choice between the two odours. Associative learning results in avoidance of the previously punished odour. We calculate a learning index (*LI*) based on the difference between odour preferences (*PREF*) of the two reciprocally trained groups. Negative LIs indicate avoidance of the learned odour. **b** Based on the reciprocal design detailed in **a**, flies are either trained and tested in dark (*DD*); trained and tested in light (*LL*); trained in light, tested in dark (*LD*) or trained in dark tested in light (*DL*). **c** In all groups, significant learning scores are found. Comparing between groups, flies perform poorest in DD and best in LL. The LD and DL conditions support intermediate performance

Results

Light facilitates both establishment and recall of olfactory memory

We compare the level of olfactory learning and recall under four conditions (Fig. 1b): training and test in dark (DD); training and test in light (LL); training in light, test in dark (LD); training in dark, test in light (DL). We find significant learning scores for each of the four conditions (Fig. 1c; one-sample sign tests: for each condition: $\alpha=0.05/4$; $P<0.001$; sample sizes $n=33, 33, 30, 34$). Comparing between conditions reveals that flies show the poorest scores when training and test happen in dark (DD) and do best when

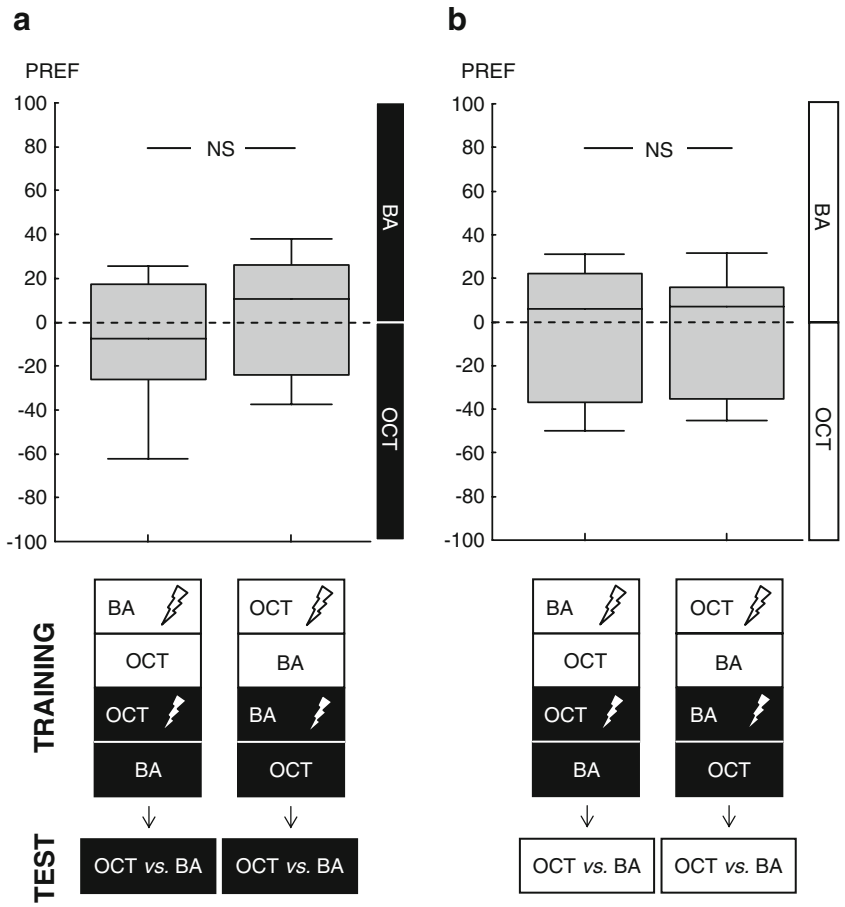

Fig. 2 No evidence for biconditional discrimination. **a** The first group of flies is trained such that in light, benzaldehyde (*BA*) is punished but 3-octanol (*OCT*) is not, whereas in dark, contingencies are reversed. The second group is trained reciprocally. When tested in dark, the first group should avoid OCT more strongly relative to the second group, which is not the case. **b** The third group of flies is trained such that in light, BA is punished but OCT is not, whereas in darkness, contingencies are reversed. The fourth group is trained reciprocally. When tested in light, the third group should avoid BA more strongly relative to the fourth group, which is not the case. **c**, **d** Flies are trained and tested in darkness with one of the odours unambiguously paired with shock, otherwise keeping the parameters of training and test as in **a** and **b**. **c** After punishment of BA, flies avoid BA stronger relative to the reciprocally trained group. **d** Learning indices calculated from these odour preferences are significantly different from zero, arguing that learning and recall are possible under these conditions

both happen in light (LL). When only training (LD) or only test (DL) happen in light, performance is intermediate [Fig. 1c; 2×2 factorial ANOVA: effect of training context: α=0.05, $F_{1,126}$=16.68, P<0.001; effect of test context: α=0.05, $F_{1,126}$=14.39, P<0.001; interaction of effects: α=0.05, $F_{1,126}$=0.81, P=0.37; each condition gives normally distributed LIs (Lilliefors test α=0.05; P>0.2, each) fulfilling the prerequisite for an ANOVA; sample sizes as above]. Thus, both olfactory learning and recall are generally enhanced by light. As it does not matter whether the visual context matches between training and test (see lack of significant interaction above), information about the visual context does not seem to be integrated into olfactory memory. Importantly, the kind of training used in this experiment allows flies to predict shock based on odours alone; in the following experiment, in contrast, we demand flies to learn about the visual context as well as about the odours.

No evidence for biconditional discrimination across visual and olfactory modalities

We run a 'biconditional discrimination' experiment where one odour is paired with shock in light but not in darkness; another odour, in turn, is paired with shock in darkness, but not in light (see sketches in Fig. 2a, b). Thus, neither the odours nor the visual context can unequivocally predict shock; only if flies were able to consider the combinations of both could they avoid danger. Biconditional discrimination should result in a difference in odour preference between reciprocally trained groups. However, neither when being tested in dark nor when tested in light do the reciprocally trained groups differ in their behaviour (test in dark: Fig. 2a, Mann–Whitney U test: α=0.05, U=94.00, P=0.32, sample sizes n=15, 16; test in light: Fig. 2b, Mann–Whitney U test: α=0.05, U=107.00, P=0.84,

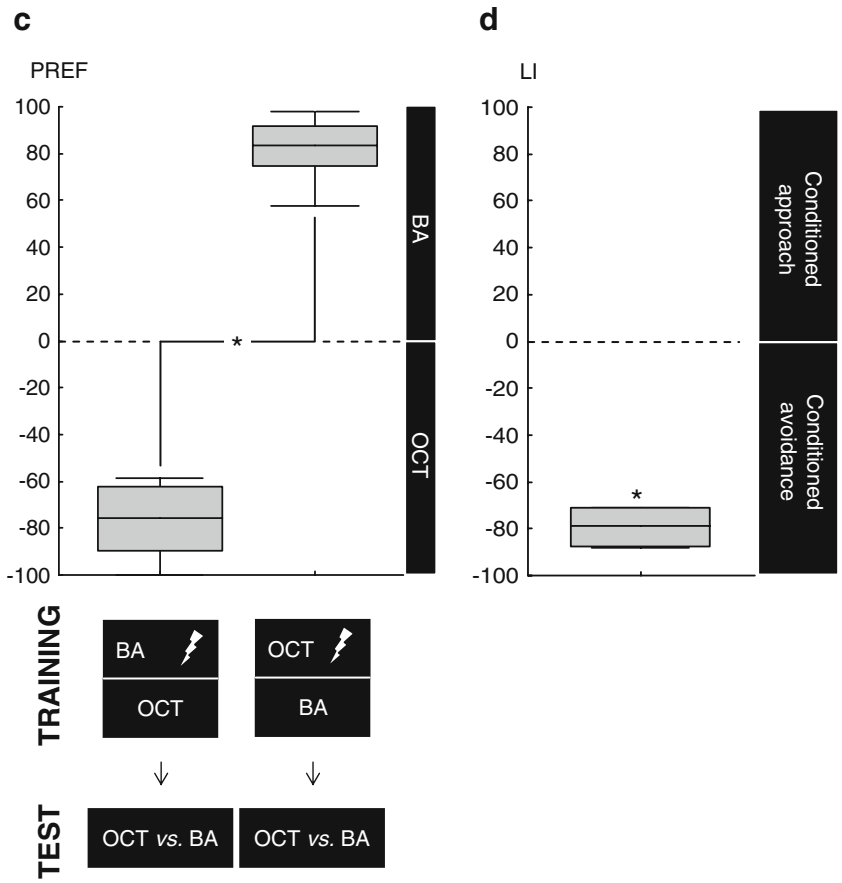

Fig. 2 (continued)

sample sizes n=15, 15). This lack of effect does not appear to be due to low statistical power because testing in light reveals no evidence for a difference between the reciprocally trained groups (the P value equals 0.84), and for testing in dark, if anything, we observe a tendency in the 'wrong direction'. Thus, we find no evidence for biconditional discrimination.

Could this reflect adverse effects of the high number of shock-pulses during training (48 instead of 12; e.g. Schwaerzel et al. 2003) or the long duration of training (~50 min instead of ~10 min)? We run a 'normal' odour-shock learning experiment (i.e. training and test are performed in darkness; one odour is reliably paired with shock), otherwise using the same training and test parameters as in the previous experiment. We find that learning and recall are possible under these conditions: The two reciprocally trained groups differ in their odour preference (Fig. 2c; Mann–Whitney U test: α=0.05, U=0.00, P=0.002, sample sizes n=6, 6), resulting in significant learning scores calculated based on this difference in preference (Fig. 2d; one-sample sign test: α=0.05, P=0.03, sample size n=6). Thus, as far as the olfactory modality is concerned, the training and test parameters of the biconditional discrimination experiment are in principle adequate. As for the visual modality, although we do not explicitly test for the learning of light versus dark, the result of the previous experiment (Fig. 1) argues that these two contexts sufficiently differ from each other to matter for the flies' behaviour.

We conclude from our experiments that there is no evidence for across-modality biconditional discrimination; clearly, absence of proof is not proof of absence. However, in principle, our experimental design seems appropriate. First, successful biconditional discrimination in crickets (Matsumoto and Mizunami 2004) and cockroaches (Sato et al. 2006) also used 'light' versus 'dark' as visual contexts. Second, the number of trials for biconditional discrimination training was chosen to match the number of trials required for asymptotic elemental learning, both in our case (Fig. 2d) and in crickets (Matsumoto and Mizunami 2000, 2004). Finally, all three experimental designs involve training with four combinations of olfactory and visual stimuli, but use only two of them at test. Testing with all four combinations, at least in larval fruit flies, does not reveal biconditional discrimination, either (Yarali et al. 2006).

Discussion

We find no evidence for biconditional discrimination using combinations of visual and olfactory cues in fruit flies,

speaking against interaction between the two modalities; however, both the establishment and the recall of olfactory memory are facilitated by light, apparently speaking in favour of such interaction. How can these findings be reconciled?

We structure this discussion considering the possible site of interaction along the sensory-motor continuum. That is, processing of different sensory modalities may interact 'truly' in the sense that the interaction is stimulus-specific, or the interaction may be 'amodal' in the sense that it happens between the behavioural tendencies or 'values' which the respective stimuli have elicited. Here, we consider only five examples for these two kinds of interaction between visual and olfactory modalities in insects.

As an example of what we here call an 'amodal' interaction, consider the case of odour-shock learning: At the site of convergence, information about the particular features of the odour is maintained (i.e. in terms of the pattern of activated mushroom body neurons; Wang et al. 2004), but information about the particular features of the shock is not. That is, in addition to the reflex responses it elicits, shock induces a reinforcement signal carried by very few dopaminergic neurons impinging onto the mushroom bodies; these neurons most likely can be activated by any negative stimulus (in fruit flies: Schwaerzel et al. 2003; Riemensperger et al. 2005; Schroll et al. 2006; in honeybees: Vergoz et al. 2007; in crickets: Unoki et al. 2005; comparably, in monkeys, dopaminergic neurons carry a reward signal; Schultz et al. 1997). In other words, they act as a 'funnel' for different kinds of negative stimuli, conveying a general 'Bad!' signal. Thus, the actual interaction is between olfactory processing and an 'amodal' value signal (Fig. 3a).

A similar interaction takes place when two stimuli relate to a common reinforcer: Honeybees learn odours as predictors for sugar more readily when these odours are accompanied by visual cues, which, in a first experimental phase, had already been learned to predict sugar (Gerber and Smith 1998). Likewise, in adult fruit flies, aversive olfactory learning during tethered flight is facilitated specifically by already learnt visual cues (Guo and Guo 2005). In both cases, it does not matter *which* particular visual stimulus is present as long as it is a previously *learnt* one. Therefore, also in these cases, the actual interaction is between olfactory processing and a value signal—specifically, the value signal triggered by the learnt visual stimulus.

As an example of a 'true' interaction, consider the association of two cues with each other such that the occurrence of one 'reminds' of the other. Guo and Guo (2005) exposed adult fruit flies, during tethered flight, simultaneously to an odour and a visual cue without any reinforcement. Then, in a second experimental phase, they trained the flies in the absence of the visual cue such that flying towards the odour resulted in heat punishment. After such training, flies not only avoided the punishment-associated odour but interestingly also that particular visual

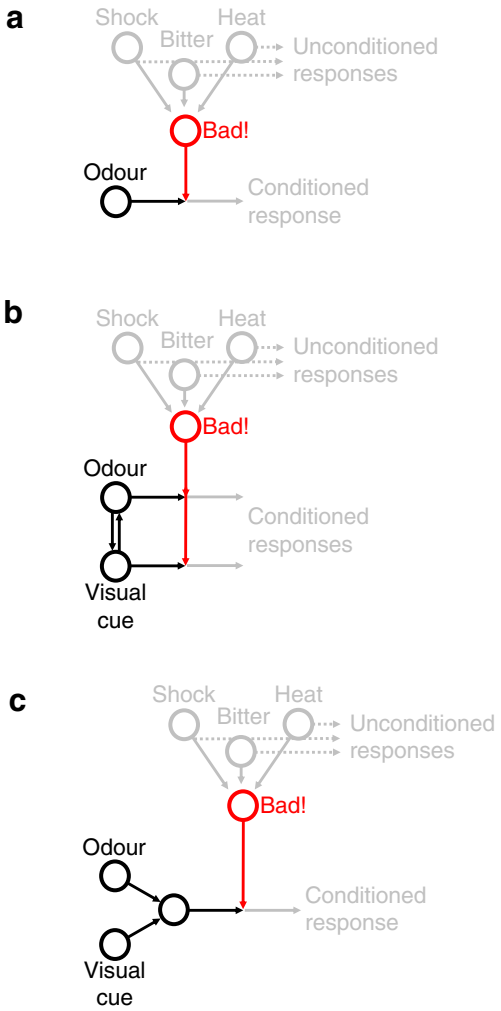

Fig. 3 'Amodal' versus 'true' interactions between sensory modalities. Processing of different sensory modalities may interact 'truly', that is, in a stimulus-specific manner, or the interaction may be 'amodal', that is, between the 'values' elicited by the respective stimuli rather than the actual stimulus features. **a** Odour-shock learning exemplifies an 'amodal' interaction: Shock and probably all other aversive stimuli feed into a common 'Bad!' signal, which interacts with the particular stimulus features of the odour. That is, the odour is associated with 'something Bad!' and thus will subsequently be avoided. **b** Sensory pre-conditioning on the other hand requires a 'true' interaction between sensory modalities: Initially, an odour and a visual cue are presented simultaneously in the absence of any reinforcer. This joint presentation endows both stimuli with the ability to 'call up' each other in a stimulus-specific manner. When in a subsequent experimental phase, one of the two is paired with aversive heat, the other is 'called up' as well and is also associated with the 'Bad!' signal. **c** Such stimulus-specific interaction is also required for biconditional discrimination, that is, learning about the combinations of odours and visual cues: Representations of both the odour and the visual cue must converge to form an additional joint representation of the two. This joint representation then can be associated with the 'Bad!' signal

cue which had previously been associated with the odour, but which itself was never associated with heat (sensory preconditioning). This suggests that the odour and the visual cue may be able to specifically 'call up' each other by virtue of their initial joint presentation; in other words, such joint presentation must endow, for example, the odour with the capacity to trigger a functional visual representation despite the actual absence of the visual stimulus (Fig. 3b). The neuronal circuitry to accomplish such a task remains to be identified.

A 'true' stimulus-specific interaction is also required for biconditional discrimination learning. In such a task, one odour is reinforced in light, but not in darkness; whereas another odour is reinforced in darkness, but not in light (see sketches in Fig. 2). Thus, neither the odours nor the visual situation alone can reliably predict reinforcement, but only the combination of both can. Crickets (Matsumoto and Mizunami 2004) as well as cockroaches (Sato et al. 2006) readily master such kind of task. On the other hand, neither in adult (this study; Fig. 2) nor in larval (Yarali et al. 2006) fruit flies any evidence for biconditional discrimination learning across sensory modalities has so far been found. This kind of learning clearly requires a combinatorial stage of olfactory and visual processing (Rudy and Sutherland 1992); in other words, there must be a stage of processing where olfaction and vision converge such that a combined signal can enter into association with reinforcement (thus, different from the situation concerning sensory preconditioning, the interaction must be *down*stream of the initial sensory representation; Fig. 3c). Indeed, in honeybees (Mobbs 1982; Ehmer and Gronenberg 2002), cockroaches (Strausfeld and Li 1999) and crickets (Honegger and Schurmann 1975), afferents from antennal lobes and optic lobes converge onto the mushroom bodies, whereas in *Drosophila*, direct visual input to mushroom bodies is not evident (Otsuna and Ito 2006). Thus, there appears a correspondence between the availability of visual input to the mushroom bodies and the ability for biconditional discrimination across the visual and olfactory modality.

In any event, the enhancing effect of light on olfactory learning and recall (Fig. 1) stands apart from both kinds of interaction discussed so far. We find that olfactory memory is recalled independent of whether the present visual context matches between training and test. Thus, neither an 'amodal' value signal nor any feature of the visual context seems to be integrated with olfactory memory. Instead, visual context may influence olfactory learning and recall indirectly, for example via altering motor activity, alertness or olfactory acuity. This kind of effect would not require a specific interaction between olfactory and visual circuits.

In summary, there does not seem to be a general rule concerning the organisation of insect behaviour across sensory modalities. Rather, whether and exactly which kinds of cross-modality interaction is found seems to depend on the particular requirements of the behavioural task and the evolutionary preparedness, that is, the available circuitry of the particular species to handle it.

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References

Ehmer B, Gronenberg W (2002) Segregation of visual input to the mushroom bodies in the honeybee (*Apis mellifera*). J Comp Neurol 451:362–373

Fauria K, Dale K, Colborn M, Collett TS (2002) Learning speed and contextual isolation in bumblebees. J Exp Biol 205:1009–1018

Gerber B, Smith BH (1998) Visual modulation of olfactory learning in honeybees. J Exp Biol 201:2213–2217

Gerber B, Tanimoto H, Heisenberg M (2004) An engram found? Evaluating the evidence from fruit flies. Curr Opin Neurobiol 14:737–744

Guo J, Guo A (2005) Crossmodal interactions between olfactory and visual learning in *Drosophila*. Science 309:307–310

Heisenberg M (1989) Genetic approach to learning and memory (mnemogenetics) in *Drosophila melanogaster*. In: Lindauer M (ed) Fortschritte der Zoologie. Fundamentals of memory formation: neuronal plasticity and brain function. G. Fischer, Stuttgart, Germany, pp 3–45

Honegger HW, Schurmann FW (1975) Cobalt sulphide staining of optic fibres in the brain of the cricket, *Gryllus campestris*. Cell Tissue Res 159:213–225

Hvorecny LM, Grudowski JL, Blakeslee CJ, Simmons TL, Roy PR, Brooks JA, Hanner RM, Beigel ME, Karson MA, Nichols RH, Holm JB, Boal JG (2007) Octopuses (*Octopus bimaculoides*) and cuttlefishes (*Sepia pharaonis*, *S. officinalis*) can conditionally discriminate. Anim Cogn 10:449–459

Liu G, Seiler H, Wen A, Zars T, Ito K, Wolf R, Heisenberg M, Liu L (2006) Distinct memory traces for two visual features in the *Drosophila* brain. Nature 439:551–556

Matsumoto Y, Mizunami M (2000) Olfactory learning in the cricket *Gryllus bimaculatus*. J Exp Biol 203:2581–2588

Matsumoto Y, Mizunami M (2004) Context-dependent olfactory learning in an insect. Learn Mem 11:288–293

Mobbs PG (1982) The brain of the honeybee *Apis mellifera*. I. The connections and spatial organization of the mushroom bodies. Philos Trans R Soc Lond B 298:309–354

Otsuna H, Ito K (2006) Systematic analysis of the visual projection neurons of *Drosophila melanogaster*. I. Lobula-specific pathways. J Comp Neurol 497:928–958

Riemensperger T, Voller T, Stock P, Buchner E, Fiala A (2005) Punishment prediction by dopaminergic neurons in *Drosophila*. Curr Biol 15:1953–1960

Rudy JW, Sutherland RJ (1992) Configural and elemental associations and the memory coherence problem. J Cogn Neurosci 4:208–216

Sato C, Matsumoto Y, Sakura M, Mizunami M (2006) Contextual olfactory learning in cockroaches. Neuroreport 17:553–557

Schroll C, Riemensperger T, Bucher D, Ehmer J, Voller T, Erbguth K, Gerber B, Hendel T, Nagel G, Buchner E, Fiala A (2006) Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. Curr Biol 16:1741–1747

Schultz W, Dayan P, Montague PR (1997) A neural substrate of prediction and reward. Science 275:1593–1599

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. J Neurosci 23:10495–10502

Strausfeld NJ, Li Y (1999) Organization of olfactory and multimodal afferent neurons supplying the calyx and pedunculus of the cockroach mushroom bodies. J Comp Neurol 409:603–625

Tully T, Quinn WG (1985) Classical conditioning and retention in normal and mutant *Drosophila melanogaster*. J Comp Physiol [A] 157:263–277

Unoki S, Matsumoto Y, Mizunami M (2005) Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. Eur J Neurosci 22:1409–1416

Vergoz V, Roussel E, Sandoz JC, Giurfa M (2007) Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. PLoS ONE 2:e288

Wang Y, Guo HF, Pologruto TA, Hannan F, Hakker I, Svoboda K, Zhong Y (2004) Stereotyped odor-evoked activity in the mushroom body of *Drosophila* revealed by green fluorescent protein-based Ca^{2+} imaging. J Neurosci 24:6507–6514

Wolf R, Heisenberg M (1991) Basic organization of operant behavior as revealed in *Drosophila* flight orientation. J Comp Physiol [A] 169:699–705

Yarali A, Hendel T, Gerber B (2006) Olfactory learning and behaviour are 'insulated' against visual processing in larval *Drosophila*. J Comp Physiol [A] 192:1133–1145

Chapter II.

Predictive learning of pain-relief in fruit flies

‘Pain-relief’ learning in fruit flies

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Abstract

We study the behavioural consequences of 'traumatic', painful experience. These consequences are fundamentally asymmetric: Fruit flies, for example, learn two kinds of prediction regarding 'traumatic' experience: If an odour *precedes* an electric shock during training, it predicts shock, and flies subsequently avoid it. If the sequence of events during training is reversed, i.e. odour *follows* shock, the odour predicts relief from shock and flies approach it. We call this latter effect 'relief' learning and show that, in terms of psychological mechanism, it establishes genuinely associative conditioned approach behaviour. We then embark upon parametric analyses and find that relief learning is reproducible across experimenters; it does not depend on gender and reaches asymptotic levels after six training trials. Out of five chosen odour-pairs, two support relief learning at all concentrations tested; for one odour-pair, we observe optimal relief learning at an intermediate odour concentration; for two odour-pairs, relief learning cannot be demonstrated. Furthermore, relief learning is maximal using relatively mild shocks, supporting stable retention for the first 2 hours after training. Knowledge of these parametric features should aid uncovering relief learning in other experimental systems. We finally return to the question of psychological mechanism and report that context-shock pre-training has no effect on subsequent relief learning, arguing that it is not mediated by context associations. These analyses further our understanding of the psychological mechanisms underlying behavioural changes after traumatic experience. They allow research into the neurobiology of pain-relief learning, enabling the implementation of truly bio-inspired learning rules for technical devices.

Introduction

Choosing correctly what to do is difficult. Obviously, having a reasonable prediction as to what may happen is helpful in this regard. This is because such predictions allow preparatory behaviour, in the simplest case moving towards or moving away from the predicted event. For example, fruit flies trained with sequential presentations of an odour and electric shock (odour-shock training) will subsequently avoid the odour because it predicts something 'bad', whereas flies trained with pairings of odour and sugar

will subsequently approach the odour because it predicts something 'good' (Tully & Quinn 1985; Tempel et al. 1983). Thus, the behaviour expressed, and the kind of learning underlying it, may be characterized as either appetitive or aversive. These kinds of learning typically are dissociated in terms of the neuronal pathways for reinforcement processing (Hammer & Menzel 1995; Mirenowicz & Schultz 1996; Schwaerzel et al. 2003; Unoki et al. 2005; Schroll et al., 2006).

Clearly, it is not only helpful to correctly predict what will happen, but it may also be helpful to predict what will *not* happen. Indeed, fruit flies can learn to predict the absence of shock, if the 'normal' timing of odour and shock during training is reversed (Tanimoto et al. 2004): If the shock comes first and only then the odour is presented (shock-odour training), flies show a relative preference for the odour during subsequent test because it signals relief (Solomon & Corbit 1974; Wagner 1981) and/ or safety (Sutton & Barto 1990; Chang et al. 2003) from shock. This asymmetry in terms of the timing of the to-be-associated events is a basic common feature of predictive learning (e.g. dog: Moscovitch & LoLordo 1968; rabbit: Plotkin & Oakley 1975; rat: Maier et al. 1976; snail: Britton & Farley 1999; pigeon: Hearst 1988; honey bees: Hellstern et al. 1998) and of synaptic plasticity (concerning insects Cassenaer & Laurent 2007; for a comprehensive review see Caporale & Dan 2008) and hence of the mnemonic organization of brain function in general. In other words, one can view learning as referring either to the presence or the absence of the respective event. This presence-absence dichotomy is 'orthogonal' to the appetitive-aversive dichotomy referred to above; thus one may actually distinguish four kinds of associative, predictive learning:

(i) predicting the presence of something good or

(ii) predicting its absence;

(iii) predicting the presence of something bad or

(iv) predicting its absence.

To contrast the latter two kinds of learning, we call them *punishment learning* and *relief learning*, respectively. Here, we focus on relief learning: We provide a detailed parametric account and the first analyses of the psychological mechanism of this behavioural effect, which is so far ill-characterized in

fruit flies. Studying relief learning is important, as understanding this 'backside' of pain is indispensable for a comprehensive understanding of the behavioural consequences of painful, 'traumatic' experience. Specifically, the parametric description of relief learning provided in this study will aid researchers of other experimental systems to uncover such relief learning in "their" preparation; the analyses into the psychological mechanisms underlying relief learning reported here should aid future studies about its neurobiological mechanisms. Last but not least, this study provides a basis for establishing a comprehensive computational model of predictive learning, including its potential implementation into a bio-inspired robot.

Materials and Methods

By and large, we use standard methods of maintaining and training flies (Tully & Quinn 1985; Schwaerzel et al. 2003; Tanimoto et al. 2004; see also Fig. 1A- C). Below we summarize the essential details and parameters as they pertain to our study.

Flies

We use flies of the Canton-Special wild-type strain, aged > 1 to < 4 days after eclosure. Flies are kept in mass culture maintained at 25 °C, 60- 70 % relative humidity and are subject to a 14 h/ 10 h light/ dark cycle. On the day prior to experiments, flies are transferred to fresh food vials and kept over-night at 18 °C and 60- 70 % relative humidity.

Learning experiments

Experiments are performed at 22- 25 °C and 70- 85 % relative humidity. Flies are trained and tested in groups of 100- 150. Training takes place under dim red light, whereas tests are done in complete darkness.

Flies receive eight training trials (unless mentioned otherwise) (see Fig. 1C). At time 0:00 min, flies are loaded to the experimental set-up, which takes approximately 1 min. After an additional accommodation period of 3 min, the control odour is presented for 15 s. Only in Experiment 2, this control

odour is omitted. At 7:30 min, the electric shock is delivered. The shock consists of 4 pulses of 100 V, each 1.2 s long and followed by the next pulse after an onset- onset interval of 5 s. The to-be-learned odour is then presented at 8:10 (unless mentioned otherwise) for 15 s. Thus, the inter-stimulus interval (ISI), between the onset of the shock and the onset of the to-be-learned odour is 40 s. At 12:00 min, flies are transferred back to food vials for 16 min until the next trial starts.

Once training is completed, the usual 16 min break is given until animals are loaded again to the set-up for the test. After an accommodation period of 5 min, animals are transferred to the choice point of a T-maze, where they can choose between the control odour and the learned odour. Only in Experiment 2, this test is between the learned odour and a non-scented maze-arm. Thus, the time interval between the end of the last training trial and the beginning of the test is 21 min (unless stated otherwise). After 2 min, the arms of the maze are closed and the number of animals (in the following denoted #) in each arm are counted. A preference index (PREF) is calculated as:

$$(1) \qquad PREF = (\#Learned\ odour - \#Control\ odour)*100 / \#Total$$

Within each group, one subgroup is trained using 3-octanol (OCT) as the control odour and benzaldehyde (BA) as the to-be-learned odour to obtain the preference score $PREF_{BA}$, while a second subgroup is trained reciprocally ($PREF_{OCT}$) (Fig. 1B). Only in Experiment 2, this reciprocal design does not apply, as only BA is used as the to-be-learned odour (see Results section). Further, in Experiment 4 different odours are used (see Results section). PREFs from the two reciprocal groups are averaged to obtain a learning index (LI):

$$(2) \qquad LI = (PREF_{BA} + PREF_{OCT}) / 2$$

Positive LIs indicate conditioned approach to the learned odour, whereas negative values reflect conditioned avoidance.

Mann-Whitney U-tests and Kruskal-Wallis tests are used to compare the scores between different groups of flies. One-sample sign tests are used to determine whether scores are significantly different from zero. When multiple one-sample or multiple pair-wise comparisons are made, we adjust significance levels using a Bonferroni correction to maintain an experiment-wide error rate of 5 %; this is done by dividing the critical *P*-value 0.05 by the number of one-sample or pair-wise comparisons. For example, if one group from a four-group experiment is compared against zero, we report the *P*-levels of the one-sample sign test as $P< 0.05/4$. Statistical analyses are performed using Statistica on a PC.

Odourants

As odourants, benzaldehyde (BA; Fluka, Steinheim, Germany); 3-octanol (OCT; Fluka, Steinheim, Germany); amylacetate (AM; Merck, Darmstadt, Germany); isoamlyacetate (IAA; Sigma-Aldrich, Steinheim, Germany); limonene (LM; Sigma-Aldrich, Steinheim, Germany); and 4-methylcyclohexanol (MCH; Fluka, Steinheim, Germany) are used. Odourants are applied either pure or 10, 100, 1000 or 2000-fold diluted in paraffin oil (PARA: Fluka, Steinheim, Germany). Teflon containers of 5 mm diameter are used for odour application for BA, AM and IAA; 14 mm diameter containers are used for OCT and MCH, and 7 mm diameter containers for LM. In Experiment 2, 15 mm diameter containers are used both for BA and for solvent PARA. Airborne odour concentrations are unknown.

Results

Experiment 1: Predictive learning is asymmetrical.

Tanimoto et al. (2004) found that electric shock can induce either conditioned avoidance or conditioned approach to an odour, depending on the relative timing between odour and shock during training. Using slightly modified parameters, we first seek to replicate these experiments.

We use four experimental groups which receive equal handling, exposure to the control odour, exposure to the to-be-learned odour as well as to the electric shock; what differs between groups is only the interval between the onset of the shock and the onset of the to-be-learned odour (Fig. 1C; inter-

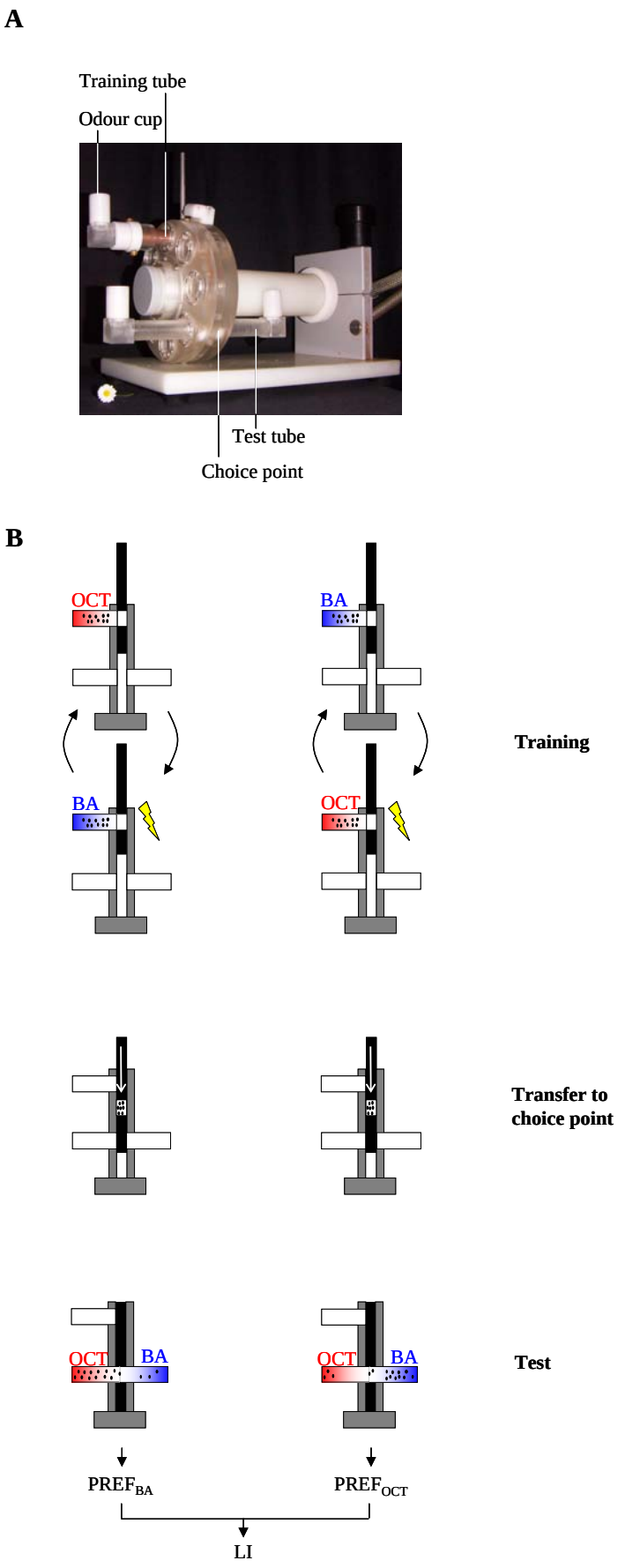

Fig. 1. See the next page for the legend.

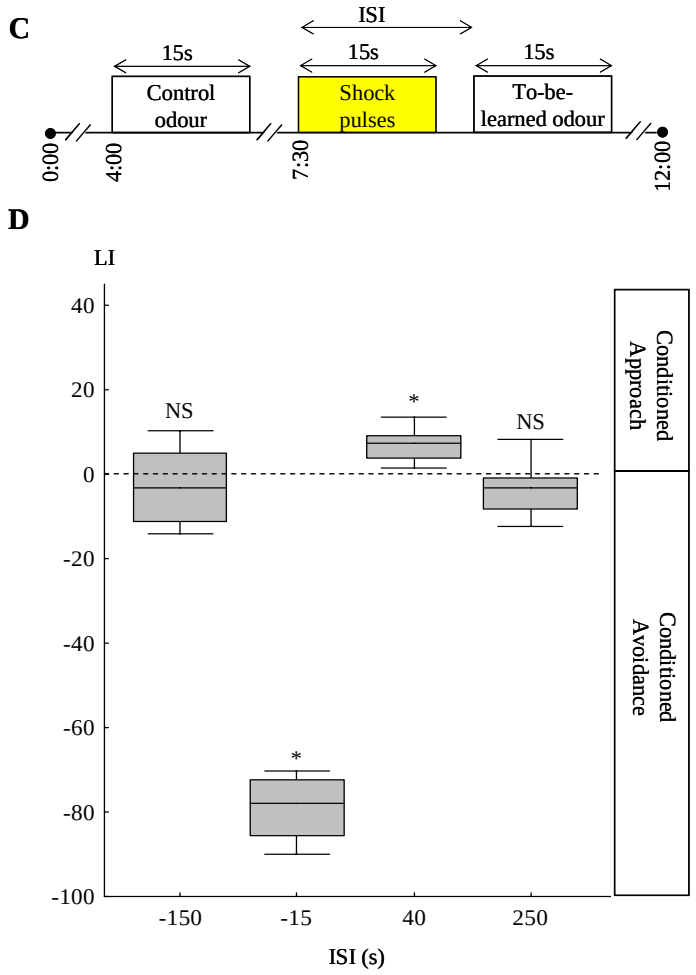

Fig. 1. Flies show conditioned avoidance or conditioned approach, depending on the relative timing of odour and shock during training

A. The experimental apparatus: The training tubes are coated inside with copper wire (not shown), which allows to apply electric shock. Odours are delivered by attaching an odour cup at one end of the training tube. Odour-saturated air is sucked through the training tube.
B. One group of flies is trained with 3-octanol (OCT) as the control odour and benzaldehyde (BA) is paired with electric shock (left); another group is trained reciprocally (right). Once the training is completed, flies are transferred to the choice point between two test tubes, each scented with one of the two odours encountered during training. A preference index (PREF) is calculated based on the distribution of the flies. A learning index (LI) is then calculated as the difference in odour preference between the reciprocally trained groups. A positive LI means that flies approach the learned odour, whereas a negative LI means that they avoid the learned odour.
C. Time-line of a single training trial. The inter-stimulus interval (ISI) is the interval between the onset of shock and the onset of the to-be-learned odour. The ISI is positive for shock-odour pairings and negative for odour-shock pairings.
D. After odour-shock training with a short ISI (-15 s), flies avoid the learned odour during test. In contrast, after shock-odour training with a short ISI (40 s), flies approach the learned odour. Flies trained with long intervals (ISI= -150 s or 250 s) do not show these effects. Sample sizes are from left to right: N= 8, 8, 9, 10. *: $P < 0.05/4$; NS: $P > 0.05/4$. The middle line represents the median, the boundaries of the box the 25 % and 75 % quantiles, and the whiskers the 10 % and 90 % quantiles, respectively.

stimulus interval: ISI). In different groups of animals, the to-be-learned odour is presented long before (ISI= -150 s), shortly before (ISI= -15 s), shortly after (ISI= 40 s), or long after (ISI= 250 s) the shock. After such training, flies´ preference between the control and the learned odour is tested in a T-maze choice assay and a learning index (LI) is calculated as detailed in the Methods section. Positive LIs indicate conditioned approach to the learned odour, whereas negative LIs reflect conditioned avoidance.

We find no learning if the to-be-learned odour had been presented either long before or long after the shock (Fig. 1D: one-sample sign tests for ISI= -150 and 250 s: N= 8, 10, $P > 0.05/4$ for each). In contrast, we find conditioned avoidance if the to-be learned odour had been presented shortly before the shock in training (Fig. 1D: one-sample sign test for ISI= -15 s: N= 8, $P < 0.05/4$). Importantly, those flies that are trained such that the to-be-learned odour closely follows shock, approach the learned odour (Fig. 1D: one-sample sign test for ISI= 40 s: N= 9, $P < 0.05/4$).

Thus, flies avoid the learned odour after odour-shock training. In contrast, after shock-odour training flies show a relative preference for the learned odour. We next consider whether this latter effect comes about by a conditioned increase in attractiveness of the learned odour, or by a decrease in its baseline, unconditioned aversiveness.

Experiment 2: Relief learning induces genuine conditioned approach.

There are two kinds of explanation for the positive learning indices reported in Experiment 1. That is, at the used concentrations, both odours are repellent to unconditioned, experimentally naïve flies (data not shown). As usual in fly learning experiments, we had initially adjusted the concentrations of the two odours such that in a choice situation naïve flies distribute equally between them (data not shown), because they are repelled equally by either odour (red and dark-blue arrows in Fig. 2A). Thus, at the moment of test both of these baseline repellent tendencies likely are present as well.

The first kind of explanation (Fig. 2A top) for the positive learning indices in Experiment 1 suggests that, as a result of shock-odour training an additional, genuinely associative attractive tendency develops for the learned odour. In other words, the learned odour predicts relief, and flies show associative

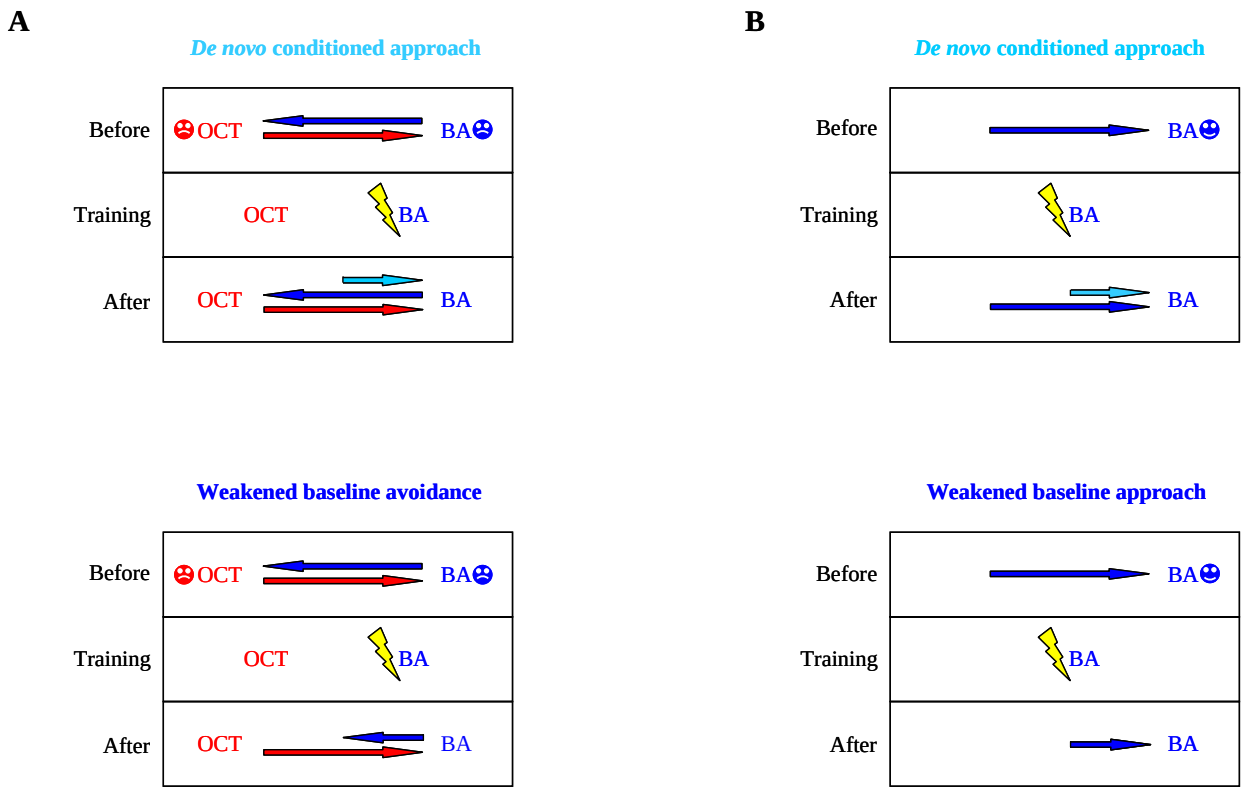

Fig. 2. Testing for the psychological mechanisms of relief learning

A. Sketches of two possible explanations for relief learning. Top: Experimentally naïve, unconditioned flies avoid both odours. Concentrations are adjusted as to obtain equally strong avoidance of both odours; this is why the red and dark-blue arrows for the control odour (OCT) and the to-be-learned odour (BA), respectively, are depicted at same length. During training, the control odour is presented alone, while the to-be-learned odour is presented shortly after shock. After such training, in addition to the baseline unconditioned aversion from both odours (red and dark-blue arrows), flies show a *de novo* genuinely associative conditioned approach to the learned odour (light-blue arrow). Thus, flies' overall preference is for the learned odour.
Bottom: Alternatively, shock may deteriorate processing of those odours that are presented shortly after it, rendering these odours less effective at the moment of test. Hence, training weakens the baseline avoidance from the learned odour (BA; truncated dark-blue arrow), leaving intact the avoidance from the control odour (OCT; red arrow). Thus, the net preference would be for the learned odour.
B. The two accounts for relief learning predict different outcomes when a single odour, benzaldehyde (BA) is used at a concentration which supports a baseline appetitive response. Top: A *de novo* conditioned approach (light-blue arrow) induced via shock-odour training would add-up with the existing baseline approach (dark-blue arrow) and thus would further increase the attractiveness of the odour. Bottom: Alternatively, a deterioration in odour processing would render the odour less attractive at the moment of test (truncated dark-blue arrow).

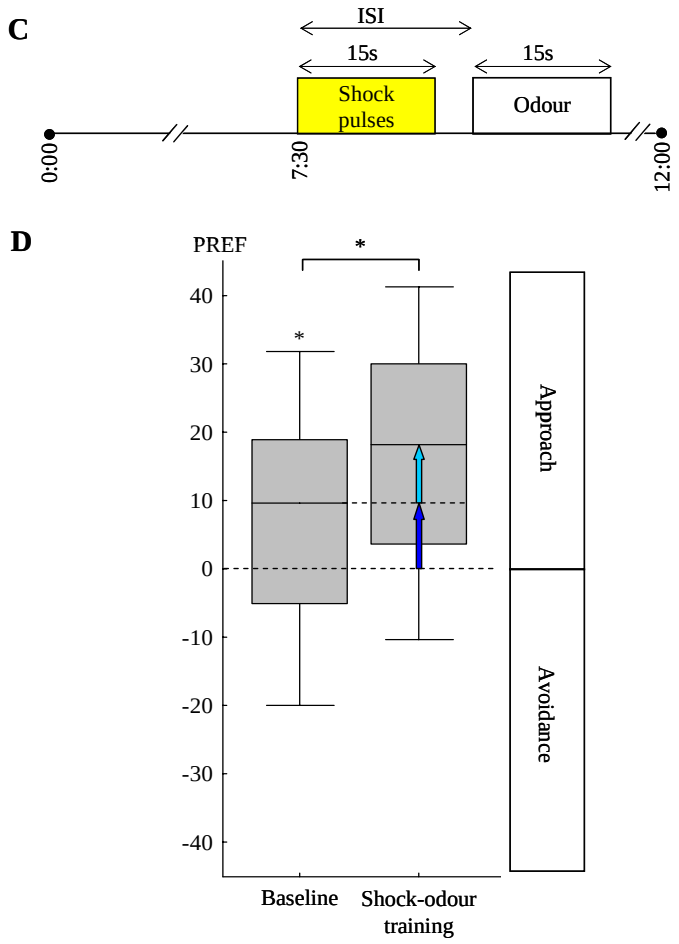

Fig. 2. Continued.

C. Time-line of a single training trial, which uses a single odour. The inter-stimulus interval (ISI) is the interval between the onset of shock and the onset of odour. The ISI is positive for shock-odour trials and negative for odour-shock trials.
D. Approach to the odour is indicated by positive preference indices (PREF), whereas negative values reflect avoidance. The baseline response to the odour is appetitive (dark-blue arrow). Shock-odour training with a short ISI further increases this approach tendency (light-blue arrow). This argues that shock-odour training induces a genuine conditioned approach towards the odour, rather than deteriorating its processing, which would have rendered it less attractive. Sample sizes are from left to right N= 123, 59. *: $P < 0.05$. Details of box plots are as in Fig. 1D.

conditioned approach to it (light-blue arrow). This attractive tendency adds to the mentioned *baseline avoidance* of both odours. Thus, the balance between the two odours is shifted in favour of the learned odour.

An alternative explanation (Fig. 2A bottom) would suggest that the positive learning indices rather come about by a decrease in the baseline avoidance of the learned odour (truncated dark-blue arrow). That is, one may postulate that the presentation of shock *per se* can, in a yet-unidentified way, weaken processing of those odours that are presented shortly afterwards to eventually render them less effective, and hence less aversive, at the moment of test. Note that such kind of process also would be specific for the learned odour; it would, however, not invoke any *de novo* conditioned approach tendency for it.

We pit these two explanations against each other using a modified experimental design, omitting the control odour. Importantly, such an experiment cannot use an odour concentration which supports baseline avoidance. This is because under such conditions both proposed mechanisms predict that preference scores for the odour will be shifted from aversion towards zero (Fig. 2A). In contrast, if we use the odour at a concentration which supports baseline appetitive responses, the two proposed mechanisms predict different experimental outcomes (Fig. 2B): An additional conditioned approach tendency that develops by shock-odour training would further increase the attractiveness of the odour, resulting in an 'up'-shift of the preference scores (Fig. 2B top); whereas an impairment in the processing of the odour would decrease its attractiveness, shifting scores towards zero (Fig. 2B bottom). We therefore reminded ourselves of the classical observation that odour responses in unconditioned, experimentally naïve flies change from avoidance to approach with decreasing odour concentration (Ayyub et al., 1990). We thus choose a very low concentration of odourant which does support appetitive baseline scores and use it in shock-odour training.

We use benzaldehyde (BA) at 2000-fold dilution as odour (Fig. 2C). One group receives shock-odour training with an inter-stimulus interval (ISI) of 40 s between odour and shock. Such training supports positive learning scores (Fig. 1D; Tanimoto et al., 2004). Three control groups receive training

with different, very long intervals between odour and shock (ISI= -210 s, -150 s or 200 s). These training conditions do not support positive learning scores (Fig. 1D; Tanimoto et al., 2004). After training, flies from all four groups are given the choice between BA and a non-scented maze-arm, and a preference index (PREF) is calculated (see Methods section). Positive PREF values indicate approach towards the odour, negative values reflect avoidance.

PREF values of the three control groups, which are trained with inter-stimulus intervals of either -210 s, -150 s, or 200 do not differ statistically (Kruskal-Wallis test: H_2= 4.49, N= 59, 32, 32, P> 0.05); data are therefore pooled and taken as a measure of the baseline response to BA at the moment of test (Fig. 2D: dark-blue arrow). As intended, we observe an appetitive baseline response (Fig. 2D: one sample sign test for 'Baseline', N= 123, P< 0.05). The critical question now is whether the group trained with a short shock-odour interval (40 s), which does support positive learning indices (Fig. 1D; Tanimoto et al., 2004), shows higher or lower preference scores than this baseline. If shock-odour training were to impair processing of the odour, this group should have below-baseline PREF values. Clearly, this is not the case. To the contrary, PREF values after shock-odour training are above baseline level (Fig. 2D: U-test: U= 2700.50, N= 123, 59, P< 0.05), arguing for an additional conditioned approach component (light-blue arrow). Thus, positive learning indices obtained by shock-odour training reflect a genuine associative conditioned approach tendency.

Experiment 3: Relief learning is robust and is found in both genders.

As relief learning is much less strong than punishment learning (approximately 1/5 of punishment learning: Tanimoto et al. 2004; 1/8 of punishment learning: Fig. 1D), we seek to bolster our confidence in this effect by testing whether it is replicable across three different experimenters. Learning indices do not differ between experimenters (Fig. 3A: Kruskal-Wallis test: H_2= 0.57, N= 12, 11, 16, P> 0.05) and are significantly different from zero in the pooled dataset (Fig. 3B: one-sample sign test for the pooled dataset: sample size as above, P< 0.05). Thus, relief learning is a reliable, yet small effect. In addition, Figure 3C shows the data from Figure 3B separated by gender of the flies. Learning indices do not differ

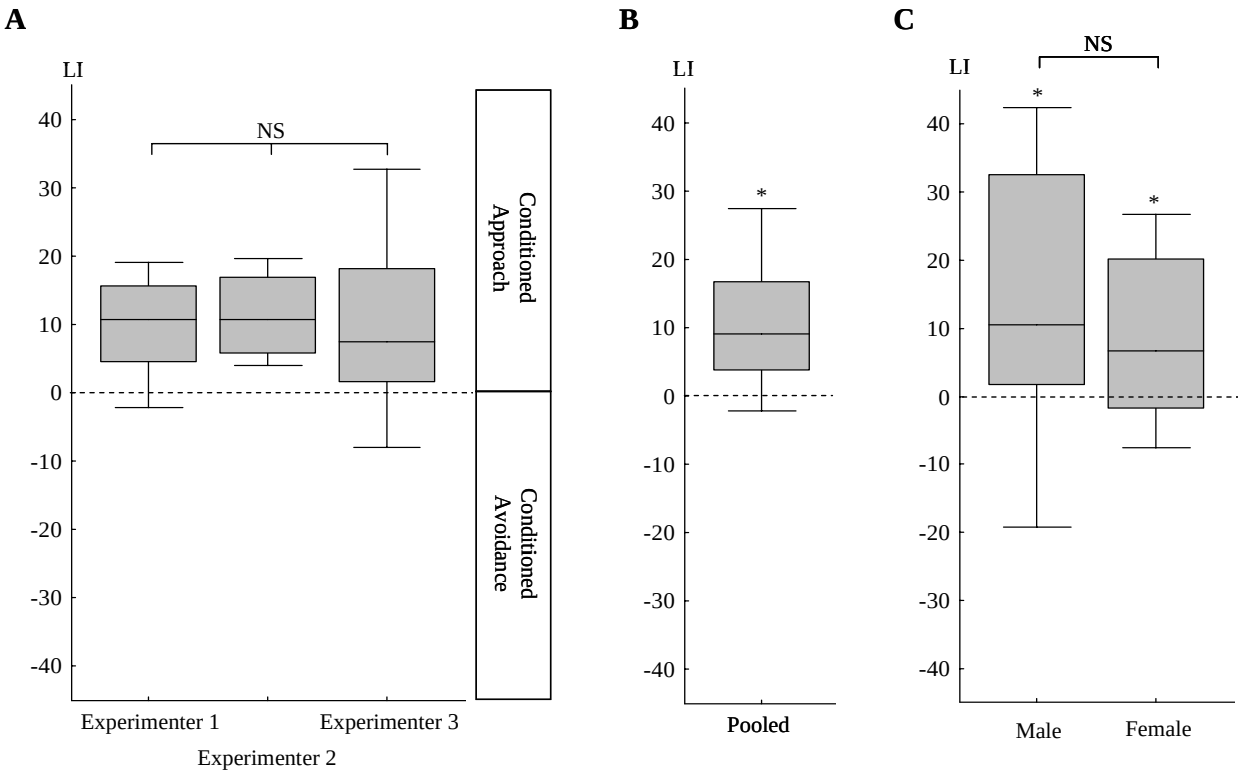

Fig. 3. Pain-relief learning is reproducible across experimenters and does not differ between male and female flies

A. After shock-odour training, all three experimenters observe conditioned approach towards the learned odour. The amount of relief learning does not differ between experimenters. Sample sizes are from left to right: N= 12, 11, 16. NS: $P > 0.05$.
B. The pooled dataset shows significant relief learning. Sample size as combined from (A) is N= 39. *: $P < 0.05$.
C. Data from (B), separated by flies' gender. There is no difference between genders in terms of relief learning. Sample sizes, as in (B) are N= 39, 39. NS: $P > 0.05$. *: $P < 0.05/2$.
Details of box plots are as in Fig. 1D.

between male and female flies (Fig. 3C: U-test: U= 680.5, sample sizes as above, $P> 0.05$); both genders do show positive learning indices, indicating relief learning (Fig. 3C: one-sample sign tests for each gender: sample size as above, $P< 0.05/2$)

Experiment 4: Relief learning requires (relatively many) repetitions.

Next, we test the effect of the number of training trials on relief learning. Different groups of flies receive one, two, four, six or eight shock-odour pairings. The number of training trials has a significant influence on relief learning (Fig. 4: Kruskal-Wallis test: $H_4= 19.58$, N= 16, 15, 20, 19, 23, $P< 0.05$). Specifically, one, two and four training trials do not yield conditioned approach to the learned odour (Fig. 4: one-sample sign tests: sample sizes as above, $P> 0.05/5$ in all three cases), whereas six and eight trials do (Fig. 4: one-sample sign tests: sample sizes as above, $P< 0.05/5$ in both cases). Relief learning after six trials is as good as after eight trials (Fig. 4: U-test: U= 208.0, sample sizes as above, $P< 0.05$). Thus, relief learning, using the current parameters and training set-up, requires at least six shock-odour pairings, with which it also reaches an asymptote.

Experiment 5: Testing for effects of odour identity and concentration.

Next, we test the effect of odour identity and concentration on relief learning, and do so for five odour-pairs: MCH-OCT, BA-LM, BA-OCT, AM-IAA, and OCT-LM. We use pure odorant as well as 10 and 100-fold dilutions, except for MCH-OCT, for which a 1000-fold dilution is used in addition. Dilutions refer to the odorant loaded to the experimental device. Airborne odour concentrations are unknown.

For three out of the five tested odour pairs, relief learning is observed: For MCH-OCT, the learning indices depend on odour concentration (Fig 5; Kruskal-Wallis test for MCH-OCT: $H_3= 8.50$, N= 20, 20, 19, 16, $P< 0.05$). A 100-fold dilution does support relief learning, whereas either higher or lower concentrations do not (Fig 5: one-sample sign tests: MCH-OCT: sample sizes as above, $P> 0.05/4$ for pure, 10-fold and 1000-fold diluted; $P< 0.05/4$ for 100-fold diluted). Thus, for MCH-OCT the range of concentrations tested uncovers optimal relief learning at an intermediate odour concentration.

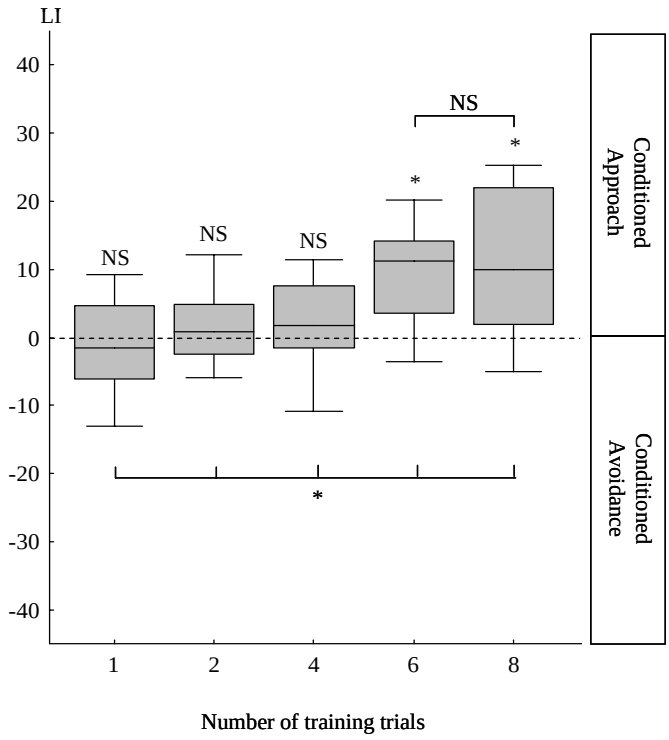

Fig. 4. Relief learning requires at least six training trials

One, two and four shock-odour training trials yield no relief learning, whereas six and eight trials do. Learning indices do not get better with more than six trials. Sample sizes are from left to right: N= 16, 15, 20, 19, 23. *: $P < 0.05/5$; NS: $P > 0.05/5$, except for the comparison across all groups, and the comparison between the six-trial and the eight-trial groups, for which *: $P < 0.05$, and NS: $P > 0.05$ is used. Details of box plots are as in Fig. 1D.

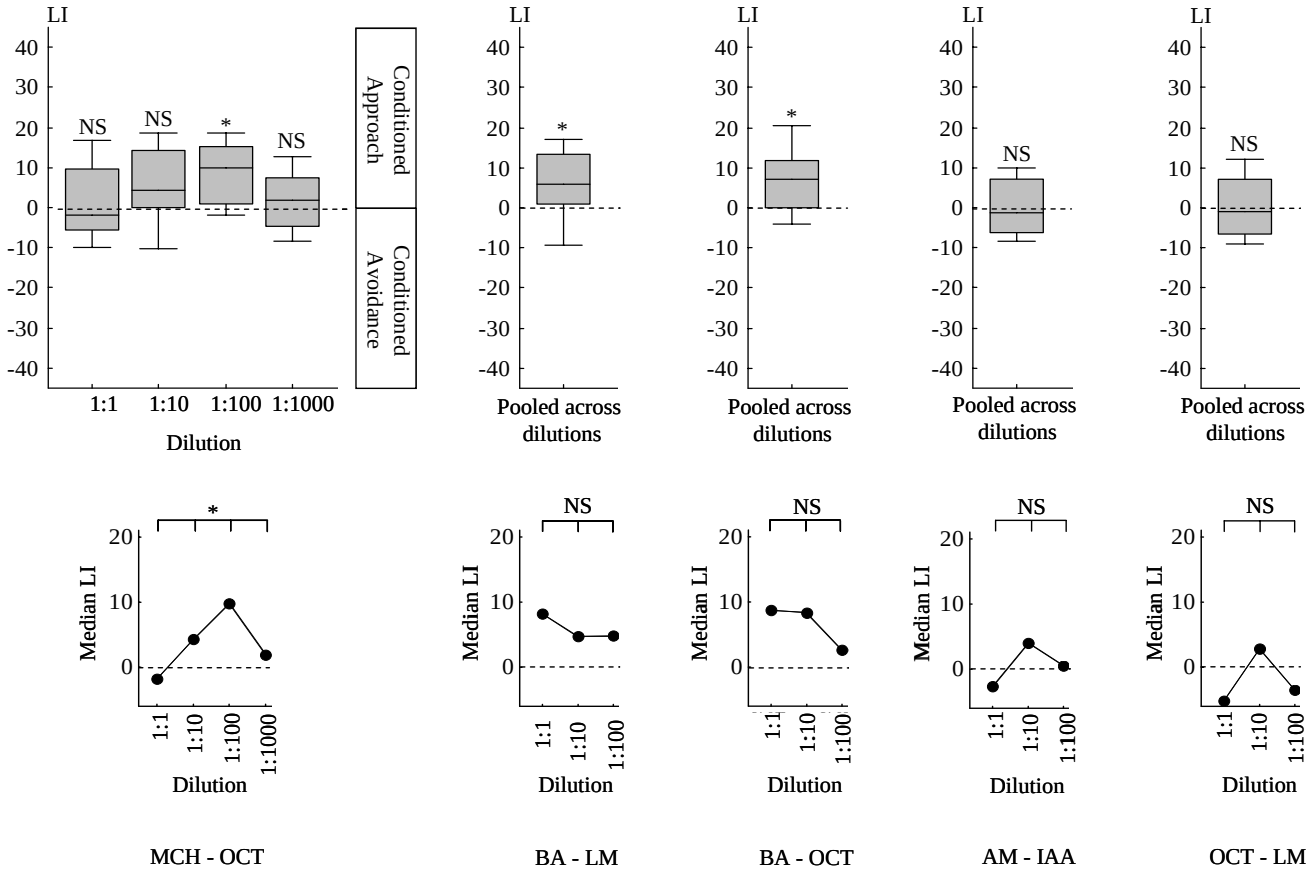

Fig. 5. Relief learning depends on odour identity and concentration

For three out of the five tested odour pairs, relief learning can be demonstrated (MCH-OCT, BA-LM, BA-OCT). For MCH-OCT, the range of concentrations tested uncovers optimal relief learning at an intermediate odour concentration (100-fold diluted); for BA-LM and BA-OCT, relief learning is found to be equally strong regardless of odour concentration. For the remaining two odour pairs (AM-IAA, OCT-LM), relief learning cannot be observed at any of the tested odour concentrations. The bottom row presents only the median learning indices for each concentration, plotted against odour concentration. Please note the truncated Y-axes. Sample sizes with respect to the box plots in the top row are from left to right: N= 20, 20, 19, 16, 60, 60, 32, 28. *: $P< 0.05$; NS: $P> 0.05$, except for the figure part at the upper left, where *: $P< 0.05/4$ and NS: $P> 0.05/4$. Details of box plots are as in Fig. 1D.

For both BA-LM and BA-OCT, learning indices are comparable across odour concentration (Fig. 5: Kruskal-Wallis tests for BA-LM: H_2= 1.66, N= 20, 20, 20, P> 0.05; for BA-OCT: H_2= 3.02, N= 20, 20, 20, P> 0.05). Therefore, for each of these odour-pairs we pool the learning indices across odour concentrations and find that both odour-pairs do support relief learning (Fig. 5: one sample sign tests: sample sizes as above, P< 0.05 in each case).

For the remaining two odour pairs, we find no effect of odour concentration on the learning indices (Fig. 5: Kruskal-Wallis tests for AM-IAA: H_2= 2.10, N= 8, 16, 8, P> 0.05; for OCT-LM: H_2= 3.99, N= 8, 12, 8, P> 0.05). Pooling across odour concentrations, there is no relief learning for either of these two odour pairs (Fig. 5: one-sample sign tests: sample sizes as above P> 0.05 in each case).

Thus, relief learning is possible with three out of five tested odour-pairs. For one odour pair (MCH-OCT), the range of concentrations tested uncovers an optimal odour concentration for relief learning. For two odour pairs, we find uniformly strong relief learning across the concentrations tested, and for two odour-pairs we find that relief learning cannot be observed at either concentration.

Experiment 6: Relief learning is found for relatively mild shocks only.

Next, we test the effect of shock intensity on relief learning. Flies are trained with six training trials using shock pulses of either 25, 50, 75, 100, or 150 V. Shock intensity does influence learning indices (Fig.6: Kruskal-Wallis test: H_4= 14.52, N= 8, 7, 12, 15, 7, P< 0.05). Specifically, relief learning is found when using 100 V (Fig.6: one-sample sign test for 100 V: sample size as above, P< 0.05/5), but not for lower or higher shock intensities (Fig. 6: one-sample sign tests for 25 V, 50 V, 75 V, 150 V: sample sizes as above, P> 0.05/5 in each case). This uncovers a relatively sharp optimum for relief learning at 100 V.

Experiment 7: Relief learning establishes relatively stable memory.

Next, we test whether memory for relief learning decays over a 2 hr retention period and compare this potential decay to the one seen for punishment memory. Four groups of flies receive six training trials; for two groups, these are odour-shock (ISI= -15 s) training trials, whereas the other two groups receive shock-

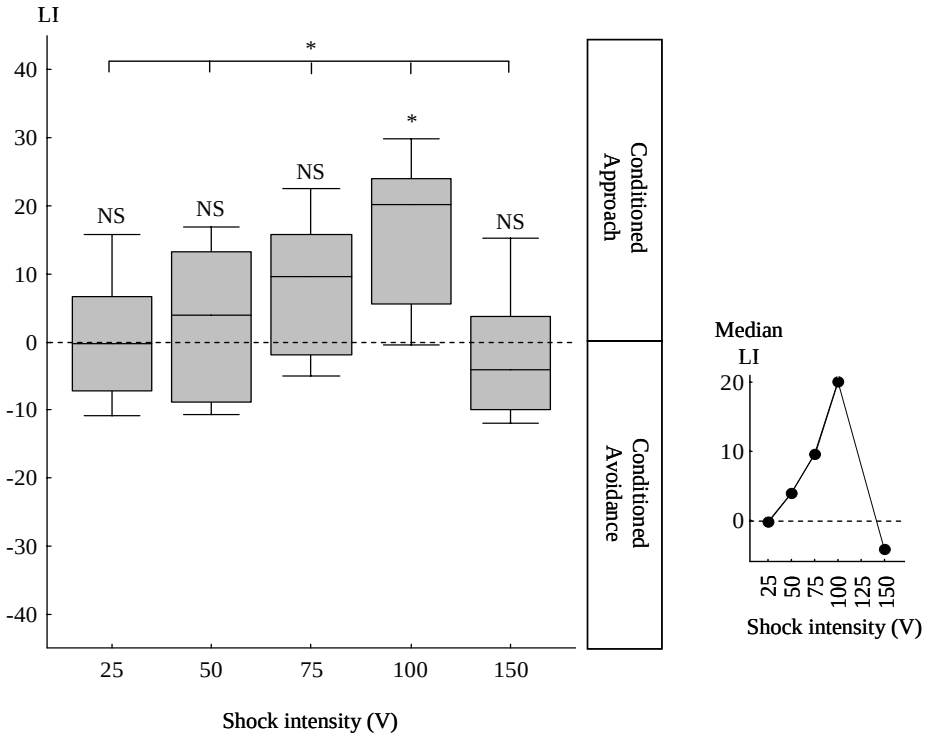

Fig. 6. Relief learning works best at an intermediate shock intensity

Relief learning is found when using 100 V for training, but neither with lower (25, 50, 75 V) nor with higher shock intensities (150 V). The inset figure presents only the median learning indices plotted against shock intensity, using a truncated Y-axis. Sample sizes are from left to right: N= 8, 7, 12, 15, 7. *: $P< 0.05/5$; NS: $P> 0.05/5$, except for the comparison across all groups which uses *: $P< 0.05$. Details of box plots are as in Fig. 1D.

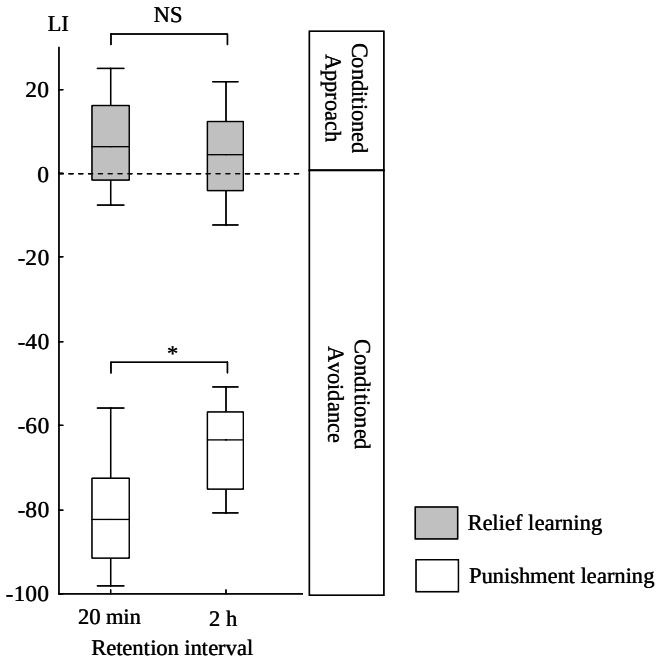

Fig. 7. Relief memory does not apparently decay across a 2 h retention period

Relief memory does not significantly decay across a 2 h retention period (grey). Punishment memory, on the other hand, does decay within this time interval (white). Sample sizes are from left to right: N= 43, 18 and 16, 13 for grey and white boxes, respectively. *: $P < 0.05/2$; NS: $P > 0.05/2$. Details of box plots are as in Fig. 1D.

odour (ISI= 40 s) trials. Once training is complete, for each training condition, one group is tested after the 'normal' retention period (20 min), while another group is tested after 2 h. Concerning punishment learning, we find that learning indices decay across the 2 h retention period (Fig. 7: U-test: punishment learning: U= 51.00, N= 16, 13, $P< 0.05/2$). Despite this approximately 25 % decay, punishment memory is still detectable after the 2 h retention period (one-sample sign tests for punishment learning: sample sizes as above, $P< 0.05/2$ for each retention interval). Concerning relief learning, learning indices do not differ significantly between the two retention intervals (Fig 7: U-test: relief learning: U= 342.00, N= 43, 18, $P> 0.05/2$). When pooled, learning indices indicate relief learning (one-sample sign test for the pooled data set: sample size as above, $P< 0.05$).

This may suggests that relief memory does not substantially decay within the respective time interval. Alternatively, such a difference may remain undetectable, due to an unfavourable signal-to-noise ratio for relief learning. In any event, we do at present have no reason to conclude that memory for relief were less stable than the one for punishment.

Experiment 8: Is relief learning mediated by context-shock associations?

Finally, we return to the issue of the psychological mechanism underlying relief learning. On one hand, it is suggested that both the onset and the offset of shock act as opposing reinforcers (Solomon & Corbit 1974; Wagner 1981). An odour that predicts the painful onset of shock is avoided. The offset of shock on the other hand induces a 'feeling of relief' and an odour that is associated with such relief is approached. Alternatively, it is suggested that the experimental context becomes associated with the shock (Sutton & Barto 1990; Chang et al. 2003), such that within this context shock is predicted. At the moment of shock offset, there arises a mismatch between the context-based prediction that the shock should be present and its actual absence; this negative 'prediction error' (Schultz 1998; Tobler et al. 2003) then could act as a reinforcer for the odour. Given that relief learning requires multiple training trials (Fig. 4), this kind of scenario would suggest that initial trials establish a context-shock association; once the context is sufficiently 'charged', the odour can be learnt by means of the prediction error mentioned above. If this

were true, odour presentation during the initial trials should be superfluous; presentation of shock within the experimental context should suffice. Here, we test this hypothesis.

First, we seek to provide a somewhat finer resolution of the number of trials necessary for relief learning than in Experiment 4; this will guide us in choosing the number of context-shock trials which may establish the context as a predictor for shock and the shock-odour trials which in turn may establish the odour as a signal for the absence of this predicted shock. Fig. 8A shows learning indices after two, four, five or six shock-odour pairings. Consistent with Experiment 4, at least six pairings are necessary to obtain relief learning (Fig. 8A: one-sample sign tests: N= 15, 44, 22, 12, $P > 0.05/4$ for two, four and five trials; $P < 0.05/4$ for six trials). Therefore, in the three follow-up experiments, we adjust the total number of trials to six.

Each of these three experiments uses two groups. One group, prior to shock-odour training, receives 'empty' trials without any odour or shock presentation. These flies are thus merely exposed to the experimental context before shock-odour training. A second group, prior to shock-odour training, receives trials in which only shock is presented. Flies in this group therefore can potentially establish a context-shock association prior to shock-odour training. If such context-shock association were essential to support relief learning, this group should have higher learning scores than the one which had been exposed merely to the context.

In Figure 8B, five shock-odour pairings are preceded by one trial of either context exposure or context-shock training. Despite a trend, learning indices do not differ statistically after these two kinds of treatment (Fig. 8B: U-test: U= 348.00, N= 32, 28, $P= 0.14$). In Figure 8C, four shock-odour pairings are preceded either by two context exposure trials or by two context-shock training trials. Again, context-shock training does not improve learning indices (Fig. 8C: U-test: U= 744.00, N= 39, 48, $P > 0.05$). In Figure 8D, we find a similar result when two shock-odour pairings are preceded by either four context exposure or four context-shock training trials (Fig. 8D: U-test: U= 93.00, N= 12, 16, $P > 0.05$). Thus, context-shock training is inconsequential for subsequent shock-odour learning.

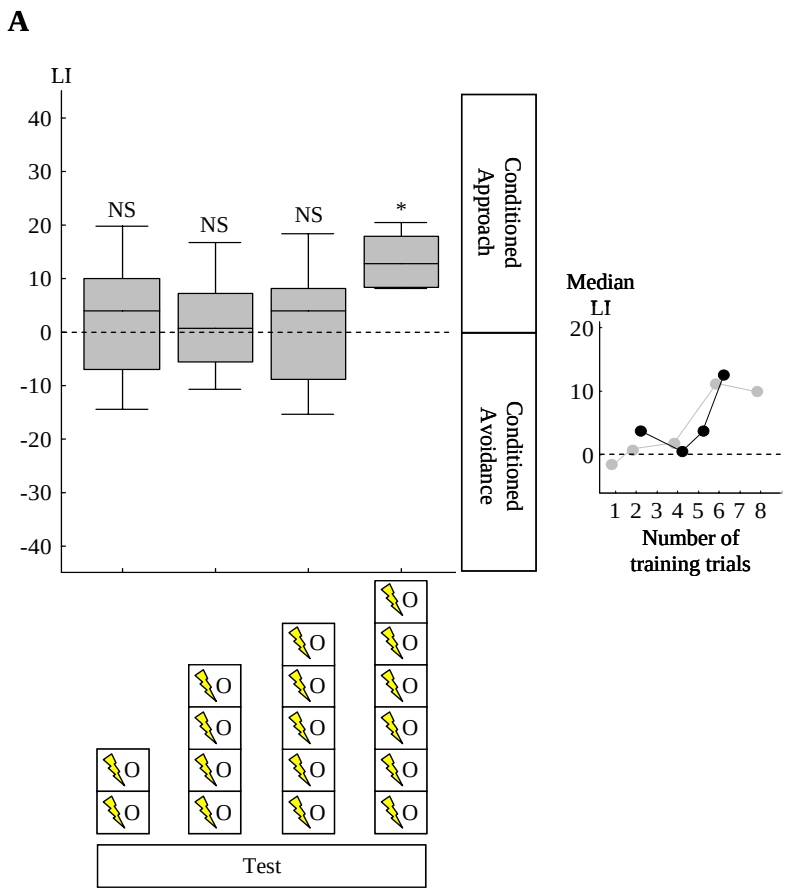

Fig. 8. No impact of context-shock pretraining on subsequent relief learning

A. Two, four or five shock-odour training trials yield no relief learning, whereas six trials do (also see Fig. 4). Sample sizes are from left to right: N= 15, 44, 22, 12. *: $P< 0.05/4$; NS: $P> 0.05/4$. The inset figure presents only the median learning indices (black) plotted against the number of training trials. Median learning indices from Fig. 4 are plotted along in grey. Please note the truncated Y-axis for the inset.

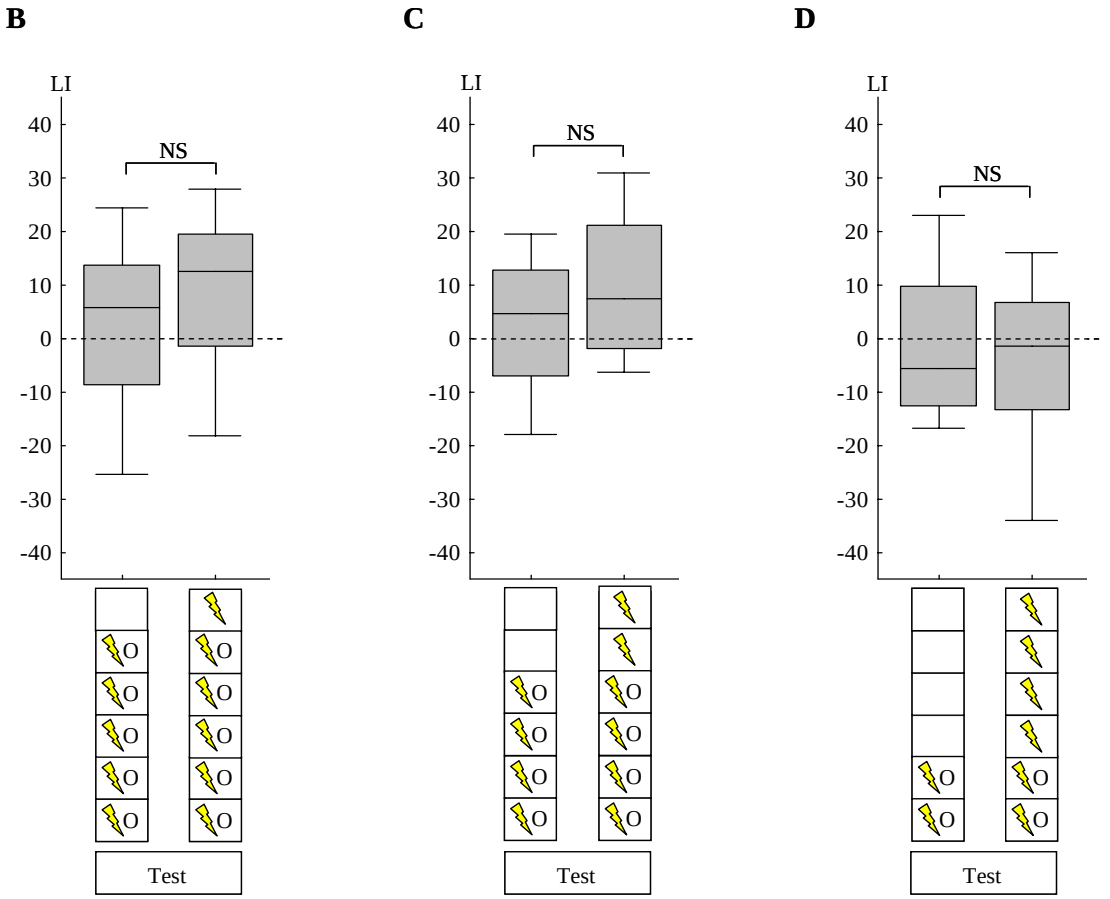

Fig. 8. Continued.

B. Five shock-odour pairings are preceded by a single trial of either context exposure or context-shock training. Despite the obvious trend, these two kinds of training result in statistically indistinguishable learning scores. Sample sizes are from left to right: N= 32, 28. NS: P= 0.14.
C. Four shock-odour pairings are preceded by two trials of either context exposure or context-shock training. Learning scores do not differ between groups. Sample sizes are from left to right: N= 39, 48. NS: P> 0.05.
D. Two shock-odour pairings are preceded by four trials of either context exposure or context-shock training. Learning indices do not differ after two kinds of treatment. Sample sizes are from left to right: N= 12, 16. NS: P> 0.05.
Details of box plots are as in Fig. 1D.

Discussion

We report eight experiments on pain relief learning in Drosophila, using a total of 51 experimental groups, with a total sample size of 1011, each sample being based on the behaviour of ~ 100 flies. We look at repeatability and effects of gender, training amount, odour identity and concentration, shock intensity, and temporal stability of the memory trace. Furthermore, we demonstrate the nature of relief learning in flies as establishing a genuinely associative conditioned approach component, and pit two alternative psychological mechanisms proposed for relief learning against each other.

After discussing the parametric features of relief learning in the light of what is known about punishment learning and reward learning in flies, we provide an outlook concerning the psychological and neurobiological mechanisms of relief learning as well as of the potential utility of relief learning for computational and robotics approaches to behaviour control.

Parametric features of relief learning

Although relatively weak, relief learning is a reproducible, robust phenomenon (Tanimoto et al. 2004; Figs 1- 8). Specifically, the strength of relief learning is about 1/5 (Tanimoto et al. 2004) to 1/8 (Fig. 1) of punishment learning if the training parameters are the same. This corresponds to introspection, which suggests that the 'bad' memories of painful events outweigh any 'good' memory concerning these same painful events; it also corresponds to one of the most influential formal psychological theories of associative learning (Wagner 1981). Furthermore, relief learning cannot be demonstrated after only one training trial (at least four [Tanimoto et al. 2004] or six [Figs 4 and 8] training trials are needed), whereas for punishment learning even a single training trial can be sufficient for asymptotic learning scores (Tully & Quinn 1985). Reward learning may also work with a single training trial (see Fig. 1A in Schwaerzel et al. 2003; Krashes et al. 2008), but usually two trials are used which support asymptotic learning indices (Tempel et al. 1983, Schwaerzel et al. 2003).

We report that gender has no effect upon relief learning (Fig. 3B). Also, neither punishment learning nor reward learning has to our knowledge been reported to depend on gender; in the Würzburg Department, at least, no such differences have been seen (unpublished).

Concerning odour identity, we find that relief learning is possible using three out of the five tested odour-pairs (Fig. 5). The odour pair AM-IAA, which as we find does not support relief learning, can readily be used for punishment or reward learning (Yarali, unpublished). A combinatorial argument may suggest that BA and MCH are largely responsible for relief learning, but in a formal sense the relative contribution of either odour within a pair has to remain unresolved. We see an effect of odour concentration on relief learning for the MCH-OCT pair (Fig. 5, upper left); specifically, within the range of concentrations covered, we observe an optimum function. This seems plausible, as very low concentrations may not be sufficiently salient to enter into association, but at too high concentrations the specificity of perception may suffer. Thus, for the other four odour-pairs, one would probably uncover an optimum function as well, if a wider range of odour concentrations were used (see the trend for BA-LM and BA-OCT in the bottom row of Fig. 5). Concerning punishment learning using MCH-OCT, Tully and Quinn (1985) reported that learning improves with increasing odour concentration, which along the same line of argument may reflect a part of an optimum function as well. Regarding reward learning, there are no systematic studies published concerning odour concentration effects.

We find that relief learning depends on shock intensity. Specifically, we observe an optimum function (Fig. 6). Punishment learning also improves with increasing shock intensity until an optimum is reached; a further increase in intensity then worsens punishment learning (Tully & Quinn 1985), but this decline is not as pronounced as in relief learning. The most plausible explanation for this decline in both kinds of learning is that high shock intensities may induce amnesia and/ or physical damage to the fly. Regarding reward learning, there are no systematic studies published concerning sugar concentration effects.

Finally, relief memory does not decay across a 2 h retention period (Fig. 7). Within this time interval, on the other hand, punishment memory does decay (Fig. 7). Interestingly, punishment memory

has been reported to decay relatively faster (within 4 h [Tempel et al. 1983] or 24 h [Tully & Quinn 1985]) than reward memory (> 24 h [Tempel et al. 1983]).

To summarize, the parametric features of relief learning presented here argue that one needs to adjust the training parameters carefully when trying to uncover this form of learning; this is in particular true if one tries to use the same parameters as are optimal for punishment learning: As a rule of thumb, one should use relatively mild shocks, and relatively many training trials. Indeed, one of the reasons why relief learning had been overlooked in earlier studies (Tully & Quinn 1985) and continues to be overlooked (Yu et al. 2006) may be that the chosen parameters are not optimal. This should be important information for researchers seeking to uncover relief learning in other experimental systems. In any event, given that the temporal asymmetry in terms of the timing of the to-be-associated events likely is a basic feature of predictive learning (see Introduction), the parametric analyses reported here may have bearings beyond the mere description of relief learning in flies and beyond serving as guide posts for its uncovery in other creatures. Rather, such analyses are indispensable to equip computational models of behaviour with truly bio-inspired learning rules, and may thus aid the development of 'intelligent' technical devices for behaviour control.

Relief learning establishes genuinely associative conditioned approach

Both the initial experiment by Tanimoto et al. (2004) and the majority of our follow-up experiments use odours at concentrations that are repellent to experimentally naïve, unconditioned flies. This unconditioned, baseline aversion complicates the interpretation of relief learning. That is, the flies' relative preference for the learned odour after shock-odour training can be explained in two ways: Training may establish an additional genuine conditioned approach tendency towards the learned odour (Fig. 2A top). Alternatively, presentation of shock *per se* may, in a yet-unknown way, weaken processing of those odours that are presented shortly afterwards, rendering these odours less effective, and hence less aversive, at the moment of test (Fig. 2A bottom). In an experiment to distinguish between these accounts,

we find that shock-odour training establishes genuinely associative conditioned approach to the odour (Figs 2B- D).

Possible mechanisms: Neurobiology

Concerning punishment learning, the current evidence suggests that the short-term memory trace is localized exclusively to the so called mushroom bodies, a third-order olfactory brain region (Heisenberg 2003; Gerber et al. 2004; Heisenberg & Gerber 2007). In contrast, for short-term reward memories, there appear to be two independent memory traces (Thum et al. 2007): One trace is laid down at the mushroom body just as for punishment memories, but an additional trace is localized to the olfactory projection neurons. It should be interesting to see whether the site of the memory trace(s) for relief learning matches either of these two patterns of memory trace localization.

Punishment learning and reward learning are dissociated with respect to how the internal reinforcing signals are carried: Dopaminergic neurons signal punishment, whereas reward is signalled by octopamine (Schwaerzel et al. 2003, Riemensperger et al. 2005, Schroll et al. 2006, see also Unoki et al. 2005, concerning crickets). Strikingly, activation of dopaminergic or octopaminergic/ tyraminergic neurons reportedly is sufficient to substitute for aversive or appetitive reinforcement, respectively (Schroll et al. 2006; see also the pioneering work in honeybees reviewed by Hammer & Menzel [1995]). Obviously, this provokes the question whether either dopamine or octopamine signalling may be necessary and/ or sufficient for relief learning. To date, no data have been published which speak to this question.

An alternative physiological mechanism to bring about opposite behavioural changes due to odour-shock *versus* shock-odour training would be to implement spike-timing-dependent plasticity at the synapse in question. That is, depending on the relative timing of two inputs, synaptic strength will be potentiated or depressed (Caporale & Dan 2008). If such a mechanism were at work at those synapses that underlie the memory traces for punishment learning and relief learning, response tendencies towards the odour may be enhanced or suppressed depending on the relative timing of odour and shock. The most

likely candidate would be the output synapses of the mushroom body Kenyon cells (see above). Indeed, as shown by Cassenaer & Laurent (2007) in the locust, it is possible to experimentally induce spike timing-dependent plasticity at these synapses. Furthermore, Drew & Abbott (2006) recently argued it were conceivable that the millisecond-timescale effects seen in spike-timing-dependent plasticity may translate into time courses that are qualitatively similar to those in behaviour. If this were so, the behavioural asymmetry of predictive learning may be rooted in the basic properties of synaptic modification.

Possible mechanisms: Psychology

In learning psychology, there is a debate as to how relief learning comes about. On one hand, it is suggested that an internal reinforcing signal is driven directly by the offset of the reinforcer (Solomon & Corbit 1974; Wagner 1981); the 'feeling of relief' at the offset of a painful event may correspond to this property of the reinforcing system. On the other hand, it is suggested that the shock becomes associated with the experimental context (Sutton & Barto 1990; Chang et al. 2003), such that within the experimental context shock is predicted. As the shock is turned off and the odour is turned on, this would lead to a mismatch between the context-based prediction that the shock should be present and its actual absence. This negative prediction error (Schultz 1998; Tobler et al. 2003; for a lucid tutorial discussion see also Hellstern et al. 1998) could then act as the reinforcing signal. This latter scenario would predict that flies, when first trained with context-shock pairings, would more readily acquire the shock-odour association. As reported here, this is not the case (Fig. 8B- D).

The possible role of context in learning about the absence of reward was also investigated in honeybees (Hellstern et al. 1998). Honeybees learn an odour as a predictor either for the *presence* or for the *absence* of sugar depending on the timing of events during training: Odour-sugar training results in proboscis extension to the odour at subsequent test, as it has become a predictor for sugar. The effect of the reversed-order sugar-odour training on the other hand needs to be assessed indirectly: In the first phase of the experiment, bees are given a sugar-odour pairing with either a short or a very long interval between the two stimuli. This is followed by 'regular' odour-sugar training. Finally, bees are tested for the

proboscis extension response to the odour. If sugar-odour training were to establish the odour as a predictor for the *absence* of sugar, further learning of this same odour as a predictor for the *presence* of sugar should be retarded. This is indeed the case: Bees trained with a short sugar-odour interval in the first experimental phase show weaker proboscis extension in the test as compared to bees trained with a very long sugar-odour interval. Does such sugar-odour learning rely on the offset of sugar as a reinforcer or does it depend on a context-sugar association? To address this question, Hellstern et al. (1998) used a very long interval between sugar and odour, but changed the experimental context during the interval between the two stimuli. This should prevent the context-sugar association from 'fading away' (see discussion in the previous paragraph); consequently sugar-odour learning would be possible even with this otherwise much-too-long interval. This, however, was not observed (Hellstern et al. 1998). Thus, in flies and in bees, contextual learning does not seem to measurably impact shock-odour and sugar-odour learning, respectively; rather, in both kinds of animal the critical aspect seems to be related to the way shock-offset and sugar-offset, respectively, are processed.

Taken together, the analyses we report provide the basis for future investigations of the psychological, neuronal, and molecular mechanisms underlying pain-relief learning. These efforts should eventually yield a comprehensive account of the behavioural consequences of painful, traumatic experience, and may help to develop a truly bio-inspired computational model of predictive learning and behavioural control.

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References

Ayyub, C., Paranjape, J., Rodrigues, V., Siddiqi, O. 1990. Genetics of olfactory behavior in Drosophila melanogaster. *J Neurogenet,* **6**, 243-262.

Britton, G. & Farley, J. 1999. Behavioral and neural bases of noncoincidence learning in Hermissenda. *J Neurosci*, **19**, 9126-9132.

Caporale, N. & Dan, Y. 2008. Spike Timing-Dependent Plasticity: A Hebbian Learning Rule. *Annu Rev Neurosci,* DOI:10.1146/annurev.neuro.31.060407.125639.

Cassenaer, S. & Laurent, G. 2007. Hebbian STDP in mushroom bodies facilitates the synchronous flow of olfactory information in locusts. *Nature*, **448**, 709-713.

Chang, R. C., Blaisdell, A. P., & Miller, R. R. 2003. Backward conditioning: Mediation by the context. *J Exp Psychol: Anim Behav Process,* **29**, 171-183.

Drew, P. J., & Abbott, L. F. 2006. Extending the effects of spike-timing-dependent plasticity to behavioral timescales. *Proc Nat Acad Sci U S A,* **103**, 8876-8881.

Gerber, B., Tanimoto, H., & Heisenberg, M. 2004. An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol,* **14**, 737-744.

Hammer, M., & Menzel, R. 1995. Learning and memory in the honeybee. *J Neurosci,* **15**, 1617-1630.

Hearst, E. 1988. Learning and cognition. In: *Stevens' handbook of experimental psychology*, *2nd Edition, Vol 2.* (Ed. by R. C. Atkinson, R. J. Herrnstein, G. Lindzey, R. D. Luce), pp 3–109. New York: Wiley.

Heisenberg, M. 2003. Mushroom body memoir: from maps to models. *Nat Rev Neurosci,* 4, 266-275.

Heisenberg, M. & Gerber, B. In press. Different associative learning tasks establish distinct local memory traces in the Drosophila brain. In: *Learning and Memory: A Comprehensive Reference.* (Ed. by J. Byrne), Elsevier.

Hellstern, F., Malaka, R., & Hammer, M. 1998. Backward inhibitory learning in honeybees: a behavioral analysis of reinforcement processing. *Learn Mem,* **4**, 429-444.

Krashes, M.J. & Waddell, S. 2008. Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in Drosophila. *J Neurosci*, **28**, 3103-3113.

Maier, S. F., Rapaport, P. & Wheatley, K. L. 1976. Conditioned inhibition and the UCS-CS interval. *Anim Learn Behav*, **4**, 217-220.

Mirenowicz, J., & Schultz, W. 1996. Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. *Nature,* **379(6564)**, 449-451.

Moskovitch, A. & LoLordo, V. M. 1968. Role of safety in the Pavlovian backward fear conditioning procedure. *J Comp Physiol Psychol*, **66**, 673-678.

Plotkin, H. C. & Oakley, D. A. 1975. Backward conditioning in the rabbit (Oryctolagus cuniculus). *J CompPhysiol Psychol*, **88**, 586-590.

Riemensperger, T., Voller, T., Stock, P., Buchner, E., & Fiala, A. 2005. Punishment prediction by dopaminergic neurons in Drosophila. *Curr Biol,* **15(21),** 1953-1960.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Völler, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E & Fiala, A. 2006. Light-induced activation of distinct modulatory neurons substitutes for appetitive or aversive reinforcement during associative learning in larval Drosophila. *Curr Biol,* **16(17),** 1741-1747.

Schultz, W. 1998. Predictive reward signal of dopamine neurons. *J Neurophysiol,* **80(1),** 1-27.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S., & Heisenberg, M. 2003. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. *J Neurosci,* **23,** 10495-10502.

Solomon, R. L., & Corbit, J. D. 1974 An opponent-process theory of acquired motivation. I. Temporal dynamics of affect. *Psychol Rev,* **81(2),** 119-145.

Sutton, R. S. & Barto, A. G. 1990. Time derivative models of Pavlovian reinforcement. In: *Learning and Computational Neuroscience: Foundations of Adaptive– Networks* (Ed. by M. R. Gabriel & J. W. Moore), pp. 497-537. Cambridge, MA: MIT Press.

Tanimoto, H., Heisenberg, M., & Gerber, B. 2004. Experimental psychology: event timing turns punishment to reward. *Nature,* **430**, 983.

Tempel, B. L., Bovini, N., Dawson, D. R., & Quinn, W. G. 1983. Reward learning in normal and mutant Drosophila. *Proc Nat Acad Sci U S A,* **80(5),** 1482-1486.

Thum, A. S., Jenett, A., Ito, K., Heisenberg, M., Tanimoto, H. 2007. Multiple memory traces for olfactory reward learning in Drosophila. *J Neurosci,* **27**, 11132-11138

Tobler, P., Dickinson, A., & Schultz, W. 2003. Coding of predicted reward omission by dopamine neurons in a conditioned inhibition paradigm. *J Neurosci,* **23,** 10402-10410.

Tully, T., & Quinn, W. G. 1985. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J CompPhysiol [A],* **157**, 263-277.

Unoki, S., Matsumoto, Y., & Mizunami, M. 2005. Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *Eur J Neurosci,* **22(6),** 1409-1416.

Wagner, A. R. 1981. SOP: A Model of Automatic Memory Processing in Animal Behavior. In: *Information Processing in Animals: Memory Mechanisms* (Ed. by N. E. Spear & R. R. Miller), pp. 5−47. Hillsdale, New Jersey: Erlbaum.

Yu, D., Akalal, D. B., Davis, R. L. 2006. Drosophila alpha/beta mushroom body neurons form a branch-specific, long-term cellular memory trace after spaced olfactory conditioning. *Neuron*, **52(5)**, 845-855.

Loss of *white* function coherently affects punishment learning and relief learning

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Abstract

The *white* gene is well-known for its effect on the eye color of fruit flies. However, *white* also has bearings on fruit fly behaviour, e.g. courtship, aggression and learning. Presently, we analyse how loss of *white* function affects olfactory associative learning. In flies, an experience with electric shock supports two opposing kinds of learning: Those odours that *precede* shock during training are subsequently avoided as predictors for *punishment*; whereas those odours that *follow* shock during training are later on approached, as they predict *relief*. Both of these kinds of learning are altered by loss of *white* function: That is, *white* mutants, as compared to wild-type flies, build stronger punishment memories for odours that precede shock and they form weaker relief memories for odours that follow shock. As one may put it, *white* mutants remember a shock-episode, overall as more 'negative'. This effect is restricted to the *learning* about shock, the reflexive responsiveness to shock itself remains unaltered. In addition, learning about *reward* is also unaffected in the *white* mutants. Having probed the *white* mutants' brains for the amounts of biogenic amines octopamine, tyramine, dopamine and serotonin, we find no difference to wild-type. Thus, the molecular basis for the *white*-effect on learning remains unresolved. Neverthless, our results have twofold significance: First, all behavioural effects of the *white* mutation should interest fruit fly behavioural neurogeneticists as a potential source of confound. Second, the fact that punishment and relief learning are *coherently* affected (e.g. by the loss of *white* function) suggests a balance between these two. Such pain *versus* relief balance most likely applies to all animals, including man. Understanding its molecular and neuronal pivots is essential for comprehending the behavioural consequences of painful, traumatic experience.

Introduction

The *white* gene, due to the prominent white eyes caused upon the loss of its function, is a basic tool for *Drosophila* geneticists (see the Discussion). It affects however more than the eye color: Ectopic, ubiquitous expression of White induces male-to-male courtship (Zhang & Odenwald 1995; Hing & Carlson 1996; Nilsson et al. 2000; An et al. 2000); whereas the loss of *white* function suppresses male-aggression (Hoyer 2007) in fruit flies. Effects of *white* are not restricted to innate behaviour: *White*

mutant flies are impaired in heat-reinforced place-learning; whereas in olfactory learning using electric shock punishment, they perform better than wild-type (Diegelmann et al. 2006).

The White protein is a 'half-size ATP-binding cassette transporter' (O'Hare et al. 1984). Heterodimers of White with two other such transporters, Scarlet (Tearle et al. 1989) and Brown (Dreesen et al. 1988), pump respectively tryptophan and guanine into cells. These are pigment-precursors in the respective cells of the eye (Sullivian & Sullivian 1975). In neurons, tryptophan is converted to serotonin, a neuromodulator regulating e.g. circadian rhythmicity, sleep (Yuan et al. 2005; Yuan et al. 2006), aggression (Dierick et al. 2007), as well as learning (Sitaraman et al. 2008). Guanine on the other hand, is converted in neurons to '6H-tetrahydrobiopterin', a cofactor for the synthesis of serotonin, dopamine, and nitric oxide (reviewed by Koshimura et al. [2000]). Dopamine, apart from signalling aversive reinforcement (Schwaerzel et al. 2003; Riemensperger et al. 2005; Schroll et al. 2006), affects arousal (Andretic et al. 2005) and regulates flies' 'decisions' between visual cues with different saliencies encountered during flight (Zhang et al. 2007). Nitric oxide is an atypical neurotransmitter in the synapses of olfactory, visual and mechanosensory systems, as well as in the neuromuscular junctions (reviewed by Bicker [2001]).

In short, the effects of the *white* gene on behaviour can be explained by its bearings on the dopaminergic and serotonergic systems on one hand and on signalling through nitric oxide on the other hand. Accordingly, *white* mutant flies reportedly have lower levels of dopamine and serotonin as compared to wild-type flies (Sitaraman et al. 2008). Effects of loss of *white* function on nitric oxide signalling remain to be experimentally probed.

In the present study, we analyse how loss of *white* function affects olfactory associative learning. Fruit flies build two opposing kinds of memory based on an experience with electric shock (Tanimoto et al. 2004; Yarali et al. In press): Those odours that precede shock are learned as predictors for *punishment* and subsequently avoided; those odours that follow the offset of shock on the other hand are learned as signals for *relief* and flies subsequently approach them. Loss of *white* function affects both of these opposing kinds of learning, coherently: White mutant flies built stronger aversive memories for odours that predict shock punishment and they form weaker appetitive memories for the odours that predict relief from shock. In other words, a shock-episode is reflected in *white* mutants'

memory overall as more 'negative' as compared to wild-type flies. Importantly, this effect is only upon the *learning* about shock; the reflexive responsiveness to shock remains unaltered. In addition, loss of *white* function also does not affect the learning of sugar *reward*. Having probed the *white* mutants' brain for the biogenic amines octopamine, tyramine, dopamine and serotonin, we find the amounts comparable to wild-type. Thus, the molecular basis for the *white*-effect on learning remains unresolved.

Our results have twofold significance: First, the effect of *white* on learning, just as its other behavioural effects, should concern all *Drosophila* behavioural neurogeneticists as a potential confound (see the Discussion). Second, the *coherent* effects of the *white* mutation on punishment and relief learning suggest that these two have common determinants to keep them in a balance. Such balance most likely governs the behaviour of also other animals, including man under normal (Solomon & Corbit 1974) and psychiatric conditions (Vincent & Kukstas 1998, Grossberg 2000). Understanding the molecular and neuronal means of balancing pain and relief is critical to comprehend the consequences of traumatic experience. Fruit flies seem to be a suitable model to do so, as the critical molecules are likely conserved through evolution (Straub et al. 1994; Croop et al. 1997; Nakamura et al. 1999).

Materials and Methods

Flies

Drosophila melanogaster are reared as mass culture at 25 °C, 60- 70 % relative humidity, under a 14: 10 h light: dark cycle. Canton-Special wild-type strain is used as a control for the w1118 strain. w1118 is a null mutant of the *white* gene and has been back-crossed with Canton-Special for several generations to restore the genetic background to wild type (Hazelrigg et al. 1984; also see Diegelmann et al. 2006).

Behavioural assays

One day prior to behavioural experiments, 1- 4 day-old flies are collected in fresh food vials and kept over night at 18 °C and 60- 70 % relative humidity. For sugar learning, flies are starved over night for

18- 20 h at 25 °C and 60- 70 % relative humidity in vials equipped with moist tissue and a moist filter paper. The experimental setup is as described by Tully and Quinn (1985) and Schwaerzel et al. (2003). Flies are trained and tested as groups of 100- 150. Trainings take place under dim red light which does not allow flies to see, tests are in complete darkness. As odourants, 90 µl benzaldehyde (BA) and 340 µl 3-octanol (OCT) (both from Fluka, Steinheim, Germany) are applied in 1 cm-deep Teflon containers of 5 and 14 mm diameters, respectively.

For electric shock-reinforced learning (Fig. 1A), flies receive 6 training trials. Each trial starts by loading the flies into the experimental setup (0:00 min). From 4:00 min on, a control odour is presented for 15 s. From 7:30 min on, electric shock is applied as 4 pulses of 100 V; each pulse is 1.2 s-long and is followed by the next with an onset-to-onset interval of 5 s. In different groups, a to-be-learned odour is presented at different times with respect to the shock; thus, the inter-stimulus interval (ISI) is varied between groups. Negative ISIs indicate first-odour-then-shock presentation; positive ISIs mean first-shock-then-odour presentation. At 12:00 min, flies are transferred out of the setup into food vials, where they stay for 16 min until the next trial starts. At the end of the sixth training trial, after the usual 16 min break, flies are loaded back into the setup. After a 5 min accommodation period, they are transferred to a T-maze, where they can choose between the two odours that they have encountered during training. After 2 min, the arms of the maze are closed and flies on each side are counted. A preference index (PREF) is calculated as:

$$(1) \qquad PREF = (\#_{\text{Learned odour}} - \#_{\text{Control odour}}) \times 100 / \#_{\text{Total}}$$

# indicates the number of flies found in the respective maze-arm. For each ISI, two subgroups of flies are trained and tested in parallel (Fig. 1A): For one of these, 3-octanol (OCT) is the control odour and benzaldehyde (BA) is to be learned; the second group is trained reciprocally, that is the roles of these two odours are switched. A learning index (LI) is calculated based on the PREF values from the two reciprocal measurements:

$$(2) \qquad LI = (PREF_{BA} + PREF_{OCT}) / 2$$

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

To test for the reflexive shock response, flies are brought to the choice point of a T-maze, 5 min after being loaded into the setup. 10 s later, one of the maze arms is electrified with 4- 1.2 s long pulses of 100 V shock with 5 s inter-pulse intervals. 10 s after the onset of the last pulse, arms of the maze are closed and flies on each side are counted. A preference index for the electrified arm ($PREF_{Shock}$) is calculated as:

$$(3) \qquad PREF_{Shock} = (\#_{Electrified\ arm} - \#_{Non\text{-}electrified\ arm}) \times 100 / \#_{Total}$$

# indicates the number of flies found in the respective maze-arm. Negative $PREF_{Shock}$ values indicate avoidance of the shock.

Sugar reward learning (Fig. 2A) uses two training trials. Each trial starts by loading the flies into the setup (0:00 min). 1 min later, flies are transferred to a tube lined with a filter paper which was soaked the previous day with 2 ml of 2 M sucrose solution, and then was dried over night. This tube is scented with the to-be-learned odour. After 45 s, this odour is removed, and after 15 further seconds flies are taken out of the tube. After a 1 min waiting period, flies are transferred into another tube lined with a filter paper which was soaked with pure water and then was dried. This second tube is scented with the control odour. After 45 s, this odour is removed and 15 s later, flies are taken out of the tube. The next trial starts immediately. For half of the cases, training trials start with the to-be-learned odour and sugar; in the other half, control odour is given precedence. Once the training is completed, after a 3 min waiting period, flies are transferred to the choice point of a T-maze between the two odours. After 2 min, the arms of the maze are closed, flies on each side are counted and a preference index (PREF) is calculated according to Equation 1. As detailed above, two groups are trained reciprocally (Fig. 2A) and a learning index (LI) is calculated based on their PREF values according to Equation 2.

All behavioural data are analysed using non-parametric statistics and are reported as box plots, showing the median as the midline and 10, 90 and 25, 75 % as whiskers and box boundaries,

respectively. For comparing scores of each group to zero, we use a one-sample sign test. To compare scores between two groups, we use a Mann-Whitney U-test. When multiple tests are done within a single experiment, we adjust the experiment-wide error-rate to 5 % by Bonferroni correction that is, we divide the critical $P< 0.05$ by the number of tests. To compare more than two groups with each other, we use a Kruskal-Wallis test. All statistical analyses are done on a PC using the software Statistica.

Quantification of biogenic amine amounts

We quantify the amounts of octopamine, tyramine, dopamine and serotonin in the fruit fly brain using High Performance Liquid Chromatography, coupled to Mass Spectrometry (LC-MS). We first explain the principle of LC-MS and the quantification method; then we give the technical particulars.

Principle of LC-MS and the quantification method: A homogenate of fruit fly brains is loaded into a liquid chromatography column. The molecules contained in this homogenate are eluted from the column at different, characteristic times, according to their chemical property. To quantify e.g. the amount of octopamine, we initially determine, in pilot measurements, its characteristic elution time. The eluates from the liquid chromatography column enter a mass spectrometer, where each molecule is broken into electrically charged fragments of characteristic mass, resulting in a mixture of fragments with different masses. These fragments are then sorted out according to mass; and finally the *relative intensity* of each mass is measured. To quantify e.g. the amount of octopamine, we need to know the mass of a characteristic fragment that is produced when octopamine is broken up. Based on the *relative intensity* of this particular mass, we deduce the *relative amount* of octopamine in the brain homogenate.

We intend however to find the *actual amount* of e.g. octopamine, rather than its *relative amount*. To do so, we use an *internal standard*: That is, we add a known amount of deuteriated octopamine (d3-octopamine) into the initial brain homogenate. As d3-octopamine and 'regular' octopamine have the same chemical property, they are eluted simultaneously from the liquid chromatography column. Likewise, in the mass spectrometer, they break up into the same characteristic fragments; except, the fragments derived from d3-octopamine are heavier than those

derived from 'regular' octopamine, enabling their separate detection. At the end of the LC-MS analysis, we thus obtain a value for the *relative amount* of d3-octopamine and another value for the *relative amount* of 'regular' octopamine. From these values, and the known amount of d3-octopamine we have added into the brain homogenate, we calculate the *actual amount* of octopamine.

This calculation however still has to be *corrected*: That is, initially, we calibrate our method of quantification. For example, with respect to octopamine, we prepare a series of samples; each sample contains 5 µg of d3-octopamine and a certain known amount of 'regular'octopamine, varying between 5 fg to 5µg. We then estimate the amount of octopamine in each sample as explained above. We plot this estimated amount against the known amount with which we have started, resulting in a close-to-linear function. We take the slope of the linear-fit is as a *correction factor*, and incorporate it into the calculation of octopamine amount in brain homogenates. The final value we obtain from this calculation is divided by the number of brains in the homogenate (i.e. 10), resulting in the pg/ brain values, which we report.

Chemicals: d3-octopamine and d4-serotonin are purchased from Medical Isotopes (Pelham, USA); d2-tyramine and d3-dopamine are obtained using acid catalyzed isotopic exchange between dopamine/ tyramine and heavy water (Pajak and Kańska 2006). Octopamine, tyramine, dopamine and serotonin are purchased as hydrochloride salts from Sigma-Aldrich Chemie Gmbh (Münich, Germany).

Sample preparation: Each sample contains 5 female and 5 male brains (2- 3 days old) from either w1118 mutant or Canton Special wild-type flies. Brains are dissected in ice-cold ringer solution and directly placed into 50 µl of ice-cold 50 mM citrate-acetate buffer (pH 4.5), which in addition contains 5 µg of each internal standard. Once 10 brains are collected (which takes ~ 30 min) they are homogenized in this solution on ice with a Teflon pestle. The homogenate is then centrifuged at 14000 rpm for 5 min at room temperature; 10 µl of the supernatant is analysed by LC-MS.

LC-MS device and conditions: We use an Agilent 1200 system (Waldbronn, Germany), coupled to a Waters Micromass Quattro Premier triple quadrupole mass spectrometer (Milford, MA, USA). Liquid chromatography is achieved using an Agilent Eclipse XDB-C18 column (150 mm x 4.6 mm, 5 µm particle size; Waldbronn, Germany). The column is eluted with a linear mobile phase gradient (0.6 ml/ min flow rate) starting from water containing 0.1% formic acid at 0 min to

acetonitrile: water: formic acid mixture (50: 50: 0.1, v/ v/ v) at 10 min. In MS, ionization is achieved using electrospray in the positive ionization mode (ESI+) with a capillary voltage of 2.5 kV. The temperature of the source block is set at 120 °C and nitrogen is used as desolvation gas and cone gas with a flow of 800 l/ h at 350 °C and 50 l/ h, respectively. In order to establish the appropriate mass spectrometric conditions for the individual compounds and their respective deuterated analogues, standard solutions are directly infused into the mass spectrometer and the cone voltage is adjusted to maximize the intensity of the protonated molecular species. Collision-induced dissociation of each compound is performed using Argon as collision gas with a flow rate of 0.3 ml /min and a pressure of $3.0x10^{-3}$ mBar and the collision energy (eV) is adjusted to optimize the signal for the most abundant product ions, which were subsequently used for Multiple Reaction Monitoring (MRM) analysis. The MRM transitions and conditions for the measurement of all compounds and their respective deuterated analogues with a dwell time of 100 ms for each reaction are as follows:

Compound	**Precursor ion (*m/z*)**	**Product ion (*m/z*)**	**Cone voltage (V)**	**Collision energy (eV)**
octopamine	154	119	10	20
d3-octopamine	157	121		
tyramine	138	103	14	20
d2-tyramine	140	105		
dopamine	154	119	16	20
d3-dopamine	157	121		
serotonin	177	160	16	24
d4-serotonin	181	164		

Results

Effects of the loss of white *function on olfactory associative learning*

We train flies with a control odour, a to-be-learned odour and pulses of electric shock (Fig. 1A), varying between groups the interval between the onsets of the to-be-learned odour and the shock (inter-stimulus interval: ISI). Negative ISIs indicate first-odour-then-shock presentation, positive ones

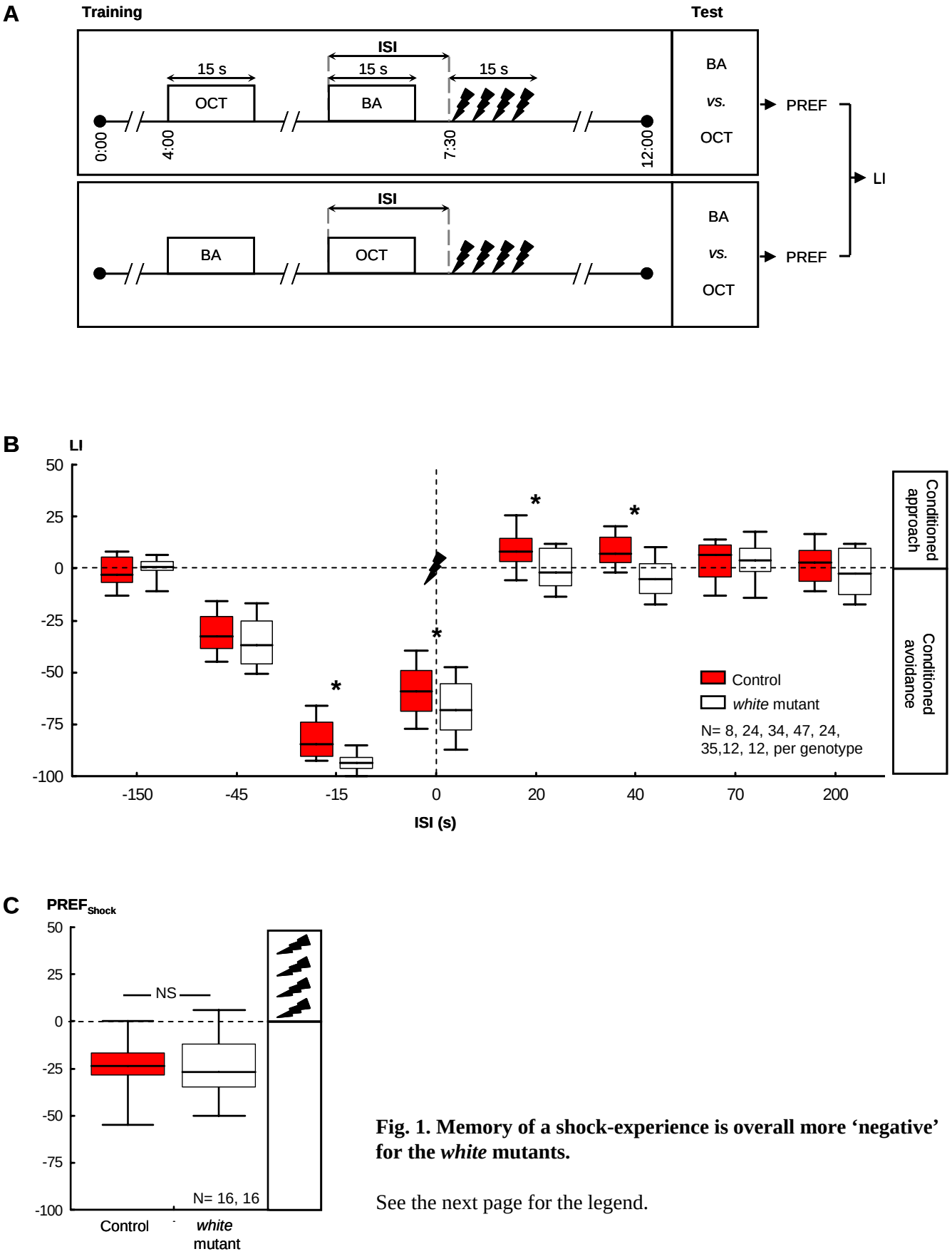

Fig. 1. Memory of a shock-experience is overall more 'negative' for the *white* mutants.

See the next page for the legend.

Fig. 1. Memory of a shock-experience is overall more 'negative' for the *white* mutants.

A. Flies are trained with two odours and pulses of electric shock. Between groups, we vary the interval between the to-be-learned odour and the shock (inter-stimulus interval: ISI). Negative ISIs indicate odour-then-shock presentation; positive values reflect shock-then-odour presentation. For each ISI, two subgroups are trained reciprocally, that is with switched-roles for the odours 3-octanol (OCT) and benzaldehyde (BA). After training, each reciprocal group is allowed to choose between the two odours; based on their odour preferences, we calculate a learning index (LI). Positive LIs indicate conditioned approach, negative values mean conditioned avoidance.
B. For wild-type Control flies, the ISI determines the conditioned behaviour: Training with very long ISIs (-150 s, 70 s, 200 s) support no learning. If during training, the odour shortly precedes or overlaps with shock (ISI= -45, -15 or 0 s), Control flies learn to avoid it (i.e. punishment learning). If during training the odour closely follows shock (ISI= 20, 40 s), Control flies learn to approach it (i.e. relief learning). As for the *white* mutants, using very long ISIs, we find no difference to the Controls. After training with short ISIs on the other hand, regardless of the sequence of odour and shock, the *white* mutants' scores are shifted southwards, that is towards conditioned avoidance.
*: $P < 0.05/8$ while comparing between genotypes (i.e. Bonferroni correction, see Methods for details). Box plots represent median as the midline; 25 and 75 % as the box boundaries and 10 and 90 % as the whiskers.
C. Control and *white* mutant flies avoid shock indistinguishably well. NS: $P > 0.05$. Box plots are as in 1B.

reflect first-shock-then-odour presentation during training. For each ISI, two subgroups are trained reciprocally and then tested for their preference between the two odours. Based on the two reciprocal preferences, we calculate a learning index. Positive values indicate conditioned approach towards the learned odour; negative values reflect avoidance.

Regarding wild-type Control flies, conforming to the previous reports (Tanimoto et al. 2004; Yarali et al. In press), the conditioned behaviour depends on the ISI (Fig. 1B: Kruskal-Wallis test: Control flies: H= 168.96, d.f.= 7, $P< 0.05$). If during training the odour is presented either long before (Fig. 1B: One-sample sign test: Control: ISI= -150 s: $P> 0.05/ 8$) or long after shock (Fig. 1B: One-sample sign tests: Control: ISI= 70 s and 200 s: $P> 0.05/ 8$ each) flies do not learn. If the odour closely precedes or overlaps with shock during training, it is subsequently avoided in the test (Fig. 1B: One-sample sign tests: Control: ISI= -45 s, -15 s and 0 s: $P< 0.05/ 8$ each), we refer to this kind of learning as *punishment learning*. Contrarily, if the odour closely follows shock during training, wild-type flies learn to approach it (Fig. 1B: One-sample sign tests: Control: ISI= 20 s, 40 s: $P< 0.05/ 8$ each); this kind of learning in turn is referred to as *relief learning*.

Next, we compare *white* mutants' learning to the wild-type situation we describe above. Very long ISIs, which support no learning in the wild-type flies, give us no difference between the genotypes (Fig 1B: U-tests: ISI= -150 s: U= 28.00, $P> 0.05/ 8$; ISI= 70 s: U= 70.00, $P> 0.05/ 8$; ISI= 200 s: U= 58.00, $P> 0.05/ 8$). In other words, when there is no learning, there is also no effect of the loss of *white* function. Contrarily, using short ISIs, which do support learning in the wild-type flies, loss of *white* function does have an effect: Namely, regardless of the sequence of the odour and the shock during training, the learning scores of the *white* mutants are shifted 'southwards' that is, towards more negative values, indicating stronger conditioned avoidance (Fig 1B: U-tests: ISI= -15 s: U= 183.00, $P<0.05/ 8$; ISI= 0 s: U= 745.00, $P< 0.05/ 8$; ISI= 20 s: U= 157.00, $P< 0.05/ 8$; ISI= 40 s: U= 226.00, $P< 0.05/ 8$). Except, the ISI of -45 s, despite supporting learning, gives us no between-genotype difference in the scores (Fig 1B: U-test: ISI= -45 s: U= 239.00, $P> 0.05/ 8$). Thus, *white* mutants form in the overall, more 'negative' memories of an experience with electric shock, than the wild-type flies.

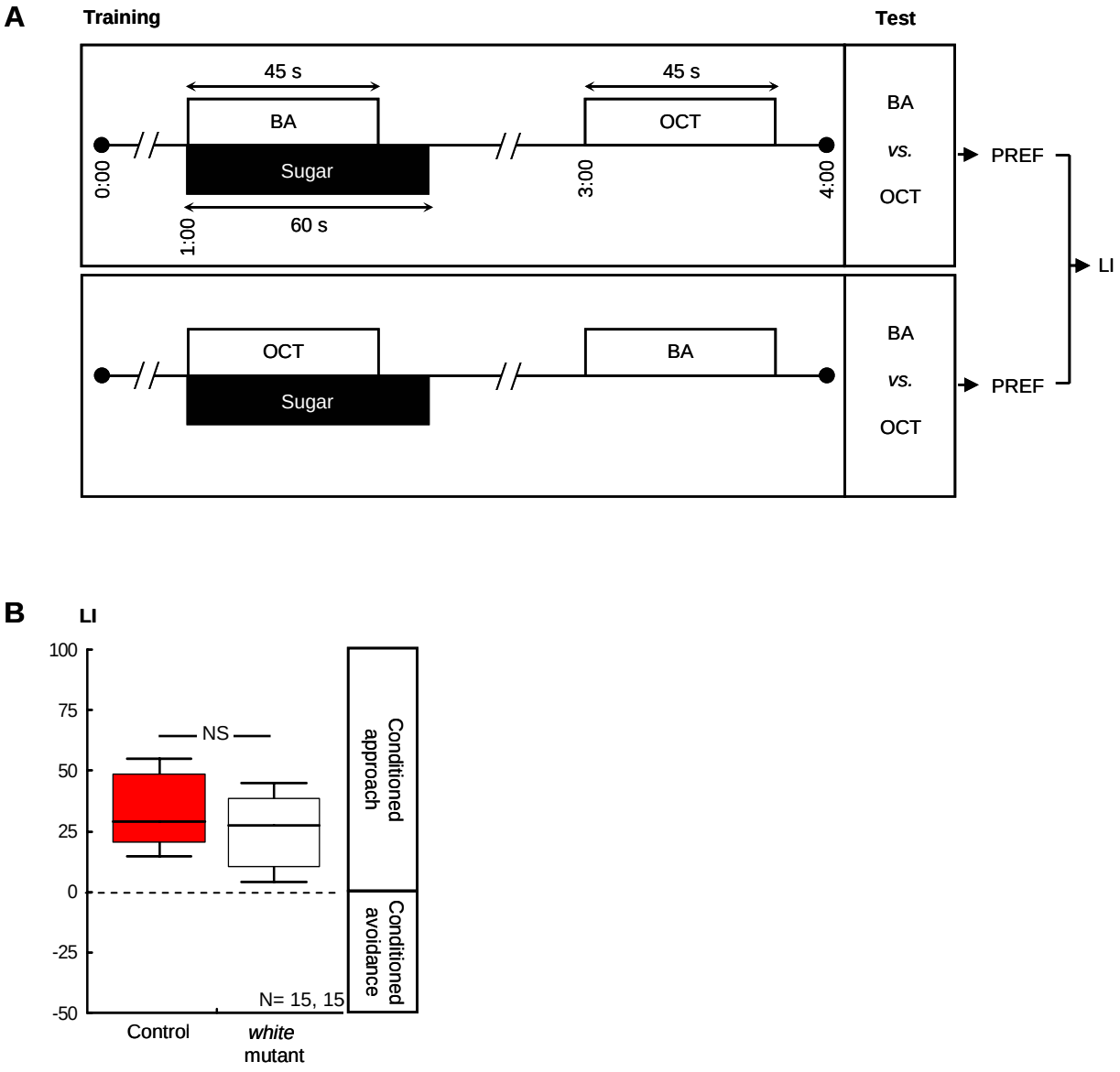

Fig. 2. Loss of *white* function does not affect sugar reward learning

A. For reward learning, flies are successively exposed to a to-be-learned odour in the presence of sugar and to a control odour without any sugar. Two subgroups are trained reciprocally, that is with switched-roles for the odours 3-octanol (OCT) and benzaldehyde (BA). Both subgroups are then given the choice between the two odours; a learning index (LI) is calculated based on their odour preferences. Positive values indicate conditioned approach towards the learned odour.
B. Control flies and *white* mutants perform equally well in reward learning. Details are as in 1C.

Wild-type Control flies and *white* mutants do reflexively avoid shock to the same extent (Fig 1C: U-test: U= 123.5, $P> 0.05$; One-sample sign test: for the pooled data set: $P< 0.05$). Furthermore, the loss of *white* leaves reward learning unaffected: After odour-sugar training (Fig. 2A), learning scores do not differ between genotypes (Fig. 2B: U-test: U= 82.00, $P> 0.05$); when pooled, they reflect conditioned approach (One-sample sign test: for the pooled data set: $P< 0.05$).

No effect of the loss of white *function on the whole-brain amounts of biogenic amines*

Next, we probe the *white* mutants' brain for abnormalities in the levels of four common biogenic amines. This is because the White protein provides neurons with the precursor for serotonin as well as the precursor for a cofactor of serotonin- and dopamine-synthesis (see the Introduction for details). In fact, Sitaraman et al. (2008) have recently reported lower whole-head levels of serotonin and dopamine in *white* mutants as compared to wild type flies.

Using high performance liquid chromatography, coupled to mass spectrometry, we quantify the amounts octopamine, tyramine, dopamine and serotonin in brain homogenates. With respect to none of these four, do we find a difference between the *white* mutants and the wild-type Control flies (Fig. 3: t-tests: $P> 0.05$ for each biogenic amine). Thus, the effect of the loss of *white* function on learning cannot be explained by any abnormality in the whole-brain amounts of biogenic amines

Discussion

Loss of white *function coherently affects punishment learning and relief learning*

Loss of *white* function has a remarkable effect on how fruit flies will remember a shock-episode (Fig. 1B). Namely, *white* mutants, as compared to wild-type flies, build stronger memories about the *painful* onset of shock, whereas they build weaker memories about its *relieving* offset. In other words, the *white* mutants remember the experience of shock as overall more 'negative' than wild-type flies. Importantly only the *learning* about shock is affected; the reflexive responsiveness to shock remains unaltered in the *white* mutants (Fig. 1C).

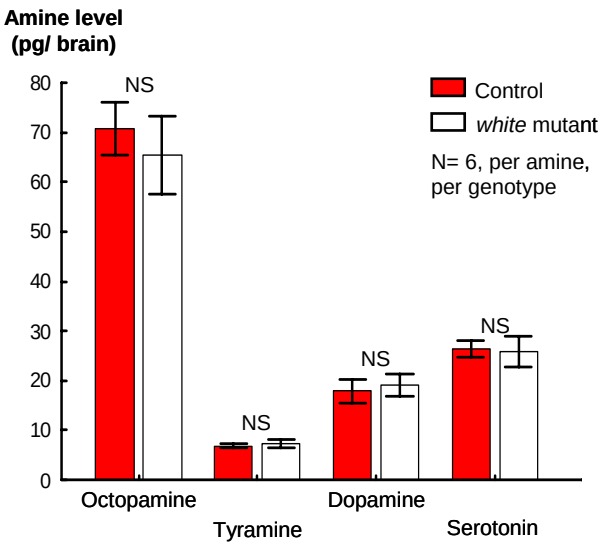

Fig. 3. Loss of *white* function does not affect the whole-brain amounts of biogenic amines

High performance liquid chromatography, coupled to mass spectrometry, reveals no difference between wilde-type Controls' and *white* mutants' brains in terms of the amounts of octopamine, tyramine, dopamine or serotonin. We report amine levels as pg/ brain; bars and whiskers respectively represent means and standard errors. NS: $P > 0.05$.

A balance between pain and relief?

These *coherent* effects on punishment learning and relief learning suggest that these two opposing kinds of learning have common determinants, which keep them in a balance. This would conform to Solomon & Corbit's (1974) psychological theory, suggesting that a painful stimulus, in addition to its primary effect, also induces with its offset, a state of relief; the balance between these two 'opponent' states supposedly govern animals' behaviour towards painful stimuli (Solomon & Corbit 1974). Such balance is suggested to be critical in psychiatric conditions such as anxiety (Vincent & Kukstas 1998) and schizophrenia (Grossberg 2000). The corresponding balance with respect to rewarding stimuli is important for addiction (reviewed by Koob [2008]).

Fruit fly seems to be an appropriate model to study the molecular and neuronal pivots of pain *versus* relief balance, because comparable paradigms are available for assessing the behavioural consequences of each. Importantly, the critical molecules may well be conserved from fly to man; e.g. the human homolog of the *white* gene is implicated in mood and panic disorders (Straub et al. 1994; Croop et al. 1997; Nakamura et al. 1999).

Molecular mechanisms of the white*-effect on fruit fly learning:*

No role for biogenic amines

In an attempt to account for the *white*-effect on learning, we probe *white* mutants' brains for the biogenic amines octopamine, tyramine, dopamine and serotonin; the amounts turn out comparable to wild-type (Fig. 3). Our result contrasts to the finding of Sitaraman et al. (2008) that *white* mutants' heads contain less serotonin and dopamine than the wild-type flies'.

In Fig. 4, we compare the mean amounts of octopamine, tyramine, dopamine and serotonin per brain/ head of wild-type/ *white* mutant flies, as reported by few selected studies. In some cases, values wildly differ. One potential source of discrepancy is the flies. All studies use strains with common origins: Canton-Special is used as wild-type, except, McClung and Hirsh [1999] use Oregon R; w1118 is used as the *white* mutant. However, differences may have arisen as these strains have long been kept separately in different laboratories. Also, fly-maintenance, (e.g. stress: Neckamayer & Weinstein 2005; diet: Schwaerzel et al. 2003) as well as fly-age (Neckamayer et al. 2000) and the time of the day

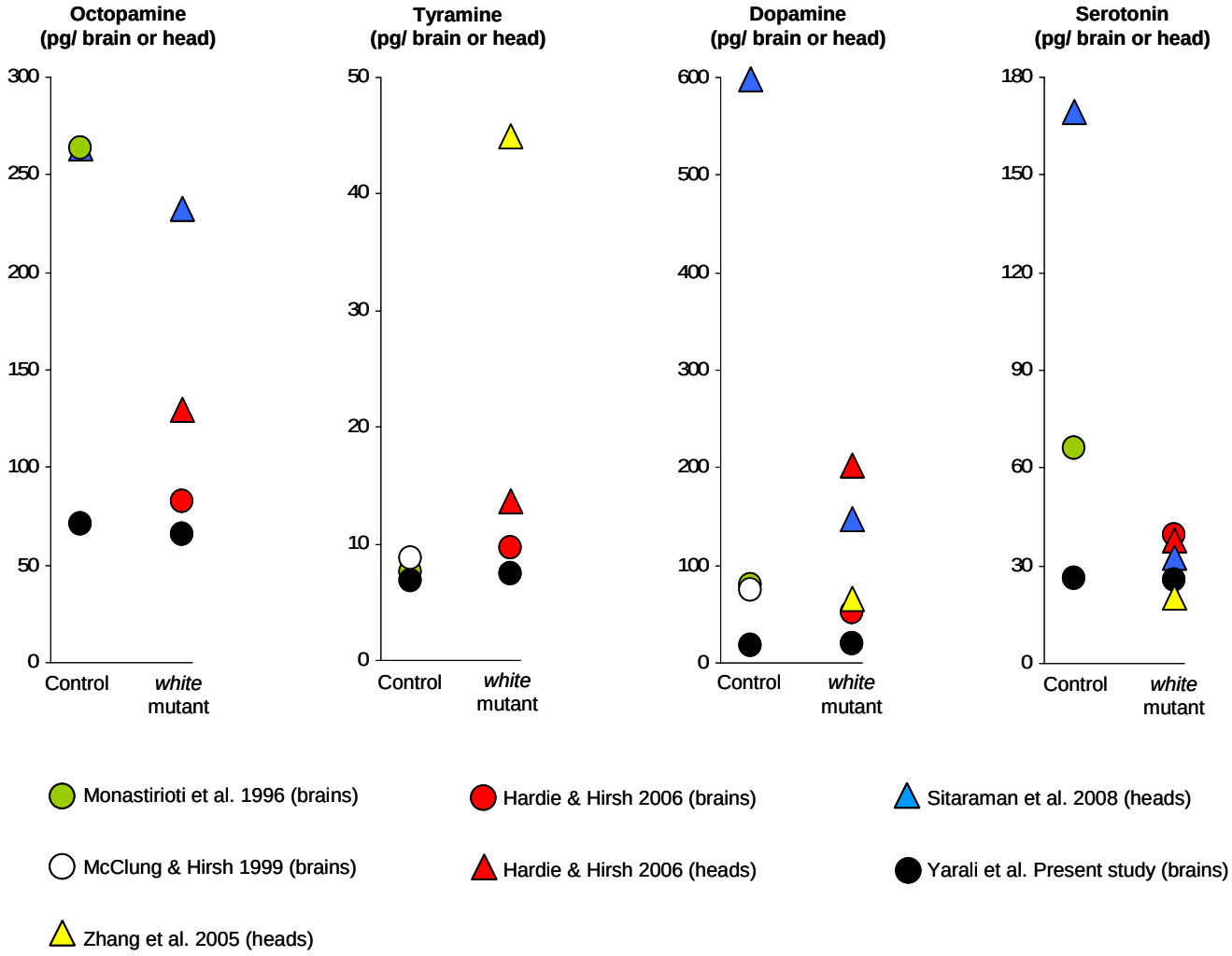

Fig. 4. Comparing the reported amine amounts between studies

We compare various studies in terms of the biogenic amine amounts they find in head- (triangles) or brain- (circles) homogenates from wild-type Control or *white* mutant flies. Mean values are shown in pg/ brain or head. Please note the different Y-axes for each amine. See the Discussion for details.

(Fowler et al. 1972) at which measurements are done seem to affect amine levels; these factors may differ between laboratories. In the present study, we keep these factors constant between the behavioural assays and the amine measurements.

Another potential source of discrepancy is the differences in sample preparation and measurement technique between studies. Using head- *versus* brain-homogenates makes a difference, even within one study (Hardie & Hirsh 2006): With respect to dopamine, this is not surprising, as dopamine is contained in the cuticle; as for octopamine, the head *versus* brain difference most likely reflects a confound in the detection method (see below). Thus, analysing brain-homogenates, as we presently do, seems more appropriate, especially for unambiguous interpretation of between-genotype differences.

As for the measurement method, all studies cited in Fig. 4 couple high performance liquid chromatography to an electrochemical detector; except, we presently use mass spectrometry for detection; and Sitaraman et al. (2008), for dopamine, use an enzyme immunoassay. Electrochemical detection has the drawback that any molecule in the sample, which co-migrates through the chromatography column with a particular amine, can be detected *as that amine*, resulting in over-estimation of the true amine level. This is indeed the case with respect to octopamine, as revealed by Hardie and Hirsh (2006). Using head- rather than brain-homogenates aggravates this confound by increasing the variety and the amount of unidentified molecules in the sample. Especially while comparing wild-type and *white* mutant heads, the contents of the wild-type eye, which are different from those of the *white* mutant eye, come into play as well. As an alternative to electrochemical detection, mass spectrometry, as we presently use, allows separate quantification even of those molecules that co-migrate through the chromatography column.

In short, with a seemingly appropriate methodology, we find no abnormality in the *white* mutants' whole-brain amine amounts to explain the abnormality in their learning. Another study, which interferes with the aminergic systems one-at-a-time, also finds no evidence for their involvement in relief learning (Chapter II.3. of this thesis).

A role for nitric oxide signalling?

We next consider the possible effects of the *white* gene on nitric oxide signalling: Guanine, which is transported into cells by the White-Brown heterodimer (Dreesen et al. 1988), is converted to '6H-tetrahydrobiopterin', which is a cofactor for nitric oxide synthesis, and in addition regulates the effects of nitric oxide (reviewed by Koshimura et al. [2000]). Guanine is also phosphorylated to GTP, the precursor for cGMP; cGMP in turn is the secondary messenger of nitric oxide signalling. In fact, White seems also to transport cGMP itself (Evans et al. 2008). Thus, effects of the *white* gene on multiple steps of nitric oxide signalling may possibly explain its effects on learning.

Nitric oxide most likely works as a retrograde neurotransmitter at the output of the mushroom body Kenyon cells, which in fruit flies harbour the key synaptic plasticity underlying both punishment and reward learning (reviewed by Gerber et al. [2004]): In crickets, output regions of Kenyon cells are innervated by arborizations, in which nitric oxide is synthesized (Bicker & Hähnlein 1995); the Kenyon cells in turn produce cGMP upon stimulation with nitric oxide (Bicker et al. 1996). In fact, the activity of the cGMP-dependent protein kinase in the Kenyon cells is critical for olfactory learning both in larval and in adult fruit flies (Kaun et al. 2007; Mery et al. 2007). Thus, the next reasonable experimental step is to probe for roles of nitric oxide signalling in punishment learning, relief learning and in the balance between these two.

The behavioural effects of white *may confound neurogenetic analysis*

Regardless of the underlying molecular biology, the behavioural effects of the *white* gene should concern all *Drosophila* behavioural neurogeneticists, as these may confound their experiments. A typical transgenic fly strain is mutant for the *white* gene; the transgene in turn is coupled to a truncated *white*-cDNA as marker. A serious problem arises when attempting to rescue a behavioural defect in a mutant by expressing the cDNA for the respective gene, using the Gal4-Uas system: In this case, the experimental flies not only express the gene of interest, but also they bear more transgenes and thus more copies of the marker *white*-cDNA, than the genetic controls. If loss of *white* function impairs the tested behaviour (e.g. relief learning), the experimental flies may indeed perform better than the controls, but not necessarily due to the rescue of the gene of interest. Similar confound arises when

attempting to impair a particular behaviour by interfering with neuronal activity or by knocking down a gene using the Gal4-Uas system: If loss of *white* function enhances the tested behaviour (e.g. punishment learning), any impairment in the experimental group may well be due to the higher level of White as compared to the genetic controls. Thus, it is well-advised to probe for effects of *white* before launching the neurogenetical analysis of a particular behaviour.

Conclusion

To summarize, we report *coherent* affects on the two opposing kinds of learning supported by an experience with electric shock, upon loss of *white* function: Learning about the *painful* onset of shock is enhanced, while learning about the *relieving* offset of shock is diminished. Although the underlying molecular mechanism remains unknown, these effects suggest a balance between pain and relief; our future research will target the molecular and neuronal pivots of such balance.

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References

An, X., Armstrong, J. D., Kaiser, K. & O'Dell, K. M. 2000. The effects of ectopic white and transformer expression on Drosophila courtship behavior. *J Neurogenet*, **14,** 227-43,271.

Andretic, R., van Swinderen, B. & Greenspan, R. J. 2005. Dopaminergic modulation of arousal in Drosophila. *Curr Biol*, **15,** 1165-75.

Bicker, G. 2001. Sources and targets of nitric oxide signalling in insect nervous systems. *Cell Tissue Res*, **303,** 137-46.

Bicker, G. & Hahnlein, I. 1995. NADPH-diaphorase expression in neurones and glial cells of the locust brain. *Neuroreport*, **6,** 325-8.

Bicker, G., Schmachtenberg, O. & De Vente, J. 1996. The nitric oxide/cyclic GMP messenger system in olfactory pathways of the locust brain. *Eur J Neurosci*, **8,** 2635-43.

Croop, J. M., Tiller, G. E., Fletcher, J. A., Lux, M. L., Raab, E., Goldenson, D., Son, D., Arciniegas, S. & Wu, R. L. 1997. Isolation and characterization of a mammalian homolog of the Drosophila white gene. *Gene*, **185,** 77-85.

Diegelmann, S., Zars, M. & Zars, T. 2006. Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in Drosophila. *Learn Mem*, **13,** 72-83.

Dierick, H. A. & Greenspan, R. J. 2007. Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat Genet*, **39,** 678-82.

Dreesen, T. D., Johnson, D. H. & Henikoff, S. 1988. The brown protein of Drosophila melanogaster is similar to the white protein and to components of active transport complexes. *Mol Cell Biol*, **8,** 5206-15.

Evans, J. M., Day, J. P., Cabrero, P., Dow, J. A. & Davies, S. A. 2008. A new role for a classical gene: White transports cyclic GMP. *J Exp Biol*, **211,** 890-9.

Fowler, D. J., Goodnight, C. J. & LaBrie, M. M. 1972. Circadian rhythms of 5-hydroxytryptamine (serotonin) production in larvae, pupae, and adults of Drosophila melanogaster (Diptera: Drosophilidae). *Ann Entomol Soc Am*, **65(1),**138-41.

Gerber, B., Tanimoto, H. & Heisenberg, M. 2004. An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol*, **14,** 737-44.

Grossberg, S. 2000. The imbalanced brain: from normal behavior to schizophrenia. *Biol Psychiatry*, **48,** 81-98.

Hardie, S. L. & Hirsh, J. 2006. An improved method for the separation and detection of biogenic amines in adult Drosophila brain extracts by high performance liquid chromatography. *J Neurosci Methods*, **153,** 243-9.

Hazelrigg, T., Levis, R. & Rubin, G. M. 1984. Transformation of white locus DNA in drosophila: dosage compensation, zeste interaction, and position effects. *Cell*, **36,** 469-81.

Hing, A. L. & Carlson, J. R. 1996. Male-male courtship behavior induced by ectopic expression of the Drosophila white gene: role of sensory function and age. *J Neurobiol*, **30,** 454-64.

Hoyer, S. C. 2007. Neuronal Correlates of Aggression in Drosophila melanogaster. Dissertation. Universität Würzburg.

Kaun, K. R., Hendel, T., Gerber, B. & Sokolowski, M. B. 2007. Natural variation in Drosophila larval reward learning and memory due to a cGMP-dependent protein kinase. *Learn Mem*, **14,** 342-9.

Koob, G. F. & Le Moal, M. 2008. Addiction and the brain antireward system. *Annu Rev Psychol*, **59,** 29-53.

Koshimura, K., Murakami, Y., Tanaka, J. & Kato, Y. 2000. The role of 6R-tetrahydrobiopterin in the nervous system. *Prog Neurobiol*, **61,** 415-38.
McClung, C. & Hirsh, J. 1999. The trace amine tyramine is essential for sensitization to cocaine in Drosophila. *Curr Biol*, **9,** 853-60.

Mery, F., Belay, A. T., So, A. K., Sokolowski, M. B. & Kawecki, T. J. 2007. Natural polymorphism affecting learning and memory in Drosophila. *Proc Natl Acad Sci U S A*, **104,** 13051-5.

Monastirioti, M., Linn, C. E., Jr. & White, K. 1996. Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *J Neurosci*, **16,** 3900-11.

Nakamura, M., Ueno, S., Sano, A. & Tanabe, H. 1999. Polymorphisms of the human homologue of the Drosophila white gene are associated with mood and panic disorders. *Mol Psychiatry*, **4,** 155-62.

Neckameyer, W. S. & Weinstein, J. S. 2005. Stress affects dopaminergic signaling pathways in Drosophila melanogaster. *Stress*, **8,** 117-31.

Neckameyer, W. S., Woodrome, S., Holt, B. & Mayer, A. 2000. Dopamine and senescence in Drosophila melanogaster. *Neurobiol Aging*, **21,** 145-52.

Nilsson, E. E., Asztalos, Z., Lukacsovich, T., Awano, W., Usui-aoki, K. & Yamamoto, D. 2000. Fruitless is in the regulatory pathway by which ectopic mini-white and transformer induce bisexual courtship in Drosophila. *J Neurogenet*, **13,** 213-32.

O'Hare, K., Murphy, C., Levis, R. & Rubin, G. M. 1984. DNA sequence of the white locus of Drosophila melanogaster. *J Mol Biol*, **180,** 437-55.

Pajak, M. & Kańska, M. 2006. Synthesis of isotopomers of dopamine labeled with deuterium or tritium. *J Labelled Comp Radiopharm*, **49,** 1061-67

Riemensperger, T., Voller, T., Stock, P., Buchner, E. & Fiala, A. 2005. Punishment prediction by dopaminergic neurons in Drosophila. *Curr Biol*, **15,** 1953-60.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Voller, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. & Fiala, A. 2006. Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae. *Curr Biol*, **16,** 1741-7.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. & Heisenberg, M. 2003. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. *J Neurosci*, **23,** 10495-502.

Sitaraman, D., Zars, M., Laferriere, H., Chen, Y. C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G. E. & Zars, T. 2008. Serotonin is necessary for place memory in Drosophila. *Proc Natl Acad Sci U S A*, **105,** 5579-84.

Solomon, R. L., & Corbit, J. D. 1974 An opponent-process theory of acquired motivation. I. Temporal dynamics of affect. *Psychol Rev,* **81(2),** 119-45.

Straub, R. E., Lehner, T., Luo, Y., Loth, J. E., Shao, W., Sharpe, L., Alexander, J. R., Das, K., Simon, R., Fieve, R. R. & et al. 1994. A possible vulnerability locus for bipolar affective disorder on chromosome 21q22.3. *Nat Genet*, **8,** 291-6.

Sullivan, D.T & Sullivan, M.C. 1975. Transport defects as the physiological basis for eye color mutants of Drosophila melanogaster. *Biochem Genet*, **13,** 603-13.

Tanimoto, H., Heisenberg, M. & Gerber, B. 2004. Experimental psychology: event timing turns punishment to reward. *Nature*, **430,** 983.

Tearle, R. G., Belote, J. M., McKeown, M., Baker, B. S. & Howells, A. J. 1989. Cloning and characterization of the scarlet gene of Drosophila melanogaster. *Genetics*, **122,** 595-606.

Tempel, B. L., Bonini, N., Dawson, D. R. & Quinn, W. G. 1983. Reward learning in normal and mutant Drosophila. *Proc Natl Acad Sci U S A*, **80,** 1482-6.

Tully, T. & Quinn, W. G. 1985. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J Comp Physiol [A]*, **157,** 263-77.

Vincent, J. D. & Kukstas, L. A. 1998. Opponent processes and anxiety: toward a neurophysiological formulation. *Acta Psychiatr Scand Suppl*, **393,** 50-5.

Yarali, A., Niewalda, T., Chen, Y., Tanimoto, H., Duerrnagel, S. & Gerber, B. In Press. 'Pain-relief' learning in fruit flies. *Anim Beh*

Yarali, A., Ritze, Y., Scholz, H. & Gerber, B. In Preparation. 'Pain-relief' learning in fruit flies: Testing for the roles of octopamine, tyramine, dopamine and serotonin.

Yuan, Q., Joiner, W. J. & Sehgal, A. 2006. A sleep-promoting role for the Drosophila serotonin receptor 1A. *Curr Biol*, **16,** 1051-62.

Yuan, Q., Lin, F., Zheng, X. & Sehgal, A. 2005. Serotonin modulates circadian entrainment in Drosophila. *Neuron*, **47,** 115-27.

Zhang, Y., Friedman, D., Wang, Z., Woodruff, E., Pan, L., O'Donnell, J. & Broadie, K. 2005. Protein expression profiling of the Drosophila fragile X mutant brain reveals upregulation of monoamine synthesis. *Cell,***107,** 591-603.

Zhang, K., Guo, J. Z., Peng, Y., Xi, W. & Guo, A. 2007. Dopamine-mushroom body circuit regulates saliency-based decision-making in Drosophila. *Science*, **316,** 1901-4.

Zhang, S. D. & Odenwald, W. F. 1995. Misexpression of the white (w) gene triggers male-male courtship in Drosophila. *Proc Nat Acad Sci U S A*, **92,** 5525-9.

‘Pain-relief’ learning in fruit flies: Testing for the roles of octopamine, tyramine, dopamine and serotonin

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& Bertram Gerber

Abstract

What is particularly worth remembering about a painful, traumatic experience is its beginning and its end. Fruit flies for example learn about the beginning and the end of an electric shock: If an odour precedes shock during training, flies subsequently avoid it as a predictor for *punishment*; if an odour follows shock during training, it is subsequently approached as a signal for *relief*. Presently, we compare such *relief learning* to *reward learning* and *punishment learning*. The internal reinforcement signals underlying reward and punishment learning are carried respectively by octopamine and dopamine. We find octopamine to be dispensible for relief learning, while we verify its necessity for reward learning. Also, when we block output from a subset of octopaminergic/ tyraminergic neurons, we find relief learning intact. We then block output from a subset of dopaminergic neurons: Such output, as we verify, is necessary for punishment learning; for relief learning on the other hand, it is dispensible. Also, blocking output from an independent subset of dopaminergic neurons leaves relief learning intact. In addition, blocking output from two independent subsets of serotonergic neurons also does not affect relief learning. Thus, relief learning is distinct from reward learning and punishment learning, with respect to the requirement for biogenic amine signalling. This dissociation may apply to other experimental systems as well.

Introduction

The brains' biological function is to organize behaviour. Behaviour is organized according to past, present and future: First, the animals' *present* needs shape the behaviour; second, the upcoming *future* is considered; and, thirdly, to predict the future, animals rely on their *past* experience. Experience contributes to behaviour organization mainly via learning about the predictive relationships in the environment. To understand such predictive, associative learning, we use the fruit fly, as it offers the fortunate combination of fine grained behavioural analysis and genetic accessibility.

Fruit flies avoid an odour, which during training had repeatedly preceded an electric shock (*punishment* learning: Tully & Quinn 1985); contrarily, they approach an odour which had been paired with a sugar reward (*reward* learning: Tempel et al. 1983). Although these kinds of learning are both

neurobiologically well-studied, one key feature has been long ignored: Associative learning is *asymmetric*. Only after first-odour-then-shock training, flies avoid the odour. After training with a 'reversed' timing of events (first-shock-then-odour), the odour is approached (Tanimoto et al. 2004; Yarali et al. In press), this is possibly because the odour in this case has become a predictor for what may correspond to the feeling of *relief* once a painful event has passed (Solomon & Corbit 1974). Thus, in fruit flies, an experience with shock changes subsequent behaviour in two opposite ways: Those stimuli that predict the beginning of shock are responded aversively; whereas those stimuli that signal the end of shock induce an appetitive response.

Such dual effects of painful stimuli apply to other animals as well: For example snails (Britton & Farley 1999), having been trained such that presentation of light repeatedly precedes an unpleasant rotation (light-rotation), show weaker phototactic behaviour than before such training; contrarily, training with a 'reversed' timing of events (rotation-light) results in increased phototaxis. Analogous results have been obtained in dogs (Moscovitch & LoLordo 1968), rabbits (Plotkin & Oakley 1975) and rats (Maier et al. 1976).

Similarly, a pleasant, rewarding experience also supports two opposing kinds of learning: Those stimuli that signal upcoming reward are responded appetitively; whereas those stimuli that predict the withdrawal of a reward release aversive responses. For example honeybees, when trained such that an odour precedes a sugar reward (odour-sugar) subsequently extend the proboscis in response to the odour; contrarily, after sugar-odour training, the odour supresses the proboscis extension (Hellstern et al. 1998). Analogous results have been obtained in pigeons (Hearst 1988).

These observations beautifully conform to the psychological theories, which suggests four kinds of 'hedonic state' induced by affective experiences (Solomon & Corbit 1974): That is, a painful state is followed by a state of relief; whereas a rewarding state is followed by a state of craving; all of these four states have behavioural as well as physiological effects in man and other animals. Further, all of these states are suggested to act as reinforcers that is, other stimuli or actions can be learned as predictors for pain, relief from pain, reward or loss of reward (Wagner 1981). Understanding the neurobiological bases for these kinds of learning is indispensable for a comprehensive account of how

behaviour is organized with respect to affective experiences. We thus study learning of pain-relief, using fruit fly as a model.

Relief learning in fruit flies is a robust and parametrically well-characterized behavioural phenomenon (Yarali et al. In press.); thus, it suits for neuronal circuit-analyses using the available genetic tools. The present study is the first step towards a neuronal circuit-account of relief learning. We compare relief learning to reward learning and to punishment learning, in terms of the requirement for biogenic amine signalling.

Punishment and reward learning are doubly dissociated in terms of the biogenic amines that signal reinforcement. Shock, and probably all other aversive stimuli, in addition to acting on their respective reflex pathways, activate the *dopamine*rgic neurons (Riemensperger et al. 2005). Output from dopaminergic neurons is necessary for punishment learning, but not for reward learning (Schwaerzel et al. 2003). Reportedly, activation of the dopaminergic neurons can alone act as aversive reinforcement (Schroll et al. 2006). A corresponding appetitive reinforcement signal is carried by the *octopamine*rgic neurons: Octopamine is necessary for reward but not punishment learning (Schwaerzel et al. 2003). Activation of octopaminergic/ tyraminergic neurons can reportedly act as appetitive reinforcement (Schroll et al. 2006; but see Schipanski 2007). This double dissociation between the signalling of appetitive and aversive reinforcement applies also to other insects (honeybee: Hammer 1993; Hammer & Menzel 1998; Farooqui et al. 2003; Vergoz et al. 2007; cricket: Unoki et al. 2005; Unoki et al. 2006; for a review on vertebrates, see Schulz 1999).

Might relief learning rely on the same biogenic amines as reward or punishment learning? Our findings suggest otherwise: We find no evidence for a requirement for either octopaminergic or dopaminergic signalling in relief learning. Importantly we do verify the key results of Schwaerzel et al. (2003) concerning the roles of octopamine and dopamine respectively in reward and punishment learning. In addition, we find no evidence that two other biogenic amines, tyramine and serotonin were required for relief learning.

Materials and Methods

Flies

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

To test for a role for octopamine, we use the mutant strain $T\beta H^{M18}$ (Monastrioti et al. 1996; also see Schwaerzel et al. 2003; Saraswati et al. 2004; Scholz 2005; Hardie et al. 2007; Brembs et al. 2007). These flies lack octopamine, due to the deficiency of the tyrosine beta hydroxylase (TβH) enzyme, which catalyzes the last step of octopamine bioysnthesis (Fig. 2). Since the original $T\beta H^{M18}$ strain (Monasitrioti et al. 1996) contains an additional mutation in the *white* gene, we instead use a recombinant strain with a $white^{+}$ allele, which was generated by Schwaerzel et al. (2003). As genetic control, we use a non-recombinant strain with $T\beta H^{+}$ and $white^{+}$ alleles, which was generated in parallel. We refer to these two strains as '*TβH* mutant' and 'Control'.

We use $shibire^{ts1}$ for temperature-controlled, reversible blockage of neuronal output (Kitamoto 2001). We direct the expression of $shibire^{ts1}$ to different kinds of neuron by crossing the males of an appropriate Gal4 strain (Table 1) with virgin females of a UAS-$shibire^{ts1}$ strain (Kitamoto 2001; 1st and 3rd chromosomes); thus the offspring are heterozygous for both the Gal4-driver and the effector $shibire^{ts1}$. We refer to these flies with the name of the Gal4-driver together with '$shibire^{ts1}$' (e.g. 'TDC / $shibire^{ts1}$'). To obtain the two kinds of genetic control, we cross each of the UAS-$shibire^{ts1}$ and the Gal4-driver strains to w1118 flies. Genetic controls are thus heterozygous either for the Gal4-driver or for the effector $shibire^{ts1}$. We refer to these flies using only the name of the Gal4-driver (e.g. TDC) and only '$shibire^{ts1}$', respectively. We evaluate the data regardless of gender; except when we use the SERT-Gal4 driver: In this case we only evaluate female progeny as the males lack the SERT-Gal4 driver.

To visualize the pattern of Gal4 expression, we use each driver (Table 1) to express the UAS-controlled transgene *mCD8GFP*, which encodes for a green fluorescent protein (GFP) variant inserted into cellular membranes. We cross males from each driver strain to virgin females of a UAS-*mCD8GFP* strain (Lee & Luo 1999) and use the progeny in immunohistochemistry, regardless of gender.

Gal4 driver		Gal4 expression in..	Chromosome	References
TDC	regulatory sequences of tyrosine decarboxylase gene	**octopaminergic / tyraminergic** neurons	2rd	**1**, 2
TH	regulatory sequences of tyrosine hydroxylase gene	**dopaminergic** neurons	3nd	2, **3**, 4, 5, 6, 7
DDC	regulatory sequences of dopa decarboxylase gene	**dopaminergic / serotonergic** neurons	3rd	7, **8**
SERT	regulatory sequences of serotonin transporter gene	**serotonergic** neurons	1st	**9**

Table 1. The Gal4 strains that are used.
(1) Cole et al. 2005; (2) Schroll et al. 2006; (3) Friggi-Grelin et al. 2002; (4) Schwaerzel et al. 2003 ; (5) Riemensperger et al. 2005; (6) Zhang et al. 2007; (7) Sitaraman et al. 2008; (8) Li et al. 2000; (9) Ritze 2007. Bold numbering indicates the original report of the respective Gal4 strain.

Immunohistochemistry

Brains are immunostained against the GFP to reflect the pattern of Gal4 expression and against the Synapsin protein to visualize neuropils. Brains are dissected in saline and fixed for 2 h in 4 % formaldehyde with PBST as solvent (phosphate-buffered saline containing 0.3 % Triton X-100). After a 1.5 h incubation in blocking solution (3 % normal goat serum [Jackson Immuno Research Laboratories Inc., West Grove, PA, USA] in PBST), brains are incubated overnight with the monoclonal anti-Synapsin mouse antibody SYNORF1, diluted 1:20 in PBST (Klagges et al. 1996) and polyclonal anti-GFP rabbit antibody, diluted 1:2000 in PBST (Invitrogen Molecular Probes, Eugene, OR, USA). These primary antibodies are detected after an overnight incubation with Cy3 goat anti-mouse Ig, diluted 1:250 in PBST (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) and Alexa488 goat anti-rabbit Ig, diluted 1:1000 in PBST (Invitrogen Molecular Probes, Eugene, OR, USA). All incubation steps are followed by multiple PBST washes. Incubations with antibodies

are done at 4 °C; all other steps are performed at room temperature. Finally, brains are mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and examined under a confocal microscope.

Behavioural assays

One day prior to behavioural experiments, 1- 4 day-old flies are collected in fresh food vials and kept overnight at 18 °C and 60- 70 % relative humidity. For sugar learning, flies are starved overnight for 18- 20 h at 25 °C and 60- 70 % relative humidity in vials equipped with a moist tissue paper and a moist filter paper. Those experiments that do not use *shibire*^{ts1} are performed at 22- 25 °C and 75- 85 % relative humidity. For inducing the effect of *shibire*^{ts1}, flies are first exposed to 34- 36 °C and 60- 70 % relative humidity for 30 min; then the experiment takes place also under these conditions, which are referred to as '@ high temperature'. The condition referred to as '@ low temperature' in turn involves exposing the flies to 20- 23 ºC and 75- 85 % relative humidity for 30 min; then the experiment follows also under these conditions. The experimental setup is as described by Tully and Quinn (1985) and Schwaerzel et al. (2003). Flies are trained and tested as groups of 100- 150. Trainings take place under dim red light which does not allow flies to see, tests are in complete darkness.

As odourants, 90 µl benzaldehyde (BA), 340 µl 3-octanol (OCT) and 340 µl methylcyclohexanol (MCH) (all from Fluka, Steinheim, Germany) are applied in 1 cm-deep Teflon containers of 5, 14 and 14 mm diameters, respectively. For those experiments that use the *TβH*^{M18} flies, MCH and OCT are diluted 100-folds in paraffin oil (Merck, Darmstadt, Germany). All other experiments use undiluted BA and OCT.

For punishment learning (Fig. 1A), flies receive 6 training trials. Each trial starts by loading the flies into the experimental setup (0:00 min). From 4:00 min on, control odour is presented for 15 s. Then, from 7:15 min on, the to-be-learned odour is presented also for 15 s, From 7:30 min on, electric shock is applied as 4 pulses of 100 V; each pulse is 1.2 s- long and is followed by the next with an onset-to-onset interval of 5 s. Thus the to-be-learned odour precedes shock with an onset-to-onset interval of 15 s. For relief learning (Fig. 1B), keeping all other parameters unchanged, we reverse the

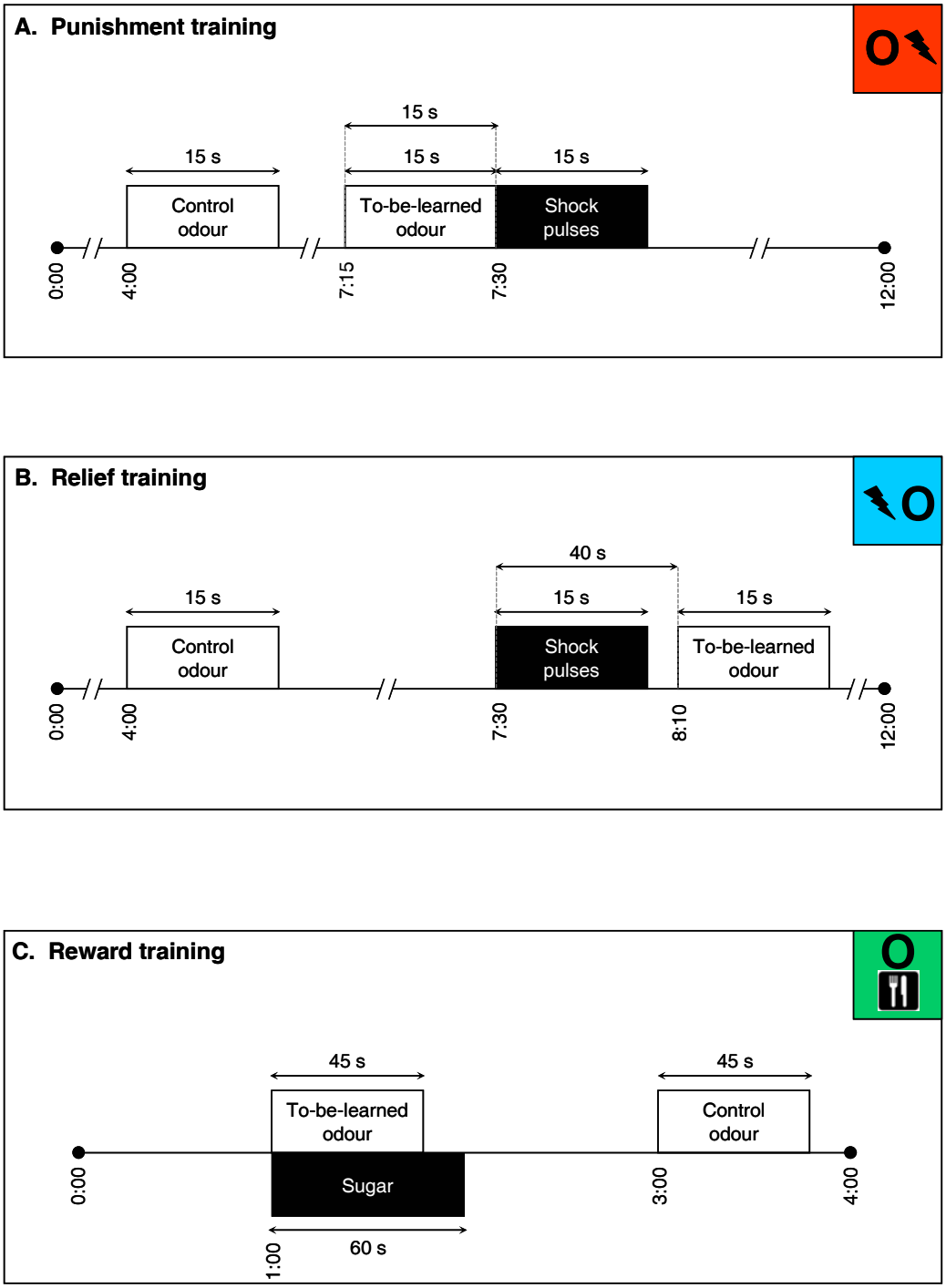

Fig. 1. Training

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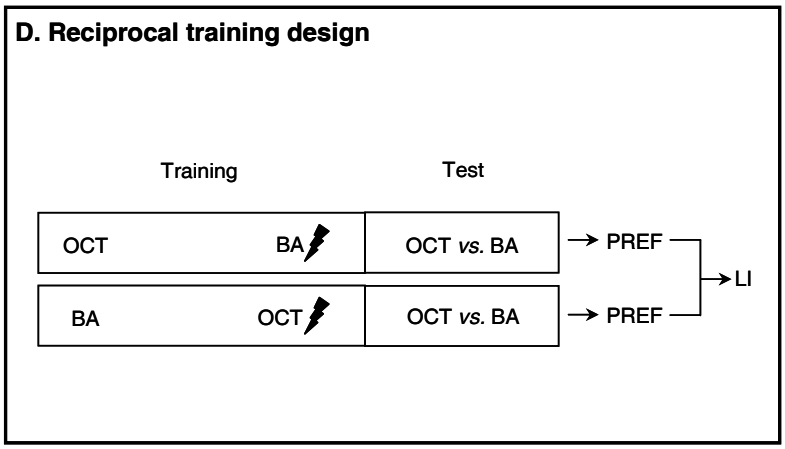

Fig. 1. Training

For punishment learning **(A)**, flies receive two odours and pulses of electric shock. A control odour is presented long before shock; a to-be-learned odour *precedes* shock with an onset-to-onset interval of 15 s. For relief learning **(B)**, while all other parameters are unchanged, the to-be-learned odour *follows* shock with an onset-to-onset interval of 40 s. For reward learning **(C)**, flies are successively exposed to a to-be-learned odour in the presence of sugar and then to a control odour without any sugar. Although not shown here, in half of the cases, training starts with the control odour instead of the to-be-learned odour and sugar. For each kind of training we use a reciprocal design **(D)**: Two groups are trained in parallel; for one of these, 3-octanol (OCT) is the control odour and benzaldehyde (BA) is to be learned; the other group is trained reciprocally. Each group is then given the choice between the two odours. Based on flies' distribution, preference indices (PREF) are calculated. Based on the two reciprocal PREF values, we calculate a learning index (LI). The situation for punishment learning is sketched; this reciprocal design applies also to relief and reward learning.

relative timing of events: That is, the to-be-learned odour is presented from 8:10 min on thus, following shock with an onset-to-onset interval of 40 s. At 12:00 min, flies are transferred out of the setup into food vials, where they stay for 16 min until the next trial. At the end of the sixth training trial, after the usual 16 min break, flies are loaded back into the setup. After a 5 min accommodation period, they are transferred to the choice point of a T-maze, where they can choose between the control odour and the learned odour. After 2 min, arms of the maze are closed and flies on each side are counted. A preference index (PREF) is calculated as:

$$(1) \qquad PREF = (\#_{\text{Learned odour}} - \#_{\text{Control odour}}) \times 100 / \#_{\text{Total}}$$

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

$$(2) \qquad LI = (PREF_{BA} + PREF_{OCT}) / 2$$

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

Reward learning (Fig. 1C) uses two training trials. Each trial starts by loading the flies into the setup (0:00 min). 1 min later, flies are transferred to a tube lined with a filter paper which was soaked the previous day with 2 ml of 2 M sucrose solution, and then was left to dry over night. This tube is scented with the to-be-learned odour. After 45 s, the to-be-learned odour is removed, and after 15 additional seconds flies are taken out of the tube. At the end of a 1 min waiting period, they are transferred into another tube lined with a filter paper which was soaked with pure water and then dried. This second tube is scented with the control odour. After 45 s, control odour is removed and 15 s later, flies are taken out of this second tube. The next trial starts immediately. For half of the cases, training trials start with the to-be-learned odour and sugar; in the other half, control odour is given precedence.

Once the training is completed, after a 3 min waiting period, flies are transferred to the choice point of a T-maze between the control odour and the learned odour. After 2 min, arms of the maze are closed, flies on each side are counted and a preference index (PREF) is calculated according to Equation 1. As detailed above (also see Fig. 1D), two groups are trained reciprocally and the learning index (LI) is calculated based on their PREF values according to Equation 2.

Statistics

All data are analysed using non-parametric statistics and are reported as box plots, showing the median as the midline and 10, 90 and 25, 75 % as whiskers and box boundaries, respectively. This is necessary since the learning scores are not normally distributed and thus the criteria for using parametric statistics are not met. For comparing scores of individual groups to zero, we use one-sample sign tests. Mann-Whitney U-tests and Kruskal-Wallis tests are respectively used for pair-wise and global between-group comparisons. When multiple tests of one kind are performed within a single experiment, we adjust the experiment-wide error-rate to 5 % by Bonferroni correction: We divide the critical $P< 0.05$ by the number of tests. All statistical analyses are performed on a PC using the software Statistica.

Results

A role for octopamine in relief learning?

Relief might resemble 'true' reward, in that it is signalled by octopamine. This would correspond to intuition: End of something bad is good! More importantly, it could readily be accommodated by the psychological theories mentioned in the Introduction (Solomon & Corbit, 1974; Wagner 1981): Shock might induce opposing internal reinforcement signals with its beginning and end. While the onset of shock is signalled by dopamine, its offset may activate the octopaminergic neurons, just as a 'true' reward would do. We scrutinize this hypothesis, by testing for a role for the octopaminergic system in relief learning.

Initially, we use the *TβH* mutant; as it lacks the critical enzyme (Fig. 2), this mutant cannot synthesise octopamine (Monastrioti et al. 1996). We first confirm the finding of Schwaerzel et al.

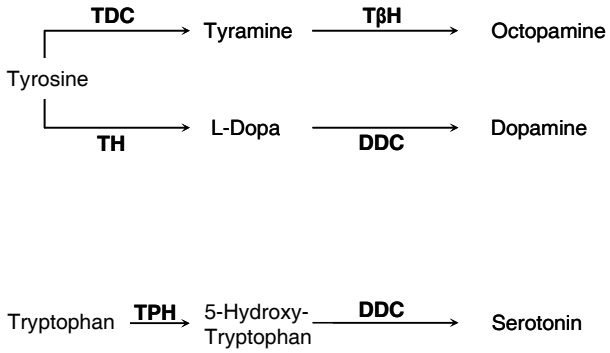

Fig. 2. Biosynthesis of tyramine, octopamine, dopamine and serotonin

TDC: tyrosine decarboxylase; TβH: tyramine β-hydroxylase; TH: tyrosine hydroxylase; DDC: dopa decarboxylase; TPH: tryptophan hydroxylase. Modified from Monastirioti (1999).

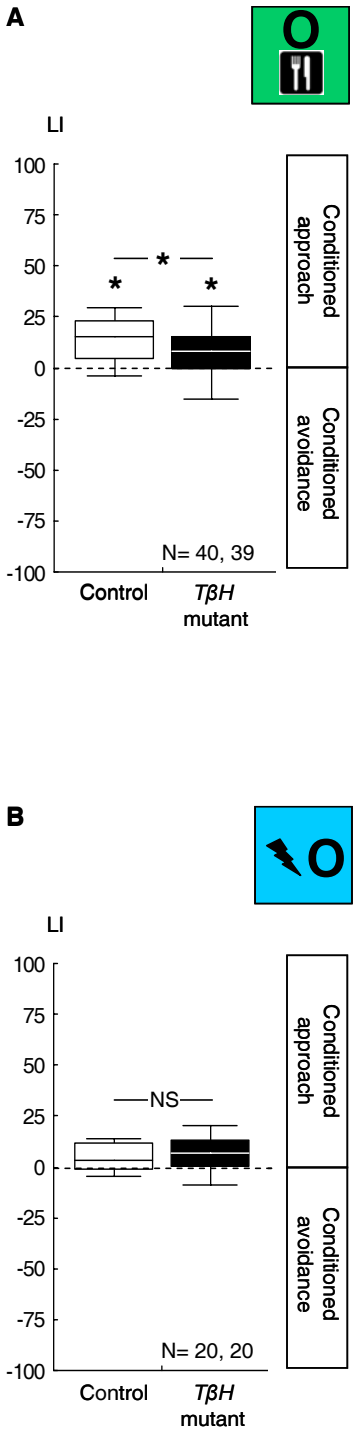

Fig. 3. Lack of octopamine impairs reward learning; relief learning remains intact.

The octopamine-deficient *TβH* mutant is partially impaired in reward learning **(A)**; relief learning remains unaffected **(B)**. *: $P< 0.05$, NS: $P> 0.05$, while comparing between genotypes. For comparing scores of each genotype to zero *: $P< 0.05/ 2$, to keep the experiment-wide error-rate at 5 % (i.e. Bonferroni correction). Box plots show the median as the midline; 25 and 75 % as the box-boundaries and 10 and 90 % as whiskers.

(2003) that in reward learning, *TβH* mutant performs significantly worse than the corresponding genetic Control (Fig 3A: U-test: U= 544.00, $P< 0.05$). Residual reward learning ability is however detectable in the *TβH* mutant (Fig. 3A: One-sample sign tests: $P< 0.05/ 2$ for each genotype), suggesting residual octopamine or a yet-unidentified, octopamine-independent compensating mechanism.

As for relief learning, the *TβH* mutant is not impaired: Learning scores are statistically indistinguishable between genotypes (Fig. 3B: U- test: U= 168.00, $P> 0.05$). Pooling the data, we find conditioned approach (One-sample sign test for the pooled data set: $P< 0.05$). Thus, in terms of the requirement for octopamine, relief learning differs from reward learning.

As an additional, independent assault towards the octopaminergic system, we block the output from a subset of octopaminergic/ tyraminergic neurons. We use UAS-*shibire*^{ts1}, which reversibly blocks neuronal output at high temperature (Kitamoto, 2001). We direct the expression *shibire*^{ts1} to octopaminergic/ tyraminergic neurons using the TDC-Gal4 driver (Cole et al., 2005; Table 1; Fig.s 2 and 4A). We first test for an effect on reward learning: When trained and tested at high temperature, TDC / *shibire*^{ts1} flies perform comparably to the genetic controls (Fig. 5A: Kruskal-Wallis test: H= 3.03, d.f.= 2, $P> 0.05$). When we pool the learning scores across genotypes, we find conditioned approach (One-sample sign test for the pooled data set: $P< 0.05$). This result may appear inconsistent with the impairment we find in the *TβH* mutant (Fig. 3A). However, it should be noted that the TDC-Gal4 driver does not target all octopaminergic neurons (personal communication: S. Busch, Universität Würzburg); those octopaminergic neurons that are not blocked may well suffice to signal reward. An alternative explanation would be that in those neurons that are indeed targeted by the TDC-Gal4 driver, the level of *shibire*^{ts1} expression may be insufficient for a complete block of output.

In any case, we probe for an impairment in relief learning, and find none: After training and test at high temperature, learning scores are statistically indistinguishable between genotypes (Fig. 5B: Kruskal-Wallis test: H= 2.43, d.f.= 2, $P> 0.05$). Pooling the data, we find conditioned approach (One-sample sign test for the pooled data set: $P< 0.05$). Thus, using two different methods of interference (Fig.s 3B and 5B), we find no evidence for a role for the octopaminergic system in relief learning. Next, we test for a role for the dopaminergic system.

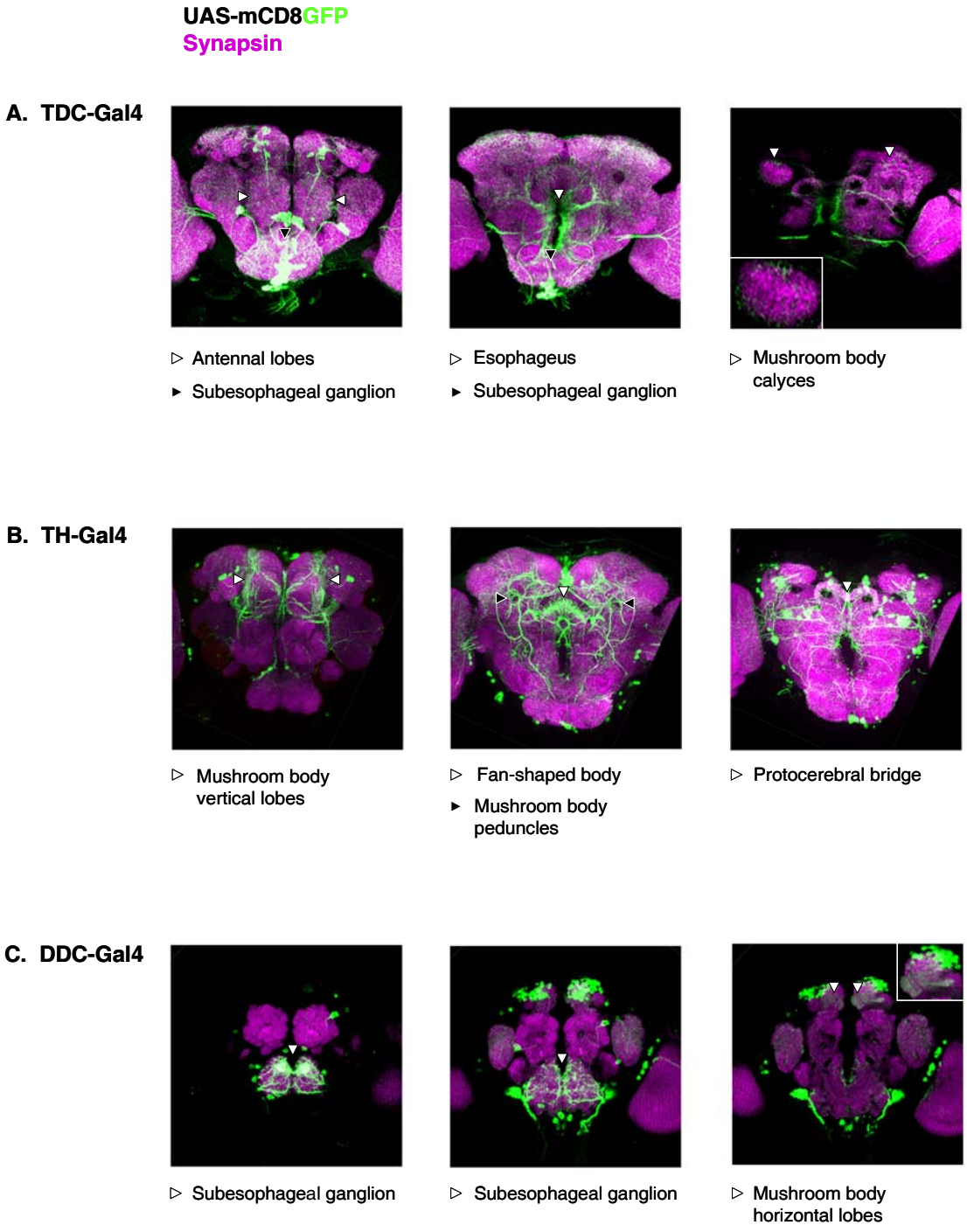

Fig. 4. Patterns of Gal4-expression driven by the used strains

See the next page for the legend.

Fig. 4. Patterns of Gal4-expression driven by the used strains

We drive the expression of a membrane bound green fluorescent protein (mCD8GFP) using three different Gal4 drivers. Patterns of GFP-immunoreactivity (green) should reflect the respective patterns of Gal4-expression; Synapsin-immunoreactivity (magenta) reflects the organization of the neuropils. We show projections of frontal optical sections of 0.9 µm, each. In each row, the leftmost panel shows the anterior-most projection; in each panel, dorsal is to top. Neurons that express GFP, driven by TDC-Gal4 **(A)** innervate the antennal lobes (left panel), the subesophageal ganglion (left and middle panels), the areas surrounding the esophageous (middle panel), and the mushroom body calyces (right panel; see also the inset). We find no innervation of the mushroom body lobes. When driven by TH-Gal4 **(B)**, GFP is expressed in neurons that innervate the mushroom body vertical lobes and peduncles (left and middle panels); also the fan-shaped body (middle panel) and the protocerebral bridge (right panel) are innervated. We find no innervation of the antennal lobes or the mushroom body calyces. Thus, as far as the olfactory neuropils are concerned, TH-Gal4 on one hand and TDC-Gal4 on the other hand seem to target complementary regions. Under the control of the DDC-Gal4 driver **(C)**, GFP is expressed in neurons that innervate the subesophageal ganglion (left and middle panels) as well as the horizontal lobes of the mushroom body (right; see also the inset).

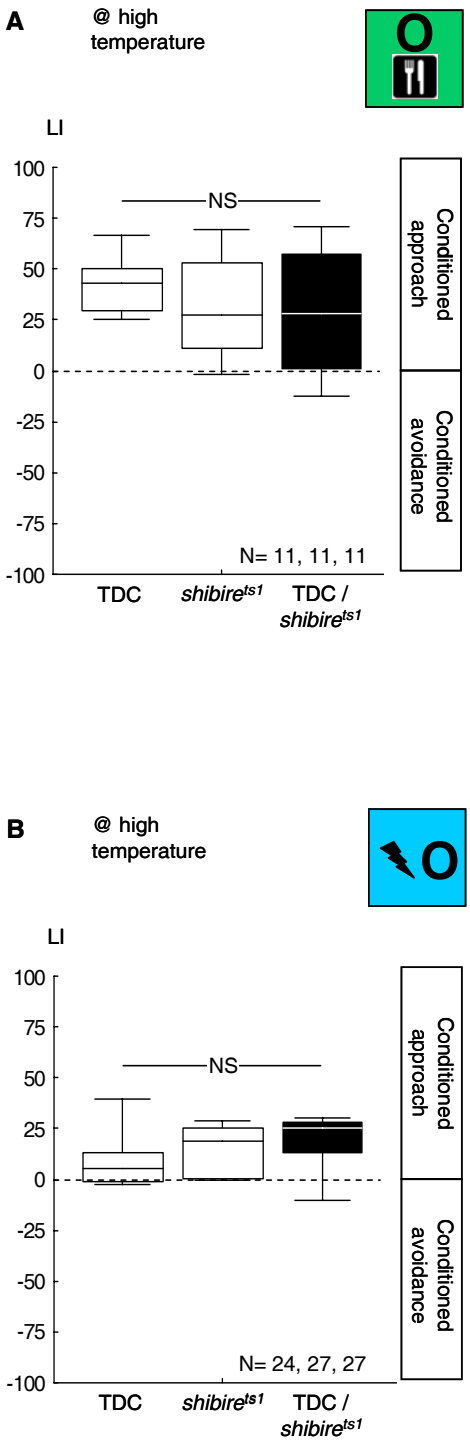

Fig. 5. Blocking the output from a subset of octopaminergic/ tyraminergic neurons impairs neither reward learning, nor relief learning.

Using *shibire*^{ts1} in combination with the TDC-Gal4 driver, we block output from a subset of octopaminergic/ tyraminergic neurons at high temperature. Neither reward learning **(A)** nor relief learning **(B)** is impaired. NS: $P > 0.05$, while comparing between genotypes. Box plots are as detailed in Fig. 3.

A role for dopamine in relief learning?

Punishment and relief might act on a common internal reinforcement signal, perhaps in opposite directions: While the onset of shock is activating the dopaminergic neurons, its offset may reduce their activity below the baseline level (e.g. in mammals, activity of the dopaminergic neurons is respectively up- and down- regulated by appetitive [Schulz 1999] and aversive [Ungless et al. 2004] stimuli). To scrutinize such a scenario, we test for a role for the dopaminergic system in relief learning.

We block the output from a subset of dopaminergic neurons, using UAS-*shibire*^{ts1} in combination with the TH-Gal4 driver (Friggi-Grelin et al. 2002; Table 1; Fig.s 2 and 4B). Conforming to the finding of Schwaerzel et al. (2003), we find punishment learning to be impaired: When trained and tested at high temperature, TH / *shibire*^{ts1} flies show less negative learning scores than the genetic controls (Fig. 6A left: Kruskal-Wallis test: @ high temperature: H= 11.44, d.f.= 2, $P< 0.05$). Residual punishment learning is however detectable in the TH / *shibire*^{ts1} flies (Fig. 6A left: One-sample sign tests: @ high temperature: $P< 0.05/ 3$ for each genotype), suggesting incomplete coverage of dopaminergic neurons by the TH-Gal4 driver and/ or incomplete block of neuronal output due to a low level of *shibire*^{ts1} expression. At low temperature, as *shibire*^{ts1} is benign, all genotypes perform indistinguishably well in punishment learning (Fig. 6A right: @ low temperature: Kruskal-Wallis test: H= 2.06, d.f.= 2, $P> 0.05$). Pooling the learning scores across genotypes, we obtain conditioned avoidance (One-sample sign test for the pooled data set: @ low temperature: $P< 0.05$).

This interference with the dopaminergic system, which clearly impairs punishment learning, leaves relief learning intact: After training and test at high temperature, we find learning scores to be indistinguishable between genotypes (Fig. 6B: Kruskal-Wallis test: H= 0.09, d.f.= 2, $P> 0.05$). Pooling the data, we find conditioned approach (One-sample sign test for the pooled dataset: $P< 0.05$). Thus, relief learning differs from punishment learning in terms of the requirement for output from this particular subset of dopaminergic neurons.

Next, we use an independent driver, DDC-Gal4 (Li et al. 2000; Table 1; Fig.s 2 and 4C), to express UAS-*shibire*^{ts1} in a subset of dopaminergic/ serotonergic neurons. Blocking output from these neurons leaves punishment learning unaffected: When trained and tested at high temperature, DDC /

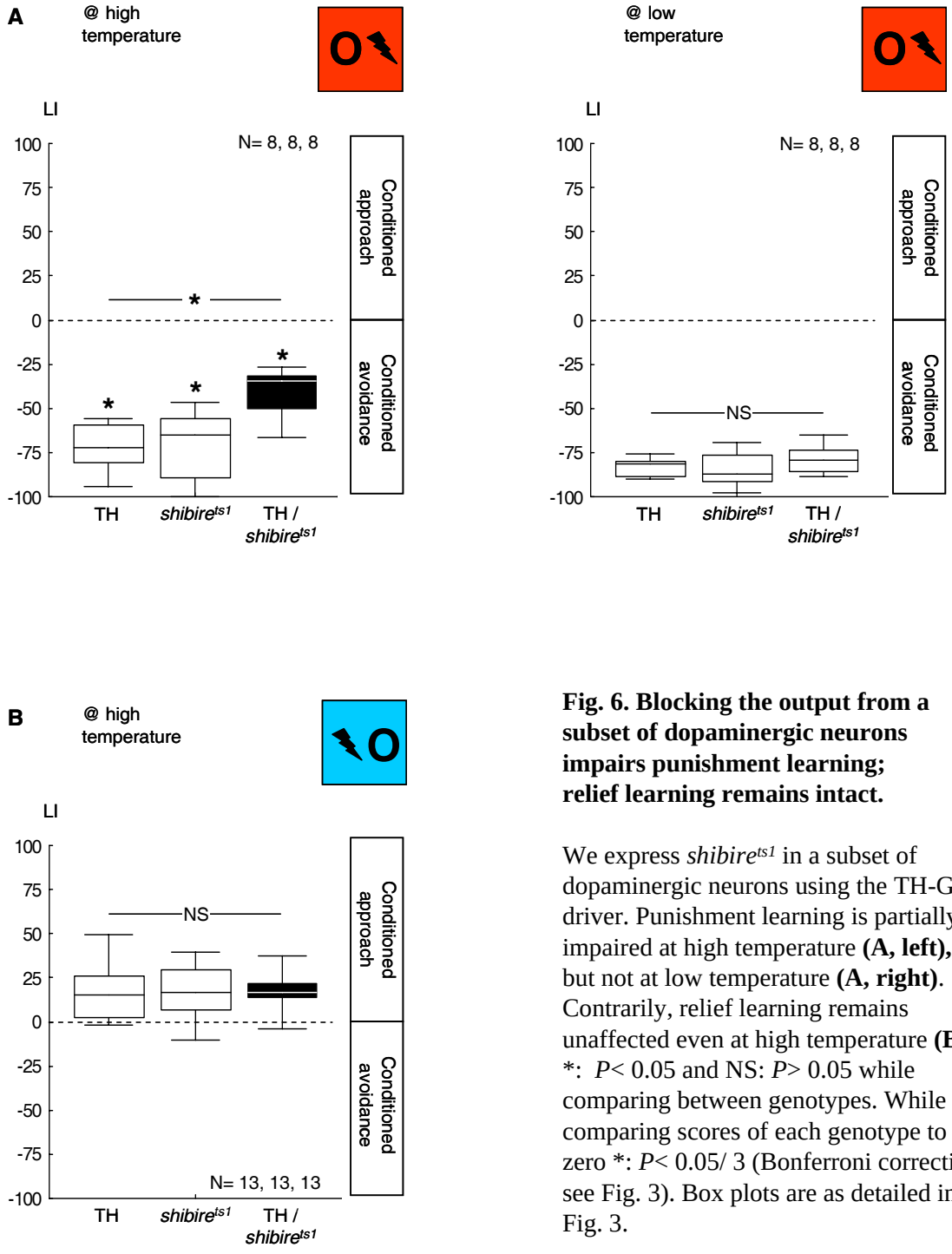

Fig. 6. Blocking the output from a subset of dopaminergic neurons impairs punishment learning; relief learning remains intact.

We express *shibire^{ts1}* in a subset of dopaminergic neurons using the TH-Gal4 driver. Punishment learning is partially impaired at high temperature **(A, left),** but not at low temperature **(A, right)**. Contrarily, relief learning remains unaffected even at high temperature **(B)**. *: $P< 0.05$ and NS: $P> 0.05$ while comparing between genotypes. While comparing scores of each genotype to zero *: $P< 0.05/ 3$ (Bonferroni correction, see Fig. 3). Box plots are as detailed in Fig. 3.

shibire^{ts1} flies show learning scores comparable to the genetic controls (Fig. 7A: Kruskal-Wallis test: H= 2.14, d.f.= 2, $P> 0.05$). When pooled across genotypes, scores indicate conditioned avoidance (One-sample sign test for the pooled data set: $P< 0.05$). The difference to the result we obtain using TH-Gal4 (Fig. 6A) argues that the respective subsets of dopaminergic neurons targeted by these two drivers do not completely overlap; alternatively, the level of *shibire*^{ts1} expression driven by DDC-Gal4 may be too low for an effective block of neuronal output.

In any case, we probe for relief learning and find it intact: After training and test at high temperature, learning scores are not different between genotypes (Fig. 7B: Kruskal-Wallis test: H= 1.24, d.f.= 2, $P> 0.05$). Pooling the data, we find conditioned approach (One-sample sign test for the pooled data set: $P< 0.05$). Thus, targeting two independent subsets of dopaminergic neurons (Fig.s 6B and 7B), we find no evidence for a role for the dopaminergic system in relief learning.

A role for serotonin in relief learning?

As we find no evidence for a role for either octopamine or dopamine in relief learning; we next consider serotonin. As detailed above, blocking output from the particular subset of serotonergic neurons, defined by the DDC-Gal4 driver, leaves relief learning unimpaired (Fig. 7B). Next, we use an additional, independent driver, SERT-Gal4 (Ritze 2007; Table 1), to target serotonergic neurons with *shibire*^{ts1}. We first test for an effect on punishment learning and find none: When trained and tested at high temperature, SERT / *shibire*^{ts1} flies perform comparable to the genetic controls (Fig. 8A: Kruskal-Wallis test: H= 0.77, d.f.= 2, $P> 0.05$). Pooling the learning scores across genotypes reveales conditioned avoidance (One-sample sign test for the pooled dataset: $P< 0.05$).

Next, we turn to relief learning, and find it unaffected, too: After training and test at high temperature, learning scores do not differ between genotypes (Fig. 8B: Kruskal-Wallis test: H= 5.01, d.f.= 2, $P> 0.05$). Pooled data reflect conditioned approach (One-sample sign test for the pooled data set: $P< 0.05$). Thus, two independent experiments (Fig.s 7 and 8) give us no evidence that the serotonergic system were involved either in punishment or in relief learning.

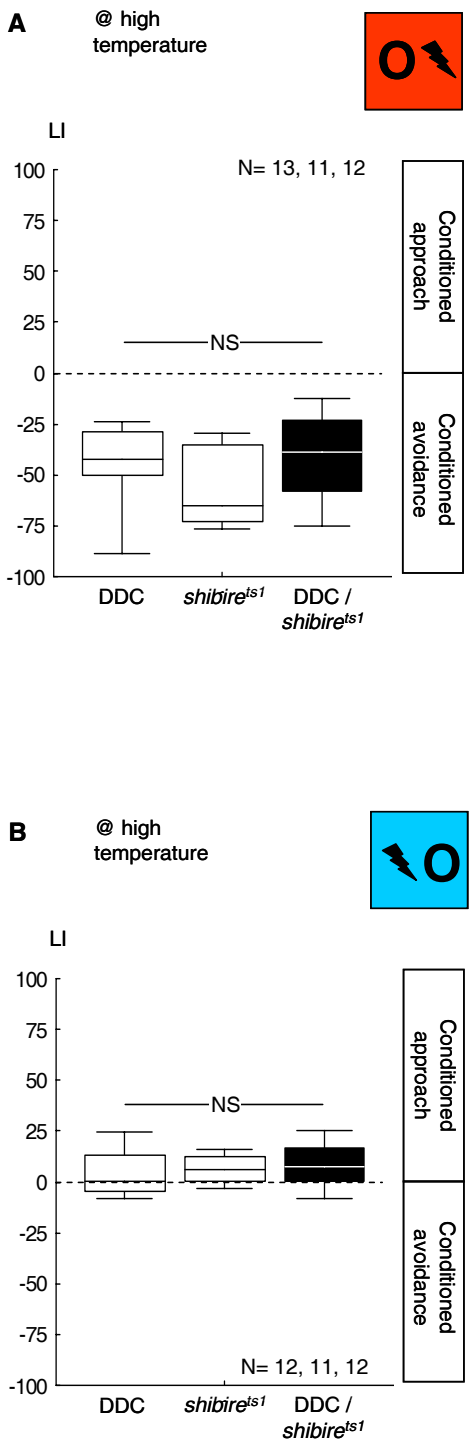

Fig. 7. Blocking the output from a subset of dopaminergic/ serotonergic neurons impairs neither punishment, nor relief learning.

We direct the expression of *shibire*^{ts1} to a subset of dopaminergic/ serotonergic neurons using the DDC-Gal4 driver. At high temperature, neither punishment learning **(A)**, nor relief learning **(B)** is affected. NS: $P > 0.05$, while comparing between genotypes. Box plots are as detailed in Fig. 3.

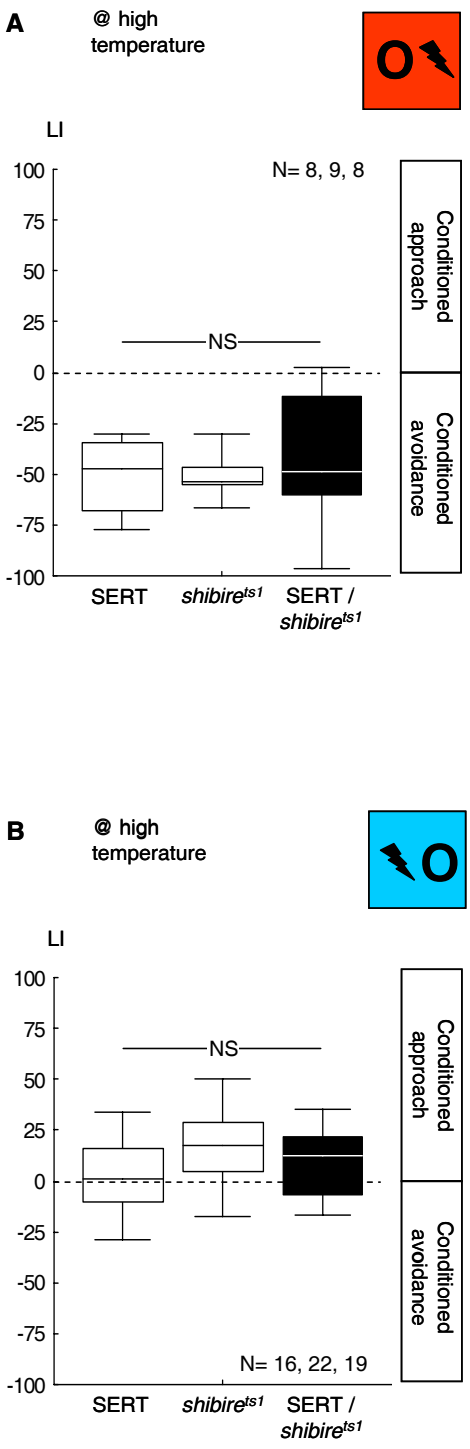

Fig. 8. Blocking the output from a subset of serotonergic neurons impairs neither punishment, nor relief learning.

Using the SERT-Gal4 driver we express *shibire*^{ts1} in a subset of serotonergic neurons. At high temperature, neither punishment learning **(A)**, nor relief learning **(B)** is impaired. NS: $P > 0.05$, while comparing between genotypes. Box plots are as detailed in Fig. 3.

Taken together, there is no evidence for a role for any of the common biogenic amines in relief learning. Importantly, relief learning is distinct from both punishment and reward learning in terms of its biogenic amine-requirements.

Discussion

Biogenic amines for signalling relief?

As the first step towards neuronal analysis of pain-relief learning, we compare it to reward learning and to punishment learning, in terms of the requirement for biogenic amines. Distinct biogenic amines, octopamine and dopamine respectively signal appetitive and aversive reinforcement in insects (fruit fly: Schwaerzel et al. 2003; Schroll et al. 2006; honey bee: Hammer 1993; Hammer & Menzel 1998; Farooqui et al. 2003; Vergoz et al. 2007; cricket: Unoki et al. 2005; Unoki et al. 2006).

First, using a mutant which lacks the key biosynthetic enzyme, we verify the requirement for octopamine in reward learning (Schwaerzel et al. 2003; Fig. 3A). For relief learning, on the other hand, octopamine turns out dispensible (Fig. 3B). As an independent approach, we block output from a subset of octopaminergic/ tyraminergic neurons and find no effect on relief learning (Fig. 5B). Next, we block the output from a subset of dopaminergic neurons and, conforming to Schwaerzel et al.'s report (2003), find punishment learning to be impaired (Fig. 6A). For relief learning on the other hand, such dopaminergic output turns out dispensible (Fig. 6B). In a follow-up experiment, we target an independent subset of dopaminergic neurons, and find relief learning to be intact (Fig. 7B). Finally, we block output from two independent subsets of serotonergic neurons and find relief learning to be unaffected also in this case (Fig.s 7B and 8B).

Thus, with respect to none of the biogenic amines octopamine, tyramine, dopamine and serotonin, do we find evidence for a role in relief learning. Thus, relief learning is distinct from both reward and punishment learning in terms of its biogenic amine requirement.

The extensive 'lack of effect' on relief learning deserves a word. Relief learning is subtle (but robust and reproducible!): Scores are typically ~ $1/2^{th}$ of reward and ~ $1/5^{th}$ of punishment learning (see Fig.s 5 and 8). Starting with such small scores, one may think it impossible to detect further decrease upon any interference. This is not true; training and test parameters (Yarali et al. In press) as

well as of the loss of the so called *white* gene (Chapter II.2. of this thesis) do affect relief learning. Also, in the present study, the lack of impairment in relief learning should not be due to low statistical power: In Fig.s 3B and 5B, we observe, not impairment, but tendencial improvement in the experimental groups. In Fig. 6B, not even a tendencial difference between genotypes comes up. Finally, in Fig.s 7B and 8B, not the experimental genotype, but one of the controls tends to perform poorer. Unfortunately, no methods of statistical power analysis to formalize these arguments are available with respect to non-parametric tests as we use (personal communication: F. Marohn, Universität Würzburg).

Neuropeptides for signalling relief?

As our results exclude the role of biogenic amines at least to some extend, how might the internal reinforcement signal underlying relief learning be carried? We consider the *neuropeptides*; similar to biogenic amines, these also function as neuromodulators, acting on G-protein coupled receptors, activating among others the cAMP signalling cascade (reviewed by Nässel [2002]). Fruit fly neuropeptides are numerous: 35 genes are confirmed to encode neuropeptide precursors, each giving rise to multiple neuropeptides; 40 genes are predicted to encode neuropeptide receptors (reviewed by Nässel & Homberg [2006]). Thus, blindly screening for a role for each neuropeptidergic system would be at best clumsy. A reasonable criterion for pre-selection is localization to neurons that provide input to the olfactory neuropils. Among the few neuropeptide families that fulfill this criterion (Nässel & Homberg 2006), the *tachykinin-related peptides* (Nässel 2002) seem particularly interesting, as they relate to mammalian Substance P, which is implicated in pain, anxiety and stress (reviewed by Rosenkratz [2007]).

The memory trace(s) underlying relief learning

Another, yet-untouched key question regarding relief learning concerns the site and the nature of the underlying neuronal plasticity. The short term memory traces underlying punishment learning seem to lay exclusively at the mushroom body Kenyon cells (reviewed by Gerber et al. [2004]): In these cells, the odour-evoked activity (Wang et al. 2004; Turner et al. 2008) on one hand, and the shock-induced

dopaminergic reinforcement signal, on the other hand coincide. This coincidence, via cAMP signalling, is thought to lead to the strengthening of the output synapses. This strengthened output then supposedly enables the odour to induce conditioned avoidance, when it is encountered again. For reward learning, two such memory traces seem to be established; one at the Kenyon cells and another independent one upstream at the olfactory projection neurons (Thum et al. 2007). The obvious question is whether relief learning might induce the same kind of memory trace(s) at the same site(s) as punishment or reward learning.

As an additional, alternative mechanism for punishment *versus* relief learning, consider the following: As detailed above, odour-shock training is thought to strengthen the Kenyon cell output to those neurons that (most probably indirectly) mediate conditioned avoidance. Shock-odour training in turn might weaken this same output, rendering the avoidance of the odour less likely than the baseline situation, thus resulting in relative approach. Such bi-directional synaptic plasticity often depends on the relative timing of pre- and post-synaptic activity (reviewed by Caporale & Dan [2008]): Typically, when the pre-synaptic action potentials happen within a temporal window of ~ 10 ms prior to the post-synaptic ones, the synapses are potentiated; a 'reversed' sequence of action potentials on the other hand depresses the synapses. Such *spike timing-dependent plasticity* can indeed be experimentally induced at the Kenyon cell output synapses of the locust (Cassenaer & Laurent 2007).

To accommodate such plasticity as a mechanism for punishment *versus* relief learning, two main assumptions are necessary (Drew & Abbott 2006): First, in addition to the odour-response in the Kenyon cells (Wang et al. 2004; Turner et al. 2008), those post-synaptic neurons that mediate the conditioned avoidance should respond to shock. If that were the case, shock-odour training would result in action potentials to occur first in the pre-synapse and then in the post-synapse, resulting in synaptic potentiation; shock-odour training on the other hand would result in a 'reversed' sequence of action potentials, resulting in synaptic depression. In addition, Drew and Abbott (2006) suggest assuming that both the Kenyon cells' odour-response and the post-synaptic neurons' shock-response persist for few seconds once the respective stimuli are actually turned off. Only if this were the case, the ms-scale synaptic plasticity rule could operate over the seconds between odour and shock presentation during training.

We need to test these assumptions, in order to put a link between spike timing-dependent plasticity and relief learning. Additionally, we can interfere with particular signalling cascades and test whether on one hand the spike timing-dependent plasticity at the Kenyon cell output synapses and on the other hand relief learning are *coherently* affected; the target cascades can be chosen based on the knowledge from vertebrates (reviewed by Caporale & Dan [2008]).

Conclusion

As this out-looking discussion also stresses, many questions await answers with respect to relief learning. The present study is the first step and should guide the next ones. Our finding that relief learning is distinct from both punishment and reward learning most likely applies to other experimental systems as well.

Acknowledgements
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References

Brembs, B., Christiansen, F., Pfluger, H. J. & Duch, C. 2007. Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. *J Neurosci*, **27,** 11122-31.

Britton, G. & Farley, J. 1999. Behavioral and neural bases of noncoincidence learning in Hermissenda. *J Neurosci*, **19,** 9126-32.

Caporale, N. & Dan, Y. 2008. Spike Timing-Dependent Plasticity: A Hebbian Learning Rule. *Annu Rev Neurosci*.

Cassenaer, S. & Laurent, G. 2007. Hebbian STDP in mushroom bodies facilitates the synchronous flow of olfactory information in locusts. *Nature*, **448,** 709-13.

Cole, S. H., Carney, G. E., McClung, C. A., Willard, S. S., Taylor, B. J. & Hirsh, J. 2005. Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. *J Biol Chem*, **280,** 14948-55.

Drew, P. J. & Abbott, L. F. 2006. Extending the effects of spike-timing-dependent plasticity to behavioral timescales. *Proc Natl Acad Sci U S A*, **103,** 8876-81.

Farooqui, T., Robinson, K., Vaessin, H. & Smith, B. H. 2003. Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J Neurosci*, **23,** 5370-80.

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J. & Birman, S. 2003. Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J Neurobiol*, **54,** 618-27.

Gerber, B., Tanimoto, H. & Heisenberg, M. 2004. An engram found? Evaluating the evidence from fruit flies. *Curr Opin Neurobiol*, **14,** 737-44.

Hammer, M. 1993. An identified neuron mediates the unconditioned stimulus in associative olfactory learning in honeybees. *Nature*, **366,** 59-63.

Hammer, M. & Menzel, R. 1998. Multiple sites of associative odor learning as revealed by local brain microinjections of octopamine in honeybees. *Learn Mem*, **5,** 146-56.

Hardie, S. L., Zhang, J. X. & Hirsh, J. 2007. Trace amines differentially regulate adult locomotor activity, cocaine sensitivity, and female fertility in Drosophila melanogaster. *Dev Neurobiol*, **67,** 1396-405.

Hearst, E. 1988. Learning and cognition. In: *Stevens' handbook of experimental psychology, 2nd Edition, Vol 2* (Ed. by R. C. Atkinson, R.J. Herrnstein, G. Lindzey & R. D. Luce), pp 3-109. New York: Wiley.

Hellstern, F., Malaka, R. & Hammer, M. 1998. Backward inhibitory learning in honeybees: a behavioral analysis of reinforcement processing. *Learn Mem*, **4,** 429-44.

Kitamoto, T. 2001. Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J Neurobiol*, **47,** 81-92.

Klagges, B. R., Heimbeck, G., Godenschwege, T. A., Hofbauer, A., Pflugfelder, G. O., Reifegerste, R., Reisch, D., Schaupp, M., Buchner, S. & Buchner, E. 1996. Invertebrate synapsins: a single gene codes for several isoforms in Drosophila. *J Neurosci*, **16,** 3154-65.

Lee, T. & Luo, L. 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*, **22,** 451-61.

Li, H., Chaney, S., Roberts, I. J., Forte, M. & Hirsh, J. 2000. Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in Drosophila melanogaster. *Curr Biol*, **10,** 211-4.

Maier, S. F., Rapaport, P. & Wheatley, K. L. 1976. Conditioned inhibition and the UCS-CS interval. *Anim Learn Behav*, **4**, 217-20.

Monastirioti, M. 1999. Biogenic amine systems in the fruit fly Drosophila melanogaster. *Microsc Res Tech*, **45,** 106-21.

Monastirioti, M., Linn, C. E., Jr. & White, K. 1996. Characterization of Drosophila tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *J Neurosci*, **16,** 3900-11.

Moskovitch, A. & LoLordo, V. M. 1968. Role of safety in the Pavlovian backward fear conditioning procedure. *J Comp Physiol Psychol*, **66**, 673-78.

Nassel, D. R. 2002. Neuropeptides in the nervous system of Drosophila and other insects: multiple roles as neuromodulators and neurohormones. *Prog Neurobiol*, **68,** 1-84.

Nassel, D. R. & Homberg, U. 2006. Neuropeptides in interneurons of the insect brain. *Cell Tissue Res*, **326,** 1-24.

Plotkin, H. C. & Oakley, D. A. 1975. Backward conditioning in the rabbit (Oryctolagus cuniculus). *J Comp Physiol Psychol*, **88**, 586-90.

Riemensperger, T., Voller, T., Stock, P., Buchner, E. & Fiala, A. 2005. Punishment prediction by dopaminergic neurons in Drosophila. *Curr Biol*, **15,** 1953-60.

Ritze, Y. 2007. Die Rolle des Neurotransmitters Serotonin bei der Entwicklung von Ethanolsensitivität und Toleranz in Drosophila melanogaster. Dissertation. Universität Würzburg.

Rosenkranz, M. A. 2007. Substance P at the nexus of mind and body in chronic inflammation and affective disorders. *Psychol Bull*, **133,** 1007-37.

Saraswati, S., Fox, L. E., Soll, D. R. & Wu, C. F. 2004. Tyramine and octopamine have opposite effects on the locomotion of Drosophila larvae. *J Neurobiol*, **58,** 425-41.

Schipanski A. 2007. Reinforcement processing in fruit fly larvae. Diploma thesis. Universität Würzburg.

Scholz, H. 2005. Influence of the biogenic amine tyramine on ethanol-induced behaviors in Drosophila. *J Neurobiol*, **63,** 199-214.

Schroll, C., Riemensperger, T., Bucher, D., Ehmer, J., Voller, T., Erbguth, K., Gerber, B., Hendel, T., Nagel, G., Buchner, E. & Fiala, A. 2006. Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in Drosophila larvae. *Curr Biol*, **16,** 1741-7.

Schultz, W. 1999. The Reward Signal of Midbrain Dopamine Neurons. *News Physiol Sci*, **14,** 249-255.

Schwaerzel, M., Monastirioti, M., Scholz, H., Friggi-Grelin, F., Birman, S. & Heisenberg, M. 2003. Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in Drosophila. *J Neurosci*, **23,** 10495-502.

Sitaraman, D., Zars, M., Laferriere, H., Chen, Y. C., Sable-Smith, A., Kitamoto, T., Rottinghaus, G. E. & Zars, T. 2008. Serotonin is necessary for place memory in Drosophila. *Proc Natl Acad Sci U S A*, **105,** 5579-84.

Solomon, R. L., & Corbit, J. D. 1974 An opponent-process theory of acquired motivation. I. Temporal dynamics of affect. *Psychol Rev,* **81(2)***,* 119-45.

Tanimoto, H., Heisenberg, M. & Gerber, B. 2004. Experimental psychology: event timing turns punishment to reward. *Nature*, **430,** 983.

Tempel, B. L., Bonini, N., Dawson, D. R. & Quinn, W. G. 1983. Reward learning in normal and mutant Drosophila. *Proc Natl Acad Sci U S A*, **80,** 1482-6.

Thum, A. S., Jenett, A., Ito, K., Heisenberg, M. & Tanimoto, H. 2007. Multiple memory traces for olfactory reward learning in Drosophila. *J Neurosci*, **27,** 11132-8.

Tully, T. & Quinn, W. G. 1985. Classical conditioning and retention in normal and mutant Drosophila melanogaster. *J Comp Physiol [A]*, **157,** 263-77.

Turner, G. C., Bazhenov, M. & Laurent, G. 2008. Olfactory representations by Drosophila mushroom body neurons. *J Neurophysiol*, **99,** 734-46.
Ungless, M. A., Magill, P. J. & Bolam, J. P. 2004. Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. *Science,* **303,** 2040-2.

Unoki, S., Matsumoto, Y. & Mizunami, M. 2005. Participation of octopaminergic reward system and dopaminergic punishment system in insect olfactory learning revealed by pharmacological study. *Eur J Neurosci*, **22,** 1409-16.

Unoki, S., Matsumoto, Y. & Mizunami, M. 2006. Roles of octopaminergic and dopaminergic neurons in mediating reward and punishment signals in insect visual learning. *Eur J Neurosci*, **24,** 2031-8.

Vergoz, V., Roussel, E., Sandoz, J. C. & Giurfa, M. 2007. Aversive learning in honeybees revealed by the olfactory conditioning of the sting extension reflex. *PLoS ONE*, **2,** e288.

Wagner, A. R. 1981. SOP: A Model of Automatic Memory Processing in Animal Behavior. In: *Information Processing in Animals: Memory Mechanisms* (Ed. by N. E. Spear & R. R. Miller), pp. 5−47. Hillsdale, New Jersey: Erlbaum.

Wang, Y., Guo, H. F., Pologruto, T. A., Hannan, F., Hakker, I., Svoboda, K. & Zhong, Y. 2004. Stereotyped odor-evoked activity in the mushroom body of Drosophila revealed by green fluorescent protein-based Ca2+ imaging. *J Neurosci*, **24,** 6507-14.

Yarali, A., Krischke, M., Divya, S., Zars, T. & Gerber, B. In Preparation. Effects of the *white* mutation on olfactory associative learning.

Yarali, A., Niewalda, T., Chen, Y., Tanimoto, H., Duerrnagel, S. & Gerber, B. In Press. 'Pain-relief' learning in fruit flies. *Anim Beh*

Zhang, K., Guo, J. Z., Peng, Y., Xi, W. & Guo, A. 2007. Dopamine-mushroom body circuit regulates saliency-based decision-making in Drosophila. *Science*, **316,** 1901-4.

Summary

Past experience contributes to behavioural organization mainly via learning: Animals learn otherwise ordinary cues as predictors for biologically significant events. This thesis studies such predictive, associative learning, using the fruit fly *Drosophila melanogaster*. I ask two main questions, which complement each other: One deals with the processing of those cues that are to be learned as predictors for an important event; the other one deals with the processing of the important event itself, which is to be predicted.

Do fruit flies learn about combinations of olfactory and visual cues?

I probe larval as well as adult fruit flies for the learning about combinations of olfactory and visual cues, using a so called 'biconditional discrimination' task: During training, one odour is paired with reinforcement only in light, but not in darkness; the other odour in turn is reinforced only in darkness, but not in light. Thus, neither the odours nor the visual conditions alone predict reinforcement, only combinations of both do. I find no evidence that either larval or adult fruit flies were to solve such task, speaking against a cross-talk between olfactory and visual modalities. Previous studies however suggest such cross-talk. To reconcile these results, I suggest classifying different kinds of interaction between sensory modalities, according to their site along the sensory-motor continuum: I consider an interaction 'truly' cross-modal, if it is between the specific features of the stimuli. I consider an interaction 'amodal' if it instead engages the behavioural tendencies or 'values' elicited by each stimulus. Such reasoning brings me to conclude that different behavioural tasks require different kinds of interaction between sensory modalities; whether a given kind of interaction will be found depends on the neuronal infrastructure, which is a function of the species and the developmental stage.

Predictive learning of pain-relief in fruit flies

Fruit flies build two opposing kinds of memory, based on an experience with electric shock: Those odours that precede shock during training are learned as predictors for *punishment* and are subsequently avoided; those odours that follow shock during training on the other hand are learned as signals for *relief* and are subsequently approached. I focus on such *relief learning.*

I start with a detailed parametric analysis of relief learning, testing for reproducibility as well as effects of gender, repetition of training, odour identity, odour concentration and shock intensity. I

also characterize how relief memories, once formed, decay. In addition, concerning the psychological mechanisms of relief learning, first, I show that relief learning establishes genuinely associative conditioned approach behaviour and second, I report that it is most likely not mediated by context associations. These results enable the following neurobiological analysis of relief learning; further, they will form in the future the basis for a mathematical model; finally, they will guide the researchers aiming at uncovering relief learning in other experimental systems.

Next, I embark upon neurogenetic analysis of relief learning. First, I report that fruit flies mutant for the so called *white* gene build overall more 'negative' memories about an experience with electric shock. That is, in the *white* mutants, learning about the painful onset of shock is enhanced, whereas learning about the relieving offset of shock is diminished. As they are coherently affected, these two kinds of learning should be in a balance. The molecular mechanism of the effect of *white* on this balance remains unresolved.

Finally, as a first step towards a neuronal circuit analysis of relief learning, I compare it to reward learning and punishment learning. I find that relief learning is distinct from both in terms of the requirement for biogenic amine signaling: Reward and punishment are respectively signalled by octopamine and dopamine, for relief learning, either of these seem dispensible. Further, I find no evidence for roles for two other biogenic amines, tyramine and serotonin in relief learning. Based on these findings I give directions for further research.

Zusammenfassung*

Vergangene Ereignisse beeinflussen die Organisation des Verhaltens hauptsächlich durch das Lernen: Tiere lernen natürlich vorkommende neutrale Reize als Signal für biologisch relevante Ereignisse zu nutzen. Diese Dissertation befasst sich mit derartigen assoziativen Lernvorgängen bei der Taufliege *Drosophila melanogaster*. Ich stelle zwei, sich ergänzende, grundlegende Fragen: Die eine Frage beschäftigt sich mit der Verarbeitung von Reizen, die als Signal für ein wichtiges Ereignis erlernt werden. Die andere Frage behandelt die Verarbeitung des Ereignisses selbst.

Lernen Taufliegen etwas über Kombinationen von olfaktorischen und visuellen Reizen?

Sowohl bei larvalen, als auch bei adulten Taufliegen wird das Lernen von Kombinationen aus olfaktorischen und visuellen Stimuli untersucht. Ich verwende einen sogenannten „bikonditionalen Diskriminierungs-Versuchsaufbau“: Während des Trainings wird ein Duft nur im Licht und nicht im Dunkeln mit Reinforcement kombiniert, während ein anderer Duft nur im Dunkeln und nicht im Licht mit Reinforcement kombiniert wird. Somit signalisieren weder die Düfte, noch die visuellen Bedingungen allein das Reinforcement, sondern nur eine Kombination aus Beiden. Ich finde keine Beweise dafür, dass larvale oder adulte Taufliegen eine solche Aufgabe lösen können. Dies spricht gegen eine Interaktion zwischen olfaktorischen und visuellen Modalitäten. Allerdings weisen frühere Studien auf derartige Interaktionen hin. Um meine Ergebnisse mit den bekannten Studien in Einklang zu bringen, ordne ich die unterschiedlichen Interaktionen zwischen den sensorischen Modalitäten nach ihrer Lage entlang des sensorisch-motorischen Kontinuums: Ich bezeichnen eine Interaktion für „echt“ cross-modal, wenn sie zwischen den spezifischen Eigenschaften der beiden Reize stattfindet. Ich halte eine Interaktion für „amodal“, wenn sie zwischen den von den Reizen induzierten Verhaltenstendenzen und „Werten“ stattfindet. Aufgrund dieser Argumentation komme ich zu der Schlussfolgerung, dass unterschiedliche Verhaltensaufgaben unterschiedliche Interaktionen zwischen den sensorischen Modalitäten erfordern. Ob eine Art von Interaktion gefunden wird oder nicht hängt von der neuronalen Vernetzung ab, welche charakteristisch für Art und Entwicklungsstadium ist.

Assoziatives Lernen von Schmerz-Erleichterung bei Taufliegen

Taufliegen entwickeln zwei unterschiedliche Arten von Gedächtnissen basierend auf Erfahrung mit Elektro-Schock: Düfte, die während des Trainings dem Schock vorausgehen, werden als Bestrafungssignale gelernt und deshalb vermieden. Düfte, die während des Trainings auf den Schock

folgen, werden als Erleichterungssignale gelernt und deshalb bevorzugt. Ich beschäftige mich mit der zweiten Art dieses assoziativen Lernens, das ich als „Erleichterungslernen“ bezeichne.

Ich beginne mit einer detaillierten parametrischen Analyse des Erleichterungslernens. Die Reproduzierbarkeit, sowie die Einflüsse des Geschlechts, der Anzahl an Trainingswiederholungen, der Duftintensität, der Duftkonzentration und der Schockintensität werden geprüft. Ich teste, wie das Erleichterungsgedächtnis, nachdem es gebildet wurde, wieder gelöscht wird. Des Weiteren gehe ich zwei wichtigen Fragen zu den psychologischen Mechanismen des Erleichterungslernen nach: Zum einen zeige ich, dass das Erleichterungslernen echtes assoziativ konditioniertes Annäherungsverhalten etabliert. Zum anderen zeige ich, dass vorausgegangenes Kontext-Schock Training das folgende Erleichterungslernen nicht beeinflusst. Das Erleichterungslernen wird also nicht durch Kontextassoziation vermittelt. Diese Ergebnisse erlauben die folgende neurobiologische Analyse des Erleichterungslernens. Außerdem werden sie in Zukunft als Grundlage für ein mathematisches Modell des Erleichterungslernens dienen. Schließlich werden die Forscher/innen, die das Erleichterungslernen in anderen experimentellen Systemen untersuchen, von diesen parametrischen Erkenntnissen profitieren.

In einer neurobiologischen Analyse des Erleichterungslernens zeige ich, dass der Verlust der Funktion des sogenannten *white* Gens die beiden unterschiedlichen Arten von Schock-Induziertem Lernen zusammenhängend beeinflusst: Das Bestrafungslernen wird verstärkt und das Erleichterungslernen wird abgeschwächt. Auf Grund dieses Ergebnisses schlagen ich vor, dass sich diese zwei Arten von Lernen in einem Gleichgewicht befinden sollen, welches vom *white* Gen beeinflusst wird. Die zugrunde liegenden molekularen Mechanismen eines solchen Gleichgewichts sind noch nicht bekannt.

Schließlich vergleiche ich das Erleichterungslernen mit dem Belohnungslernen und dem Bestrafungslernen. Ich zeige, dass das Erleichterungslernen anders ist als beide: Bestrafung und Belohnung werden entsprechend von Dopamin und Octopamin vermittelt. Für das Erleichterungslernen sind beide diese biogenen Aminen unnötig. Ebenso finde ich beim Erleichterungslernen keinen Beleg für die Rolle von zwei weiteren Aminen: Tyramin und Serotonin. Aufgrund dieser Ergebnisse schlage ich vor weitere Forschungsrichtungen.

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Curriculum vitae

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2004	MSc in Neural and Behavioral Sciences, University of Tuebingen, Germany
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Research

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Predictive learning of pain-relief in fruit flies.

Cross-modal behaviour in larval and adult fruit flies.

Learning of odour concentration in adult fruit flies.

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March - May 2004.

Max-Planck-Institute for Developmental Biology, Tuebingen, Germany. Supervisor: Prof. M. Kiebler

Investigation of the function of Barentz in zebrafish development.

Rotations

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Live time-lapse video imaging of axon growth in fruit fly embryos by confocal microscopy.

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Isolation and characterization of Staufen-containing ribonucleoprotein particles from rat brain.

BSc

September 2001 - May 2002.

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Differential expression of pro- and anti-apoptotic genes during rat liver development.

Internships

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Medical Research Center, Cambridge, UK. Supervisor: Dr. B. Davletov

Regulation of SNARE-complex formation in presynaptic nerve terminals.

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Cedars-Sinai Medical Center, LA, USA. Supervisors: Dr. M. Arditi and Dr. O. Equils

Role of Toll-like receptors in HIV-LTR transactivation.

Workshops

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Methods in Computational Neuroscience,

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List of publications

Yarali, A., Niewalda, T., Chen, Y., Tanimoto, H., Duerrnagel, S. & Gerber, B. In Press. 'Pain-relief' learning in fruit flies. *Anim Beh*

Schipanski, A., Yarali, A., Niewalda, T. & Gerber, B. In press. Behavioural analyses of sugar processing in choice, feeding, and learning in larval Drosophila. *Chem Senses*

Yarali, A., Mayerle, M., Nawroth, C. & Gerber, B. 2008. No evidence for visual context-dependency of olfactory learning in *Drosophila*. *Naturwissenschaften,* **DOI** 10.1007/s00114-008-0380-1.

Yarali, A., Hendel, T. & Gerber, B. 2006. Olfactory learning and behaviour are 'insulated' against visual processing in larval Drosophila. *J Comp Physiol (A),* **192,** 1133-45.

Equils, O., Schito, M. L., Karahashi, H., Madak, Z., Yarali, A., Michelsen, K. S., Sher, A. & Arditi, M. 2003. Toll-like receptor2 (TLR2) and TLR9 signaling results in HIV-long terminal repeat trans-activation and HIV replication in HIV-1 transgenic mouse spleen cells: implications of simultaneous activation of TLRs on HIV replication. *J Immunol,* **170,** 5159-64.

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