Neurogenetic analyses of pain-relief learning in the fruit fly

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Thomas Niewalda

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Gutachter : PD Dr. Bertram Gerber

Gutachter: Prof. Wolfgang Rössler

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gemäß §4 der Promotionsordnung für die Fakultät für Biologie der

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Die vorliegende Dissertation enthält drei Publikationen und zwei Manuskripte, sowie zusätzlich eine allgemeine Einleitung und Diskussion. Der Beitrag des Promovierenden wird auf den folgenden Seiten genau kenntlich gemacht.

Hiermit erkläre ich, daß ich die vorliegende Dissertation selbständig angefertigt habe und keine anderen Hilfsmittel als die angegebenen angewandt habe. Alle aus der Literatur entnommenen Stellen und Abbildungen sind als solche kenntlich gemacht.

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 zu erwerben versucht.

Würzburg,

Thomas Niewalda

Yarali A, Niewalda T, Chen Y, Tanimoto H, Dürrnagel S, Gerber B (2008) 'Pain relief' learning in fruit flies. Animal Behavior

I performed part of experiment 3, took part in designing experiment 4 and 8, performed those experiments and analysed the data.

T. Niewalda . .....

Signatures of coauthors agreeing to the statement of my contribution:

A. Yarali .....

Y. Chen ....

H. Tanimoto .....

S. Dürrnagel .....

B. Gerber ....

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B. Gerber .....

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I together with B. Gerber formulated the project, contributed significantly to the design of all experiments, performed and analyzed all behaviour experiments, part of the immunohistochemistry and western blotting, and contributed to the writing of the manuscript.

T. Niewalda .....

Signatures of coauthors agreeing to the statement of my contribution:

B. Michels .. ..

A. Yarali .....

B. Gerber .. ..

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T. Niewalda .....

Signatures of coauthors agreeing to the statement of my contribution:

B. Michels .....

A. Yarali ....

B. Gerber .....

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T. Niewalda ....

Signatures of coauthors agreeing to the statement of my contribution:

T. Völler .....

J. Ehmer .....

A. Fiala .....

B. Gerber .. ..

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J. Ehmer ……

A. Fiala ……

B. Gerber ……

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T. Niewalda .....

Signatures of coauthors agreeing to the statement of my contribution:

T. Völler .....

J. Ehmer .. ..

A. Fiala .....

B. Gerber .....

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T. Niewalda .....

Signatures of coauthors agreeing to the statement of my contribution:

N. Singhal .....

A. Fiala ... .

T. Saumweber .....

S. Wegener .....

B. Gerber .....

Niewalda T, Singhal N, Fiala A, Saumweber T, Wegener S, Gerber B (2008) Salt processing in larval Drosophila: choice, feeding, and learning shift from appetitive to aversive in a concentration-dependent way. Chem Senses

Together with B. Gerber I identified the research topic; I performed the majority of the experiments, analyzed all data, and participated significantly in the writing of the manuskript.

T. Niewalda .....

Signatures of coauthors agreeing to the statement of my contribution:

N. Singhal ... .

A. Fiala .....

T. Saumweber . .....

S. Wegener .....

B. Gerber ....

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Together with B. Gerber I identified the overall research topic; I performed the majority of the experiments, analyzed most data, and participated significantly in the writing of the manuscript. N. Singhal and A. Fiala established, performed and analyzed the experiments on quantification of food uptake in larvae. T. Saumweber and S. Wegener performed behavioural experiments.

T. Niewalda .....

Signatures of coauthors agreeing to the statement of my contribution:

N. Singhal .....

A. Fiala ....

T. Saumweber .....

S. Wegener .....

B. Gerber .....

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T. Niewalda .....

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A. Fiala .....

T. Saumweber .....

S. Wegener ....

B. Gerber .....

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I performed part of the feeding experiments.

T. Niewalda . .....

Signatures of coauthors agreeing to the statement of my contribution:

A. Schipanski .....

A. Yarali .....

B. Gerber ....

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B. Gerber .....

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T. Niewalda .....

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A. Schipanski .....

A. Yarali .....

B. Gerber .....

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General Introduction and Discussion

This Thesis uses the fruitfly *Drosophila melanogaster* for research into learning and memory. Learning and memory is a universal ability across the animal kingdom and some of the principles that govern such behaviour are common to all animals. Examples of these principles are the observations that spaced training leads to a better and longer-lasting memory than massed training, and that only these longer-lasting memories require protein synthesis. By studying a genetically tractable model organism such as *Drosophila* one hopes to gain a mechanistic understanding of such principles, which hopefully are applicable to other animals, and man as well.

This Thesis comprises three publications and two manuscripts, accordingly organized into five chapters. Chapter I contains parametric studies for characterizing relief-learning, which are then used in chapter II to conduct experiments with a Synapsin null mutant strain in the punishment-and relief-learning paradigm. Chapter III investigates the relationship between perception and physiology in terms of odour similarity. Chapters IV and V are about the behavioural analysis of salt and sugar processing in the *Drosophila* larva using choice, feeding and learning assays.

In nature, the life of *Drosophila* revolves around rotten fruit. It uses its sense of smell to find food sources, mates, and egg laying substrates. It has evolved to discriminate flavours accurately and is able to use them to make predictions about future events by attributing meaning to smells through associative learning. In one type of such associative learning, an originally neutral cue (conditioned stimulus, CS), such as an odorant, is associated with a meaningful event, such as electric shock (unconditioned stimulus, US). Association comes about if the CS occurs together with the US. After association, the conditioned stimulus has acquired meaning stemming from its pairing with the unconditioned stimulus and organizes behaviour characteristic for the expectation of the US (Pavlov 1927).

The fruitfly is able to learn a variety of tasks under tightly controlled laboratory conditions. Examples from adult *Drosophila* are odour-electroshock conditioning (Quinn et al. 1974, Tully and Quinn 1985, Schwaerzel et al. 2003), and odour-sugar conditioning (Tempel et al. 1983, Schwaerzel et al. 2003). Operant conditioning has been demonstrated in the flight simulator (Wolf and Heisenberg 1991), and in the so-called heatbox paradigm (Wustmann et al. 1996, Putz and Heisenberg 2002). Larval *Drosophila* can associate odours with sugar reward (Michels et al. 2005, Schipanski et al. 2008), with electric shock punishment (Khurana et al. 2009, Pauls et al. 2010), and with aversive feeding substrates

(Gerber and Hendel 2006, Niewalda et al. 2008). Visual learning has also been reported in the larva (Gerber et al. 2004).

Drosophila, especially at the larval stage, has a relatively simple nervous system in terms of cell number. Yet larvae and adults show notable similarity in the general organization of the olfactory nervous system (Python and Stocker 2002). The larval nervous system is accessible with transgenic techniques just as is the case for adults, facilitating research on molecular, cellular and behavioural levels. One method particularly useful is the Gal4-UAS-system (Brand and Perrimon 1993) for spatially and/or temporally restricted expression of transgenes. This system allows for the constituent or induced expression of transgenes in defined groups of cells, such that these cells can be manipulated dependent on the nature of the transgene; for example, protein levels can be decreased via transgenic expression of RNAi constructs, or protein expression in a given mutant can be restored; cells can be induced to undergo apoptosis, or synaptic output can be reversibly interrupted by the temperature-dependent dominant-negative allele of the dynamin gene (shibire^{ts}: Kitamoto 2001, Chen et al. 1991).

Regarding learning, genetic screens have isolated learning mutants and many of the mutated genes have been identified. Those mutants are used to genetically investigate behaviour (Benzer 1967, Dudai 1988). Notably, many mutants of learning and memory have defective genes that are part of the cAMP pathway. A mutation in an allele of the X-linked gene *dunce*, the first learning mutant discovered in *Drosophila*, encodes a cAMP-degrading enzyme (Byers et al. 1981). In turn, the cAMP-producing enzyme is coded for by the *Rutabaga* gene, an allele of which also has been identified as causing defects in associative function (Dudai et al. 1983, Livingstone et al 1984). The gene *amn* encodes a neuropeptide that stimulates cAMP synthesis (Feany and Quinn 1995) and that is strongly expressed in the dorsal paired medial (DPM) neurons (Keene et al. 2004), and likely responsible for the consolidation of immediate memory into more permanent memory. That is, a mutant allele of the *amnesiac* gene leads to more rapid memory decline (Quinn et al. 1979, Waddell et al. 2000). Output of DPM neurons is required during the consolidation period, but not during acquisition or recall (Keene et al. 2004). A mutation of DCO, a catalytic subunit gene of protein kinase A has mild effects on learning but no effect on memory (Skoulakis et al. 1993). Also, disrupting cAMP signalling with constitutively active G_{α} subunit abolishes olfactory learning (Connolly et al. 1996). Significantly, the genes mutated in the described mutants encode proteins that participate in the cAMP signalling cascade that is important for the experience-dependent regulation of transmitter release upon an incoming action potential (Lee

et al. 2008). Together, all these data indicate the importance of this second messenger pathway for learning in *Drosophila*. Also in other animals, the importance of the cAMP-pathway has been demonstrated, e.g. in Aplysia (Silva and Murphy 1999) and mouse (Abel and Nguyen 2008). These findings about long-term memory storage are yet another example of the conserved nature of mechanisms of learning.

The question where in the brain the underlying molecular and cellular processes are occurring prompted memory localization studies. The search for memory trace localization is extensively done in many organisms. Memory trace localization attempts in humans, for example, may be undertaken by making use of lesion patients to study the impact of known brain damages on behaviour. For example, the well-known amnesia patient HM who suffered a lesion in the hippocampal area lacks short-term declarative memory (Scoville and Milner, 1957). A disadvantage of this method is that such lesions are mostly unique and cannot be reproduced. But even in cases when systematic lesion studies are possible, as in rodents, the information gained from lesion patients is coarse, as no statement about the precise location of memory can be made: the memory for the studied task could be located at the lesion area or either up- or downstream of it. One step forward is brain imaging to monitor the activity of specific brain areas before versus after a learning task. Changes in the activity of the monitored area imply that changes in synaptic strength have occurred upstream of the point of measurement. Another step forward is the UAS-shibire technique that can be used for blocking the neuronal output reversibly, either during training or during retrieval. This tool allows to differentiate whether the memory trace is located upstream or downstream from the point of intervention. If the neuronal output is blocked during training but not during test and the animals show memory during test, the memory trace is located upstream of the blocked synapse. If the animals do not show memory in test, the location of the memory trace for that task is downstream.

In *Drosophila* there exists an independent approach to identify groups of neurons that are involved in a learning and memory task: in a mutant strain that has reduced learning ability, the defective protein can be restored in specific neurons and the sufficiency of that protein in that learning task confirmed (Thum et al 2007; Zars et al. 2000). Knock-down with RNAi in the same neurons can be done to check whether that protein is necessary there. Clearly, this method only relates to those aspects of the memory trace that indeed require the respective protein.

Relief learning in fruit flies

My main project deals with the relief-learning process. That is, fruit flies learn from experience. This is necessary to make predictions regarding upcoming events and improve survival. Predictions are made by detecting temporal patterns in the environment, which have the potential to significantly influence the well-being of the organism and often arise repeatedly to qualify as a base for predictions to be made. In nature, flies that have been exposed to a food source flavoured with a certain odourant, will show attractive behaviour towards that odorant if encountering it again later. The opposite also applies: after having experienced a painful stimulus in presence of a certain odorant, flies show aversive behaviour if that odourant is encountered again. To study associations between odorants and electric shock, the Tully-Quinn paradigm has been developed (Tully and Quinn 1985, Schwaerzel et al. 2003). Using this paradigm, it turned out that flies do learn associating these stimuli, but what they learn is critically dependent on timing of the stimuli: if shock is presented shortly after the odour, flies avoid that odour later in test. This is reasonable, because due to the odour-shock training, the odour predicts punishment. This punishment learning is powerful and conditioned avoidance is already seen after a single training trial. If the sequence of odour and shock is reversed such that the shock is presented prior to the odourant, flies approach the odour in test (Tanimoto et al. 2004). Such shock-odour training turns the odour into a predictor of a safety period (Sutton and Barto 1990; Chang et al. 2003) or of relief from electric shock (Solomon and Corbit 1974; Wagner 1981). This relief learning is relatively weak, i.e. it reaches only approximately one sixth of punishment learning levels, requires a higher number of training trials than punishment learning, and is optimal for intermediate shock intensities (Yarali et al. 2008). Due to this specificity of the required parameters, relief learning has often been overlooked (Tully and Quinn 1985). As we show, relief learning is critically dependent on timing and is truly associative in nature, and leads to an increase in attractiveness of the trained odourant. Also, context-shock pairings prior to shock-odour training did not enhance successive relief-learning, arguing that relief-learning does not appear to be mediated by a contextually-mediated prediction error (Sutton and Barto 1990, Chang et al. 2003). Relief-learning depends on the quality and intensity of the used odours; specifically, for one odour pair, relief learning is best at an intermediate odour concentration, whereas two other odour pairs support relief learning at all concentrations tested. For two further odour pairs, relief-learning could not be observed. Lastly, the memory after relief learning is practically stable over a two hour retention period, a time window during which punishment memory decays relatively fast.

A recent study by Andreatta et al. (2010) explores punishment-learning and relief-learning using human subjects, again showing the universal nature of learning principles in different species. The authors used simple visual stimuli, such as geometrical shapes, as conditioned stimuli. These CS were paired with mild electric shocks. After the subjects had undergone repeated CS-US pairings, i.e. punishment learning, the startle response in presence of the CS was potentiated, indicating that the CS has acquired negative valence. After US-CS pairings, i.e. relief-learning, in contrast, the startle response was attenuated, meaning that the CS has acquired positive valence. Interestingly, this implicit (behavioural) rating does not conform to the explicit ratings (verbal statements) about the valence of the CS: after both punishment- and relief-learning, the CS is explicitly rated as negative. These findings may ultimately help to understand mismatches between what people say and what they do, maybe even under psychiatric conditions.

Punishment learning and relief learning in Synapsin mutant and rescue

Given this universal behaviour feature, we were wondering if a similar universal molecular feature is underlying these types of learning and focused on a Synapsin mutant. Synapsins are evolutionarily conserved phosphoproteins associated with synaptic vesicles and required for regulation of vesicle release. In the mammalian genome, multiple isoforms are expressed arising from three different genes (Südhof et al.1989, Porton et al. 1999). In flies, only a single Synapsin gene exists, which is expressed in the whole nervous system (Klagges et al. 1996). Synapsins are thought to control vesicle flow from the reserve pool to the releaseable pool by tethering the vesicles to the cytoskeleton and releasing them for potential release in a phosphorylation-dependent manner (Hilfiker et al. 1999, Gitler et al. 2008).

Due to this proposed function, Synapsin is likely involved in learning processes, and the Synapsin mutant syn^{97}, a 1.4 kb deletion mutant lacking the Synapsin protein, is a likely candidate for a phenotype in learning and memory. Syn^{97} has indeed already been shown to be impaired in odour-shock learning in adult fruit flies (Godenschwege et al. 2004; Knapek et al. 2010) and in larval odour-sugar learning (Michels et al. 2005). As part of my main project, detailed in chapter II of this Thesis, I confirm that punishment-learning is significantly reduced; notably, relief-learning is undetectable in syn^{97}. These observed defects in associative function are not due to impairments in sensory or motor function: no differences in response between wildtype and mutant to the odours or to electric shock are observed. Considering non-associative learning as the cause for the observed phenoptype seems inevitable (Préat 1998). However, training-like exposure of flies to CS or US (sham-training;

Michels et al 2005) shows that the observed phenoptype can not be attributed to handling or exposure effects. Knock down of Synapsin with RNAi, using the pan-neuronal driver-line *elav*-Gal4, results in mutant-like phenotypes in both punishment- and relief-learning. The mutant phenotypes can be fully rescued by expression of Synapsin with the mushroom body-specific driver *mb247*-Gal4. These two approaches, rescue with *mb247*-Gal4 and RNAi, indicate that the learning phenotype is not due to genetic background effects or to a side effect of the deletion.

Thus, Synapsin is necessary for both punishment- and-relief learning; restoring Synapsin in the mushroom bodies is sufficient for relief-learning and punishment-learning, arguing that this structure harbours both a punishment- and a relief-learning memory trace. In this context it is significant that on the cellular level punishment learning and sugar-reward learning are dissociated in regard to the modulating neurotransmitter required. Dopamine and octopamine are proposed to represent the reinforcing properties of electric shock and sugar, respectively. Appetitive learning requires octopamine, whereas aversive learning requires dopamine (Schwaerzel et al. 2003; Schroll et al. 2006). Identifying a neurotransmitter specifically required for relief-learning so far remains elusive (Yarali and Gerber, personal communication).

Similarity of odorants in behaviour and physiology

Chapter III deals with the input of olfactory stimuli in the periphery of the fly. *Drosophila* detects olfactory stimuli through receptors located in sensilla on the antenna and maxillary palps. Olfactory sensory neurons (OSN) express mostly a single type of ligand-binding receptor of the *Or* gene family (OR, Hallem et al. 2004) in addition to Or83b, which is expressed as co-factor in most OSNs (Larsson et al. 2004). Another type of chemosensory receptors are the ionotropic receptors (IRs) (Benton et al. 2009). Sensory neurons project to the antennal lobes, where sensory neurons expressing a particular receptor type converge onto a single glomerulus (Vosshall et al. 2000, Gao et al. 2000). In the antennal lobe, glomeruli are connected by local interneurons (Ng et al. 2002), a large portion of which are GABA-ergic (Stocker 1994). Projection neurons, which receive input from mostly a single glomerulus (Jefferis et al 2001), carry the signal to other brain regions, most notably to the mushroom body calyx and to the lateral horn (Yasuyama et al. 2003; Marin et al 2002).

Depending on the odour and its intensity, it may function as attractant or repellent to various degrees (Rodrigues 1980). I use behavioural methods, based on associative recognition of odours, to characterize odour likeness in flies, and relate it to olfactory

physiology. Odour intensities are first adjusted for equal learnability. This seems necessary to achieve mutually corresponding measures of generalization between odour pairs. Three different training and testing procedures are used: (1) Animals are trained to avoid one odour, and subsequently are tested for their preference towards another odour. If the tested odour is similar to the trained odour, it should elicit a share of the previously acquired memory score. (2) Flies trained as in (1) are tested for their choice between the trained and a novel odourant. If those two odours are similar, flies are expected to distribute equally between them. (3) Flies are trained and tested discriminatively, i.e. during training one but not the other odour is paired with a punishment and animals are tested for their choice between both odours. These different procedures yield qualitatively similar results and hence are combined into a comprehensive score of behavioural odour similarity. In collaboration with the work group of Prof. André Fiala we then look at how such odours are represented physiologically. Calcium imaging reveals that odours detected as similar in behaviour possess similar activation patterns in projection neurons, but not in sensory neurons. Interestingly, the similarity measures obtained in this study correspond with a metric for odourant comparison obtained from evaluating about 1600 molecular descriptors (Haddad et al. 2008), arguing that the processing step in the antennal lobe classifies the odours according to their molecular properties and that the flies´ perception corresponds to the odours´ molecular properties.

Effect of salt and sugar on larval behaviour

Sodium and chloride play an important role in the well-being of animals. In humans, overconsumption of salt is associated with many health problems, such as hypertension (Ritz 2010, Savica et al. 2010); also, salt intake is an important factor for the regulation of the water balance of bodily fluids. *Drosophila* larvae, which live on their food feeding continuously, should have a basic interest to seek or avoid salt-containing substrates according to their physiological needs. In chapter IV of my Thesis, I investigate the effect of NaCl on larval reflexive behaviour and compare it to its capacity as a reinforcer in associative learning. At low salt concentrations larvae prefer the salt-containing substrate over the salt-free substrate. At high concentrations, larvae avoid salted substrates. In other words, the animals modulate their choice behaviour according to the salt concentration of the substrate. If choosing the substrate according to salt concentration is not possible, larval *Drosophila* can still regulate salt intake by decreasing or increasing the intake of the salt-containing medium. Assays that measure the amount of food intake from a substrate with constant salt concentration can monitor this. The results using this feeding assay are qualitatively similar to the results using the choice assay: larvae feed slightly more at low salt concentration compared to salt-free

substrates, whereas at high salt concentration, feeding is substantially inhibited. Salt also can be used as reinforcer in associative conditioning. Low concentrations of salt support appetitive learning, but high concentrations of salt support aversive learning. Although the dose-effect curves for choice, feeding and learning share a common shape, the curve for learning is shifted to higher concentrations.

A related study documented in chapter V focuses on various sugars (glucose, trehalose, fructose and sucrose) with respect to choice, feeding behaviour and learning in larval *Drosophila*. *Drosophila* larvae show preference towards all these sugars, although at different intensities. For glucose and trehalose, we find only a weak preference. For fructose and sucrose, preference is strong, feeding is slightly enhanced at low concentrations and strongly down-regulated at high concentrations. Both fructose and sucrose support learning at medium and high concentrations. The dose-effect curve for learning is shifted toward higher concentrations relative to choice and feeding.

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Chapter I

‘Pain relief’ learning in fruit flies

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'Pain relief' learning in fruit flies

AYSE YARALI, THOMAS NIEWALDA, YI-CHUN CHEN, HIROMU TANIMOTO, STEFAN DUERRNAGEL & BERTRAM GERBER
Lehrstuhl für Genetik und Neurobiologie, Universität Würzburg

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We studied the behavioural consequences of 'traumatic', painful experiences. These consequences were fundamentally asymmetric. Fruit flies, *Drosophila melanogaster*, learned two kinds of prediction regarding a 'traumatic' experience. If an odour preceded an electric shock during training, it predicted shock, and flies subsequently avoided it. When the sequence of events during training was reversed, that is odour followed shock, the odour predicted relief from shock and flies approached it. We call this latter effect 'relief' learning and showed that, in terms of psychological mechanisms, it established genuinely associative conditioned approach behaviour. Parametric analyses showed that relief learning was reproducible across experimenters; it did not depend on the flies' gender and reached asymptotic levels after six training trials. Of five chosen odour-pairs, two supported relief learning at all concentrations tested; for one odour-pair, we observed optimal relief learning at an intermediate odour concentration; for two odour-pairs, relief learning could not be demonstrated. Furthermore, relief learning was maximal with relatively mild shocks, supporting stable retention for the first 2 h after training. Knowledge of these parametric features should aid uncovering relief learning in other experimental systems. In terms of psychological mechanism, context–shock pretraining had no effect on subsequent relief learning, suggesting that it is not mediated by context associations. These analyses may further our understanding of the psychological mechanisms underlying behavioural changes after traumatic experience. They facilitate research into the neurobiology of pain relief learning, enabling the implementation of truly bioinspired learning rules for technical devices.

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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 away from the predicted event. For example, fruit flies, *Drosophila melanogaster*, 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' (Tempel et al. 1983; Tully & Quinn 1985). Thus, the behaviour expressed, and the kind of learning underlying it, may be characterized as either aversive or appetitive. These kinds of learning are typically 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 helpful not only to predict correctly what will happen, but also 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 the odour is then presented (shock–odour training), flies show a relative preference for the

Correspondence: A. Yarali or B. Gerber, Lehrstuhl für Genetik und Neurobiologie, Biozentrum, Am Hubland, Universität Würzburg, D97074 Würzburg, Germany (email: ayse.yarali@biozentrum.uni-wuerzburg.de; bertram.gerber@biozentrum.uni-wuerzburg.de).

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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 two events to be associated is a basic common feature of predictive learning (e.g. dog, *Canis familiaris*: Moscovitch & LoLordo 1968; rabbit, *Oryctolagus cuniculus*: Plotkin & Oakley 1975; rat, *Rattus norvegicus*: Maier et al. 1976; snail, *Hermissenda crassicornis*: Britton & Farley 1999; pigeon, *Columba livia*: Hearst 1988; honeybee, *Apis mellifera*: Hellstern et al. 1998) and of synaptic plasticity (insects: Cassenaer & Laurent 2007; reviewed in Caporale & Dan 2008) and hence of the mnemonic organization of brain function in general. In other words, one can view learning as referring to either 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 distinguish four kinds of associative, predictive learning: (1) predicting the presence of something good or (2) predicting its absence; (3) predicting the presence of something bad or (4) 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 so far is poorly 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 own 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 bioinspired robot.

GENERAL METHODS

By and large, we used 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 used flies of the Canton-Special wild-type strain, aged 2–3 days after eclosure. Flies were kept in mass culture maintained at 25 °C, 60–70% relative humidity and were subject to a 14:10 h light:dark cycle. Flies were reared on standard cornmeal–molasses food (Guo et al. 1996). On the day prior to the experiments, flies were transferred to fresh food vials and kept overnight at 18 °C and 60–70% relative humidity.

Learning Experiments

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

Flies received eight training trials (unless mentioned otherwise; see Fig. 1c). At time 0 min, flies were loaded on to the experimental set-up, which took approximately 1 min. After an additional accommodation period of 3 min, the control odour was presented for 15 s. In experiment 2, this control odour was omitted. At 7 min 30 s, the electric shock was delivered. The shock consisted of four pulses of 100 V, each 1.2 s long and followed by the next pulse after an onset–onset interval of 5 s. The odour to be learned was then presented at 8 min 10 s (unless mentioned otherwise) for 15 s. Thus, the interstimulus interval (ISI) between the onset of the shock and the onset of the odour to be learned was 40 s. At 12 min, flies were transferred back to the food vials for 16 min until the next trial started.

Once training was completed, the usual 16 min break was given until the flies were loaded on to the set-up for the test. After an accommodation period of 5 min, the flies were transferred to the choice point of a T-maze, where they could choose between the control odour and the learned odour. In experiment 2, this test was between the learned odour and a nonscented maze arm. Thus, the interval between the end of the last training trial and the beginning of the test was 21 min (unless stated otherwise). After 2 min, the arms of the maze were closed and the number of flies (N) in each arm were counted. A preference index (PI) was calculated as:

$$\mathrm{PI} = (N_{\text{Learned odour}} - N_{\text{Control odour}}) \times 100 / N_{\text{Total}} \qquad (1)$$

Within each group, one subgroup was trained with 3-octanol (OCT) as the control odour and benzaldehyde (BA) as the odour to be learned to obtain the preference score $\mathrm{PI_{BA}}$, while a second subgroup was trained reciprocally ($\mathrm{PI_{OCT}}$; Fig. 1b). In experiment 2, this reciprocal design did not apply, as only BA was used as the odour to be learned (see below). In experiment 4, different odours were used (see below). The PIs from the two reciprocal groups were averaged to obtain a learning index (LI):

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

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

Mann–Whitney U tests and Kruskal–Wallis tests were used to compare the scores between different groups of flies. One-sample sign tests were used to determine whether scores were significantly different from zero. When multiple one-sample or multiple pairwise comparisons were made, we adjusted significance levels using a Bonferroni correction to maintain an experimentwide error rate of 5%; this was done by dividing the critical P value 0.05 by the number of one-sample or pairwise comparisons. For example, if one group from a four-group experiment was compared against zero, we report the P level of the one-sample sign test as $P < 0.05/4$. For statistical analyses we used Statistica (Statsoft, Tulsa, OK, U.S.A.) on a PC.

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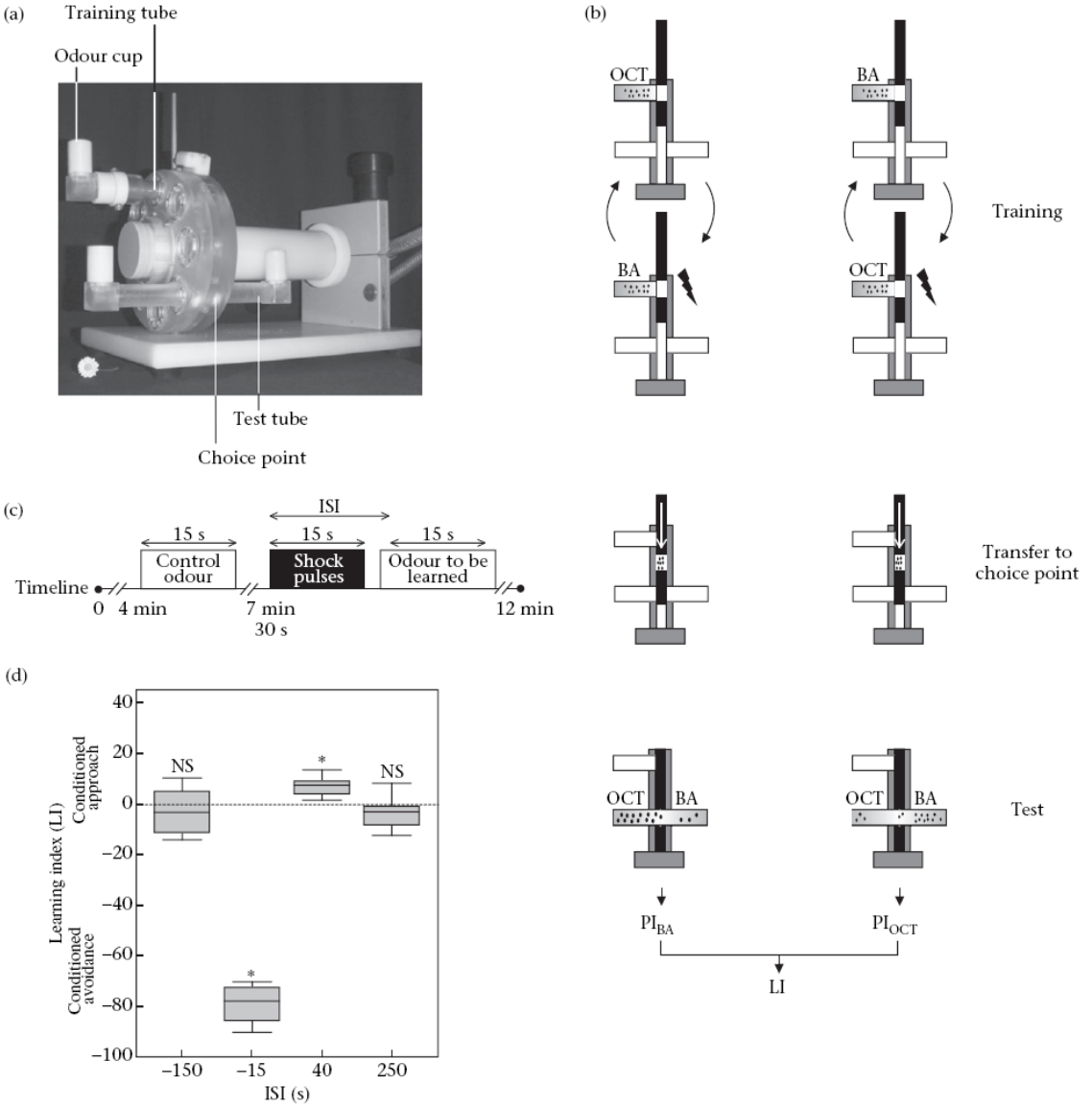

Figure 1. (a) The experimental apparatus. The training tubes were coated inside with a copper grid (not shown), which allowed an electric shock to be applied. Odours were delivered by attaching an odour cup at one end of the training tube. Odour-saturated air was sucked through the training tube. (b) One group of flies was trained with 3-octanol (OCT) as the control odour and benzaldehyde (BA) was paired with electric shock (left); another group was trained reciprocally (right). Once the training was completed, flies were transferred to the choice point between two test tubes, each scented with one of the two odours encountered during training. A preference index (PI) was calculated based on the distribution of the flies. A learning index (LI) was then calculated as the difference in odour preference between the reciprocally trained groups. A positive LI means that flies approached the learned odour, whereas a negative LI means that they avoided the learned odour. An LI of zero (dashed line in d) means that flies were equally attracted/repelled by the control and learned odours. (c) Timeline of a single training trial. The interstimulus interval (ISI) is the interval between the onset of shock and the onset of the odour to be learned. The ISI is positive for shock–odour pairings and negative for odour–shock pairings. (d) Conditioned behaviour of flies after odour–shock or shock–odour training with various ISIs. 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% quartiles, and the whiskers the 10% and 90% quantiles, respectively.

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Odorants

As odorants, 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) were used. Odorants were applied either pure or 10-, 100-, 1000- or 2000-fold diluted in paraffin oil (PARA: Fluka, Steinheim, Germany). Teflon containers 5 mm in diameter were used for odour application for BA, AM and IAA; containers 14 mm in diameter were used for OCT and MCH, and containers 7 mm in diameter for LM. In experiment 2, containers 15 mm in diameter were used for both BA and the solvent PARA. Airborne odour concentrations were unknown.

EXPERIMENT 1: TIMING MATTERS

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 sought to replicate these experiments.

Methods

We used four experimental groups which received equal handling and exposure to the control odour, the odour to be learned and the electric shock; only the ISI, that is, the interval between the onset of the shock and the onset of the odour to be learned differed between groups (Fig. 1c). In different groups, the odour to be learned was 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, the flies' preference between the control and the learned odour was tested in a T-maze choice assay and a learning index (LI) was calculated as detailed in the General Methods. Positive LIs indicate conditioned approach to the learned odour, whereas negative LIs reflect conditioned avoidance.

Results and Discussion

We found no learning when the odour to be learned had been presented either long before or long after the shock (one-sample sign tests for ISI = −150 and 250 s: $N_1 = 8$, $N_2 = 10$, $P > 0.05/4$ for each; Fig. 1d). In contrast, we found conditioned avoidance if the odour to be learned had been presented shortly before the shock in training (one-sample sign test for ISI = −15 s: $N = 8$, $P < 0.05/4$; Fig. 1d). On the other hand, those flies that were trained such that the odour to be learned closely followed shock approached this odour (one-sample sign test for ISI = 40 s: $N = 9$, $P < 0.05/4$; Fig. 1d).

Thus, flies avoided the learned odour after odour–shock training. In contrast, after shock–odour training flies showed a relative preference for the learned odour. We next considered whether this latter effect came about by a conditioned increase in attractiveness of the learned odour, or by a decrease in its baseline, unconditioned aversiveness.

EXPERIMENT 2: INCREASED ATTRACTIVENESS?

There are two kinds of explanation for the positive LIs reported in experiment 1. That is, at the concentrations used, both odours were 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 distributed themselves equally between them (data not shown), because they were repelled equally by the two odours (red and dark-blue arrows in Fig. 2a, b). Thus, at the moment of test both of these baseline repellent tendencies were probably present as well.

The first kind of explanation (Fig. 2a) for the positive LIs in experiment 1 suggests that, as a result of shock–odour training, an additional, genuinely associative attractive tendency developed for the learned odour. In other words, the learned odour predicted relief, and flies showed an associative conditioned approach to it (light-blue arrow). This attractive tendency added to the baseline avoidance of both odours. Thus, the balance between the two odours was shifted in favour of the learned odour.

An alternative explanation (Fig. 2b) would suggest that the positive LIs rather came about by a decrease in the baseline response to 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 render them eventually less effective, and hence less aversive, at the moment of test. Such a process would also be specific for the learned odour; it would not, however, invoke any de novo conditioned approach tendency for it.

We pitted these two explanations against each other using a modified experimental design, omitting the control odour. Importantly, such an experiment should not use an odour concentration that 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, b). In contrast, if we use the odour at a concentration that supports baseline appetitive responses, the two proposed mechanisms predict different experimental outcomes (Fig. 2c, d). An additional conditioned approach tendency that develops by shock–odour training would further increase the attractiveness of the odour, resulting in a shift upwards of the preference scores (Fig. 2c), whereas an impairment in the processing of the odour would decrease its attractiveness, shifting scores towards zero (Fig. 2d). Odour responses in unconditioned, experimentally naïve flies typically change from avoidance to approach with decreasing odour concentration (Ayyub et al. 1990). We thus chose a very low concentration of odorant that supported appetitive baseline scores and used it in shock–odour training.

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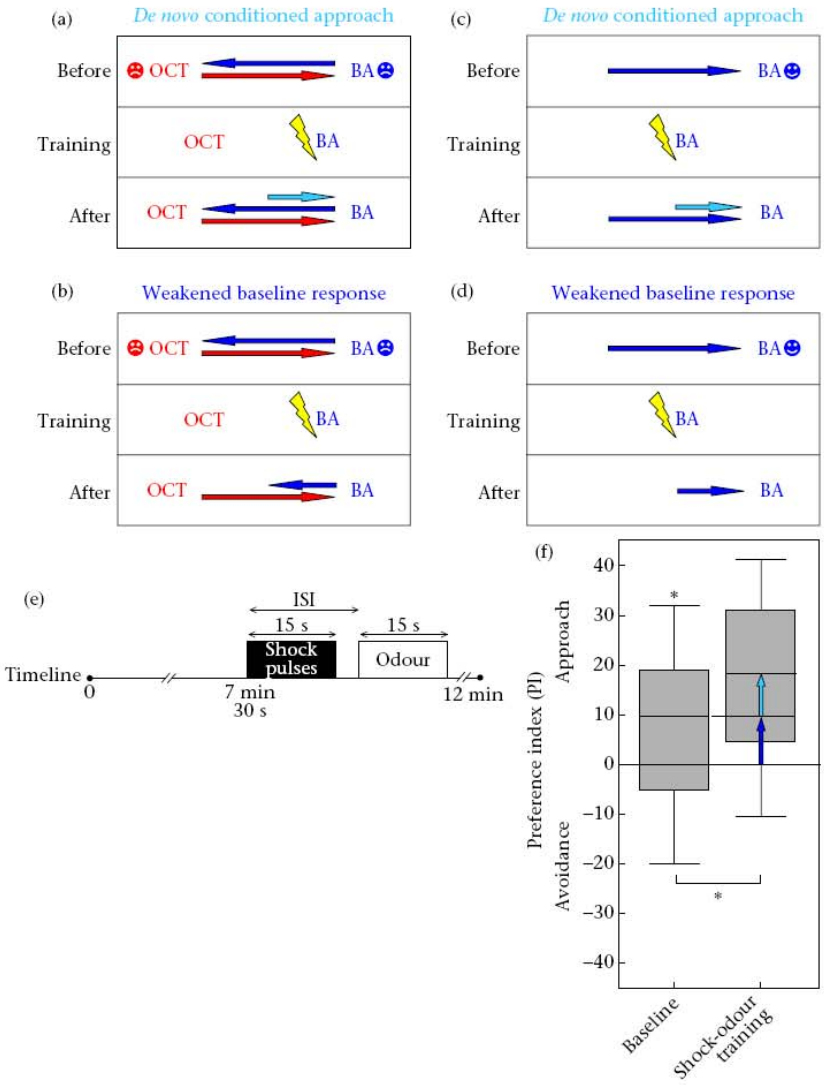

Figure 2. (a, b) Sketches of two possible explanations for relief learning. (a) Experimentally naïve, unconditioned flies avoid both odours. Concentrations are adjusted to obtain equally strong avoidance of both odours; hence the red and dark-blue arrows for the control odour (OCT) and the odour to be learned (BA), respectively, are depicted at the same length. During training, the control odour is presented alone, while the odour to be learned 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, the flies' overall preference is for the learned odour. (b) Alternatively, shock may reduce 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 response from the learned odour (BA; truncated dark-blue arrow), leaving intact the avoidance response from the control odour (OCT; red arrow). Thus, the net preference would be for the learned odour. (c, d) The two accounts for relief learning predict different outcomes when a single odour, benzaldehyde (BA), is used at a concentration that supports a baseline appetitive response. (c) A de novo conditioned approach (light-blue arrow) induced via shock–odour training would add to the existing baseline approach (dark-blue arrow) and thus would further increase the attractiveness of the odour. (d) Alternatively, a deterioration in odour processing would render the odour less attractive at the moment of test (truncated dark-blue arrow). (e) Timeline of a single training trial, which used a single odour. The interstimulus 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. (f) Approach to the odour is indicated by positive preference indexes (PI), whereas negative values reflect avoidance. A PI of zero means that the flies were indifferent to the odour. The baseline appetitive response to BA and the increased appetitive response to BA after shock–odour training are shown. Sample sizes are, from left to right: $N = 123, 59$. $^*P < 0.05$. Details of box plots are as in Fig. 1d.

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Methods

We used benzaldehyde (BA) at 2000-fold dilution as the odour (Fig. 2e). One group received shock–odour training with an ISI of 40 s between odour and shock. Such training supports positive learning scores (Fig. 1d; Tanimoto et al. 2004). Three control groups received 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 were given the choice between BA and a nonscented maze arm, and a preference index (PI) was calculated (see General Methods). Positive PI values indicate approach towards the odour, negative values reflect avoidance.

Results and Discussion

The PI values of the three control groups, which were trained with ISIs of either −210 s, −150 s, or 200 s did not differ statistically (Kruskal–Wallis test: $H_2 = 4.49$, $N_1 = 59$, $N_2 = 32$, $N_3 = 32$, $P > 0.05$); data were therefore pooled and taken as a measure of the baseline response to BA at the moment of test (Fig. 2f: dark-blue arrow). As intended, we observed an appetitive baseline response (one-sample sign test for 'Baseline': $N = 123$, $P < 0.05$; Fig. 2f). The critical question was then whether the group trained with a short shock–odour interval (40 s), which does support positive LIs (Fig. 1d; Tanimoto et al. 2004), would show 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 PI values. Clearly, this was not the case. To the contrary, PI values after shock–odour training were above baseline level (Mann–Whitney U test: $U = 2700.50$, $N_1 = 123$, $N_2 = 59$, $P < 0.05$; Fig. 2f), suggesting an additional conditioned approach component (light-blue arrow). Thus, positive LIs obtained by shock–odour training reflect a genuine associative conditioned approach tendency.

EXPERIMENT 3: REPRODUCIBILITY AND GENDER

As relief learning is much less strong than punishment learning (approximately a fifth of punishment learning in Tanimoto et al. 2004; an eighth of punishment learning in Fig. 1d), we sought to bolster our confidence in this effect by testing whether it is replicable across three different experimenters. The LIs did not differ between experimenters (Kruskal–Wallis test: $H_2 = 0.57$, $N_1 = 12$, $N_2 = 11$, $N_3 = 16$, $P > 0.05$; Fig. 3a) and were significantly different from zero in the pooled data set (one-sample sign test for the pooled data set: sample size as above, $P < 0.05$; Fig. 3b). Thus, relief learning was a reliable, yet small effect. The LIs did not differ between male and female flies (Mann–Whitney U test: $U = 680.5$, sample sizes as above, $P > 0.05$; Fig. 3c); both genders showed positive LIs indicating relief learning (one-sample sign tests for each gender: sample size as above, $P < 0.05/2$; Fig. 3c).

EXPERIMENT 4: NUMBER OF TRIALS

Next, we tested the effect of the number of training trials on relief learning. Different groups of flies received one, two, four, six or eight shock–odour pairings. The number of training trials had a significant influence on relief learning (Kruskal–Wallis test: $H_4 = 19.58$, $N_1 = 16$, $N_2 = 15$, $N_3 = 20$, $N_4 = 19$, $N_5 = 23$, $P < 0.05$; Fig. 4). Specifically, one, two and four training trials did not yield conditioned approach to the learned odour (one-sample sign tests: sample sizes as above, $P > 0.05/5$ in all three cases; Fig. 4), whereas six and eight trials did (one-sample sign tests: sample sizes as above, $P < 0.05/5$ in both cases; Fig. 4). Relief learning after six trials was as good as after eight trials (Mann–Whitney U test: $U = 208.0$, sample sizes as above, $P < 0.05$; Fig. 4). Thus, relief learning, using the current parameters and training set-up, required at least six shock–odour pairings, with which it also reached an asymptote.

EXPERIMENT 5: ODOUR IDENTITY AND CONCENTRATION

Methods

We tested the effect of odour identity and concentration on relief learning for five odour-pairs: MCH–OCT, BA–LM, BA–OCT, AM–IAA and OCT–LM. We used pure odorant as well as 10- and 100-fold dilutions, except for MCH–OCT, for which also a 1000-fold dilution was used. Dilutions refer to the odorant loaded on to the experimental device. Airborne odour concentrations were unknown.

Results and Discussion

For three of the five odour-pairs, relief learning was observed. For MCH–OCT, the LIs depended on odour concentration (Kruskal–Wallis test: MCH–OCT: $H_3 = 8.50$, $N_1 = 20$, $N_2 = 20$, $N_3 = 19$, $N_4 = 16$, $P < 0.05$; Fig. 5f). A 100-fold dilution supported relief learning, whereas either higher or lower concentrations did not (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; Fig. 5a). Thus, for MCH–OCT the range of concentrations tested uncovered optimal relief learning at an intermediate odour concentration.

For both BA–LM and BA–OCT, LIs were comparable across odour concentrations (Kruskal–Wallis tests: BA–LM: $H_2 = 1.66$, $N_1 = 20$, $N_2 = 20$, $N_3 = 20$, $P > 0.05$; BA–OCT: $H_2 = 3.02$, $N_1 = 20$, $N_2 = 20$, $N_3 = 20$, $P > 0.05$; Fig. 5g, h). Therefore, for each of these odour-pairs we pooled the LIs across odour concentrations. Both odour-pairs supported relief learning (one-sample sign tests: sample sizes as above, $P < 0.05$ in each case; Fig. 5b, c).

For the remaining two odour-pairs, we found no effect of odour concentration on the LIs (Kruskal–Wallis tests: AM–IAA: $H_2 = 2.10$, $N_1 = 8$, $N_2 = 16$, $N_3 = 8$, $P > 0.05$; OCT–LM: $H_2 = 3.99$, $N_1 = 8$, $N_2 = 12$, $N_3 = 8$, $P > 0.05$; Fig. 5i, j). When we pooled across odour concentrations, there was no relief learning for either of these two

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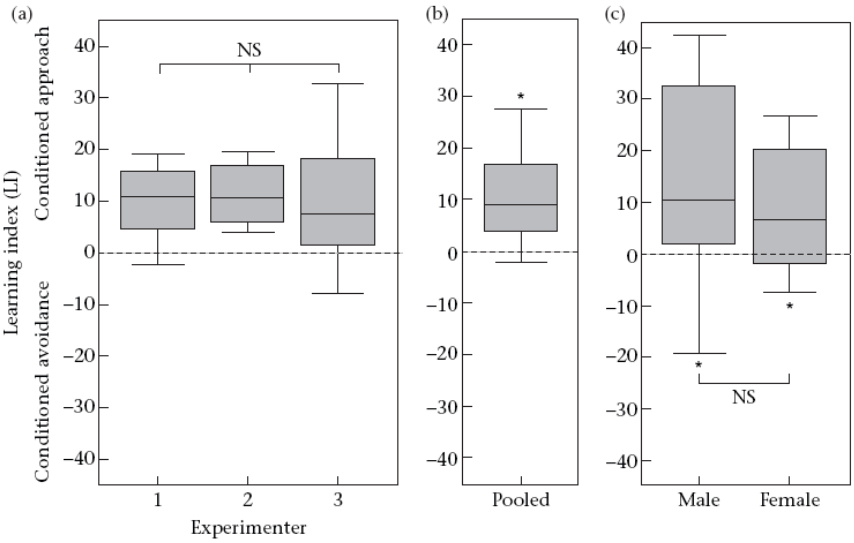

Figure 3. (a) Relief learning in relation to experimenter. Sample sizes are, from left to right: $N = 12, 11, 16$. NS: $P > 0.05$. (b) The pooled data set from (a). $N = 39$. *$P < 0.05$. (c) Data from (b), separated by flies' gender. Sample sizes, as in (b), are: $N = 39, 39$. *$P < 0.05/2$; NS: $P > 0.05$. Details of box plots are as in Fig. 1d.

odour-pairs (one-sample sign tests: sample sizes as above $P > 0.05$ in each case; Fig. 5d, e).

Thus, relief learning was possible with three of five odour-pairs. For one odour-pair (MCH–OCT), the range of concentrations tested uncovered an optimal odour concentration for relief learning. For two odour-pairs, we found uniformly strong relief learning across the concentrations tested, and for two odour-pairs relief learning could not be observed at either concentration.

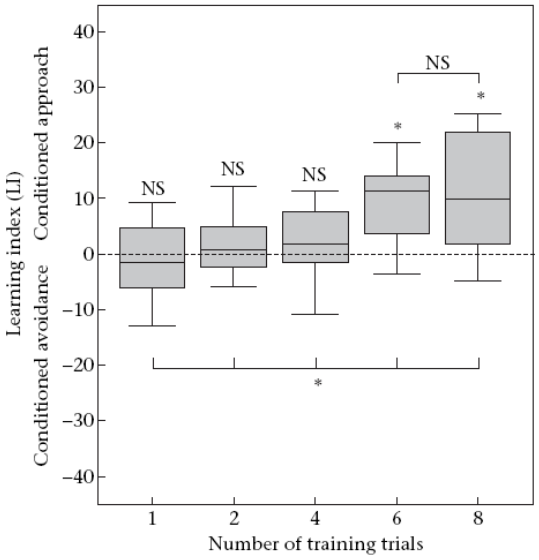

Figure 4. Relief learning in relation to number of shock–odour training trials. Sample sizes are, from left to right: $N = 16, 15, 20, 19, 23$. *$P < 0.05/5$; NS: $P > 0.05/5$, except for the comparison across all groups, and the comparison between the six-trial and the eight-trial groups, for which NS: $P > 0.05$. Details of box plots are as in Fig. 1d.

EXPERIMENT 6: SHOCK INTENSITY

We tested the effect of shock intensity on relief learning. Flies were trained with six training trials using shock pulses of either 25, 50, 75, 100, or 150 V. Shock intensity influenced LIs (Kruskal–Wallis test: $H_4 = 14.52$, $N_1 = 8$, $N_2 = 7$, $N_3 = 12$, $N_4 = 15$, $N_5 = 7$, $P < 0.05$; Fig. 6). Specifically, relief learning was found when we used 100 V (one-sample sign test for 100 V: sample size as above, $P < 0.05/5$), but not for lower or higher shock intensities (one-sample sign tests for 25 V, 50 V, 75 V, 150 V: sample sizes as above, $P > 0.05/5$ in each case; Fig. 6). Thus there was a relatively sharp optimum for relief learning at 100 V.

EXPERIMENT 7: STABILITY OF MEMORY

Next, we tested whether memory for relief learning decays over a 2 h retention period and compared this potential decay to the one seen for punishment memory. Four groups of flies received six training trials; for two groups, these were odour–shock (ISI = −15 s) training trials, whereas the other two groups received shock–odour (ISI = 40 s) trials. Once training was complete, for each training condition, one group was tested after the 'normal' retention period (20 min), while another group was tested after 2 h. For punishment learning, LIs decayed across the 2 h retention period (Mann–Whitney U test: $U = 51.00$, $N_1 = 16$, $N_2 = 13$, $P < 0.05/2$; Fig. 7). Despite this

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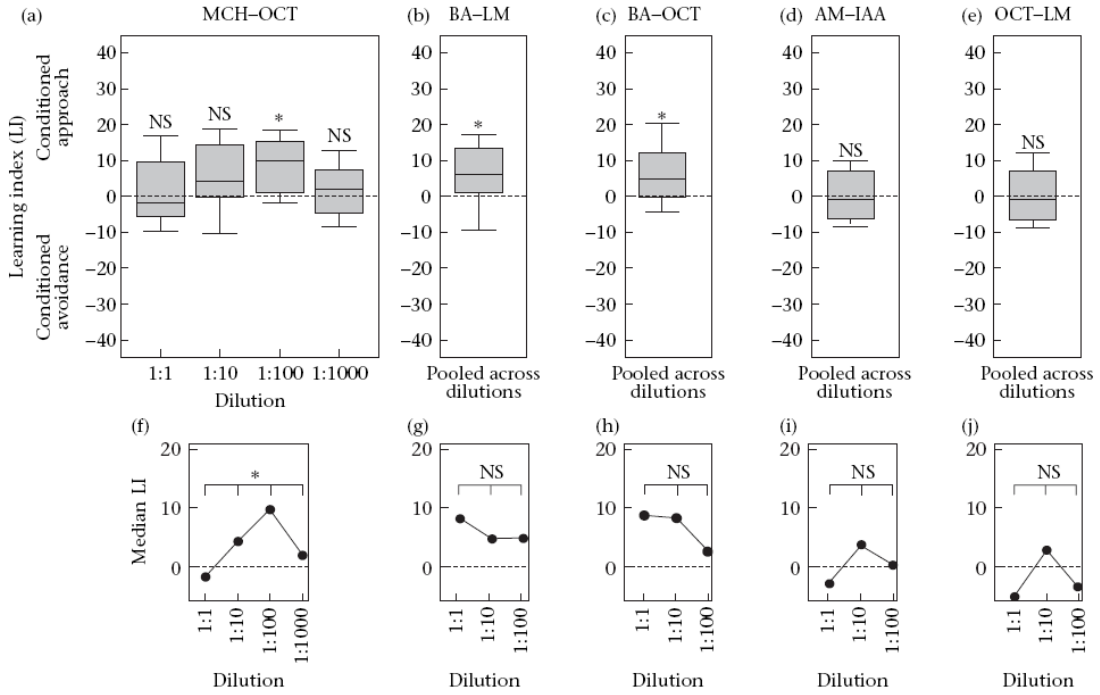

Figure 5. Relief learning in relation to various odour-pairs and a range of concentrations. (a–e) Learning indices (LIs). MCH: 4-methylcyclohexanol; OCT: 3-octanol; BA: benzaldehyde; LM: limonene; AM: amylacetate; IAA: isoamylacetate. (f–j) Median LIs for each concentration, plotted against odour concentration, with truncated *Y* axes. (a–e) Sample sizes are, from left to right: $N = 20, 20, 19, 16, 60, 60, 32, 28$. *$P < 0.05$; NS: $P > 0.05$, except for (a) where *$P < 0.05/4$; NS: $P > 0.05/4$. Details of box plots are as in Fig. 1d.

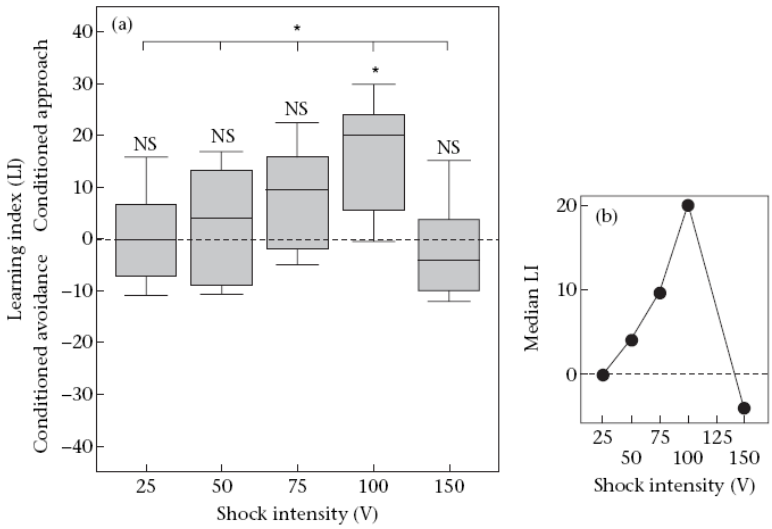

Figure 6. Relief learning in relation to shock intensity. (a) Learning index (LI). (b) Median LIs plotted against shock intensity, with a truncated *Y* axis. Sample sizes are, from left to right: $N = 8, 7, 12, 15, 7$. *$P < 0.05/5$; NS: $P > 0.05/5$, except for the comparison across all groups where *$P < 0.05$. Details of box plots are as in Fig. 1d.

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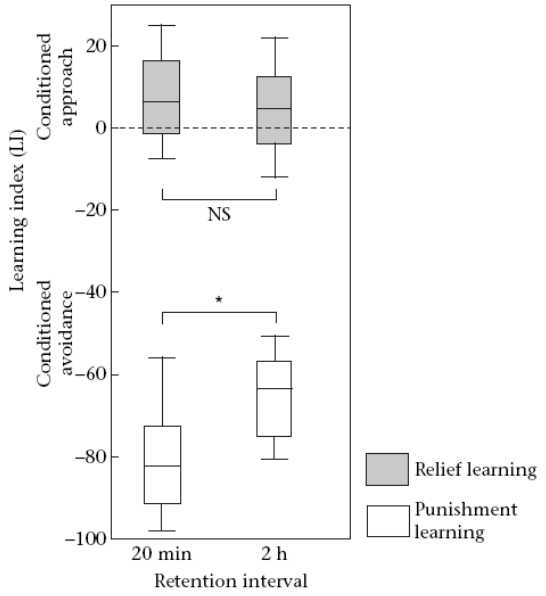

Figure 7. Relief memory (grey boxes) and punishment memory (white boxes) at 20 min and 2 h after training. Sample sizes are, from left to right: $N = 43$, 18 and 16, 13 for grey and white boxes, respectively. $^*P < 0.05/2$; NS: $P > 0.05/2$. Details of box plots are as in Fig. 1d.

approximately 25% decay, punishment memory was 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). For relief learning, LIs did not differ significantly between the two retention intervals ($U = 342.00$, $N_1 = 43$, $N_2 = 18$, $P > 0.05/2$; Fig. 7). When pooled, LIs indicated relief learning (one-sample sign test for the pooled data set: sample size as above, $P < 0.05$). This may suggest that relief memory did not substantially decay within the respective time interval. Alternatively, such a difference may remain undetectable, owing to an unfavourable signal-to-noise ratio for relief learning. In any event, at present we have no reason to conclude that memory for relief was less stable than that for punishment.

EXPERIMENT 8: A ROLE FOR CONTEXT?

Finally, we returned to the issue of the psychological mechanism underlying relief learning. On the one hand, both the onset and the offset of shock may 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, the experimental context may become 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) could then 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. In experiment 8 we tested this hypothesis.

First, we sought a finer resolution of the number of training trials necessary for relief learning than provided in experiment 4. This information would guide us in choosing the number of trials in the rest of experiment 8. That is, how many context–shock trials might establish the context as a predictor for shock and how many shock–odour trials might in turn establish the odour as a predictor for the absence of shock? As in experiment 4, at least six pairings were necessary to obtain relief learning (one-sample sign tests: $N_1 = 15$, $N_2 = 44$, $N_3 = 22$, $N_4 = 12$, $P > 0.05/4$ for two, four and five trials; $P < 0.05/4$ for six trials; Fig. 8a). We therefore adjusted the total number of trials to six for the rest of experiment 8.

Each of the following three subexperiments used two groups. One group, prior to shock–odour training, received trials without any odour or shock presentation. These flies were thus merely exposed to the experimental context before shock–odour training. A second group, prior to shock–odour training, received trials in which only shock was presented. Flies in this group could therefore 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 merely exposed to the context.

First, we used five shock–odour pairings, which were preceded by one trial of either context exposure or context–shock training. Despite a trend, LIs did not differ statistically after these two kinds of treatment (Mann–Whitney U test: $U = 348.00$, $N_1 = 32$, $N_2 = 28$, $P = 0.14$; Fig. 8c). Then we used four shock–odour pairings, preceded by either two context exposure trials or two context–shock training trials. Again, context–shock training did not improve LIs ($U = 744.00$, $N_1 = 39$, $N_2 = 48$, $P > 0.05$; Fig. 8d). Finally we found a similar result when two shock–odour pairings were preceded by either four context exposure or four context–shock training trials ($U = 93.00$, $N_1 = 12$, $N_2 = 16$, $P > 0.05$; Fig. 8c). Thus, context–shock training was inconsequential for subsequent shock–odour learning.

GENERAL DISCUSSION

In these eight experiments on pain relief learning in *Drosophila*, we used 51 experimental groups, with a total sample size of 1011, each sample being based on the behaviour of approximately 300 flies. We looked at repeatability and effects of gender, training amount, odour identity and concentration, shock intensity, and temporal

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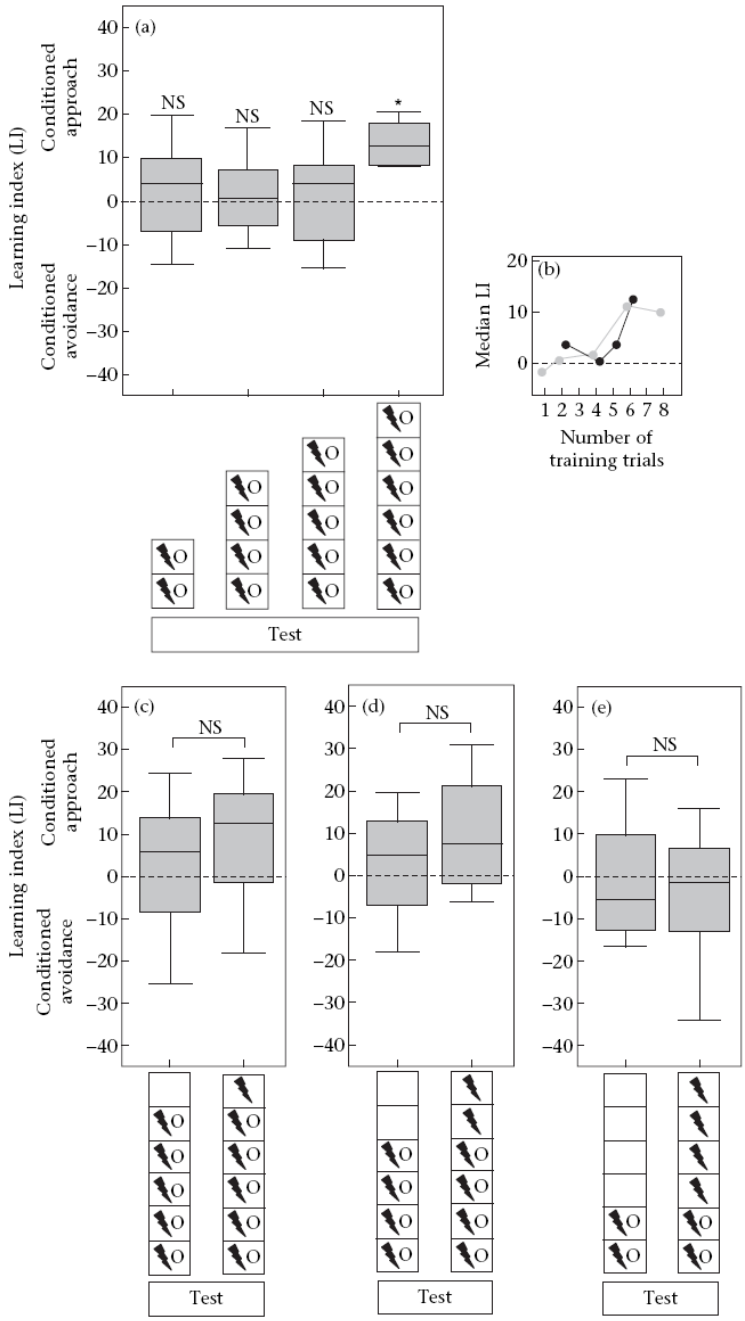

Figure 8. (a, b) Relief learning in relation to number of shock–odour training trials. (a) Learning indices (LIs). Sample sizes are from left to right: $N = 15, 44, 22, 12$. *$P < 0.05/4$; NS: $P > 0.05/4$. (b) Median LIs (black) plotted against the number of training trials with a truncated Y axis; median LIs from Fig. 4 are plotted in grey. (c–e) Relief learning in relation to whether shock–odour pairings were preceded by either context exposure or context–shock training. (c) Five shock–odour pairings preceded by a single trial of either context exposure or context–shock training. Sample sizes are, from left to right: $N = 32, 28$. NS: $P = 0.14$. (d) Four shock–odour pairings preceded by two trials of either context exposure or context–shock training. Sample sizes are, from left to right: $N = 39, 48$. NS: $P > 0.05$. (e). Two shock–odour pairings preceded by four trials of either context exposure or context–shock training. Sample sizes are, from left to right: $N = 12, 16$. NS: $P > 0.05$. Details of box plots are as in Fig. 1d.

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stability of the memory trace. Furthermore, we demonstrated the nature of relief learning in flies as establishing a genuinely associative conditioned approach component, and pitted 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 consider below the psychological and neurobiological mechanisms of relief learning and 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 a fifth (Tanimoto et al. 2004) to an eighth (Fig. 1) of that 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 Figure 1a in Schwaerzel et al. 2003; Krashes & Waddell 2008), but usually two trials are used to obtain asymptotic LIs (Tempel et al. 1983; Schwaerzel et al. 2003).

Gender had no effect upon relief learning in the present study (Fig. 3c). 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 data).

Relief learning was possible with three of the five tested odour-pairs (Fig. 5). The odour-pair AM–IAA, which did not support relief learning, can readily be used for punishment or reward learning (A. Yarali, unpublished data). A combinatorial argument may suggest that BA and MCH were largely responsible for relief learning, but in a formal sense the relative contribution of either odour within a pair remains unresolved. We saw an effect of odour concentration on relief learning for the MCH–OCT pair (Fig. 5a); specifically, within the range of concentrations covered, we observed 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 Fig. 5g, h). Using MCH–OCT, Tully & Quinn (1985) reported that punishment learning improves with increasing odour concentration, which along the same line of argument may reflect part of an optimum function as well. There are no systematic studies published on effects of odour concentration on reward learning.

Relief learning depended on shock intensity. Specifically, we observed an optimum function (Fig. 6). Punishment learning also improved with increasing shock intensity until an optimum was reached; a further increase in intensity then worsened punishment learning (Tully & Quinn 1985), but this decline was 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. There are no systematic studies published on effects of sugar concentration on reward learning.

Finally, relief memory did not decay across a 2 h retention period (Fig. 7). Within this time interval, in contrast, punishment memory did decay (Fig. 7). Punishment memory has been reported to decay relatively faster (within 4 h: Tempel et al. 1983; within 24 h: Tully & Quinn 1985) than reward memory (>24 h: Tempel et al. 1983).

To summarize, the parametric features of relief learning presented here suggest that one needs to adjust the training parameters carefully when trying to uncover this form of learning; in particular, this is 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 events to be associated is probably 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 discovery in other animal taxa. Rather, such analyses are indispensable to equip computational models of behaviour with truly bioinspired 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 used 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). 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. 2b). In our experiment to

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distinguish between these accounts, shock–odour training established genuinely associative conditioned approach to the odour (Fig. 2c–f).

Possible Mechanisms: Neurobiology

Evidence suggests that the short-term memory trace for punishment learning is localized exclusively to the so-called mushroom bodies, a third-order olfactory brain region (Heisenberg 2003; Gerber et al. 2004; Heisenberg & Gerber 2008). 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 on crickets, *Gryllus bimaculatus*). Strikingly, activation of dopaminergic or octopaminergic/tyraminergic neurons is reportedly 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 raises the question whether either dopamine or octopamine signalling may be necessary and/or sufficient for relief learning. To date, no data have been published on this question.

An alternative physiological mechanism to bring about opposite behavioural changes as a result of 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, *Schistocerca americana*, it is possible to induce spike-timing-dependent plasticity at these synapses experimentally. Furthermore, Drew & Abbott (2006) recently argued that it is 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 the one hand, an internal reinforcing signal may be 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, the shock may become 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; 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. 8c–e).

A possible role of context in learning about the absence of reward has also been investigated in honeybees (Hellstern et al. 1998). Honeybees learned 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 resulted in proboscis extension to the odour at subsequent test, as it had become a predictor for sugar. The effect of the reversed-order sugar–odour training, in contrast, had to be assessed indirectly. In the first phase of the experiment, bees were given a sugar–odour pairing with either a short or a very long interval between the two stimuli. This was followed by 'regular' odour–sugar training. Finally, bees were tested for the proboscis extension response to the odour. If sugar–odour training established 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 was indeed the case: bees trained with a short sugar–odour interval in the first experimental phase showed weaker proboscis extension in the test than 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 have prevented the context–sugar association from 'fading away' (see discussion in the previous paragraph); consequently sugar–odour learning would have been 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 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, our analyses 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 bioinspired

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computational model of predictive learning and behavioural control.

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Chapter II

Common requirement of Synapsin in punishment- and pain relief-learning

Niewalda T, Michels B, Yarali A, Gerber B

Common requirement of Synapsin in punishment- and pain relief-learning

Thomas Niewalda, Birgit Michels, Ayse Yarali, Bertram Gerber

Abstract

Shock can induce negative memories for stimuli preceding it, but also positive memories for stimuli presented upon its cessation: after odour-shock training, fruit flies subsequently avoid the shock-predicting odour, whereas after presentations of the odour after shock offset (shock-odour training), they subsequently approach the odour. Do these associative processes, which we call punishment- and pain relief-learning, share molecular determinants? We focus on the role of Synapsin, an evolutionarily conserved presynaptic phosphoprotein regulating the balance between reserve-pool and readily releaseable-pool of synaptic vesicles. We find that lack of Synapsin in the syn^{97CS} deletion mutant leaves all sensory and motor faculties required to perform in these learning tasks unaffected. In contrast, punishment-learning is significantly reduced, and relief-learning is fully abolished in the mutant. Both these defects are also observed upon an RNAi-mediated partial knock-down of Synapsin, and are fully rescued by transgenically restoring Synapsin in mutant flies. We conclude that punishment- and relief-learning, despite their opposing effects upon behaviour, both require the Synapsin protein, and in this sense share genetic and molecular determinants. Corresponding molecular commonalities between punishment- and relief-learning in humans would constrain pharmacological attempts to selectively interfere with excessive punishment-memories e.g. after trauma.

Introduction

Painful, traumatic experiences can have a moulding influence on behaviour. In terms of associative processing, research is largely concerned with the negative memories that such experiences induce: those stimuli perceived right before a painful event become predictors of danger and consequently will be avoided when encountered again. While in principle such danger-predictions are adaptive, they may under unfavourable conditions also contribute to maladaptive behaviour and undesired psychological states (e.g. panic, anxiety, stress), so that any means to counteract these effects may be of value. Therefore, we look at the backside of painful events by investigating memories related to stimuli perceived after a painful event. Interestingly, these stimuli will be approached when subsequently encountered: in fruit flies odour-shock training leads to conditioned avoidance of the odour during subsequent test,

whereas repeated shock-odour training leads to conditioned approach (Tanimoto et al. 2004). Thus, regarding the same painful, traumatic experience there apparently are two kinds of memory, one negative for preceding events, which supports conditioned avoidance, and one, typically much less strong, positive memory for following events, which supports conditioned approach (see Hellstern et al. (1998) for related results in bees and Andreatta et al. (2010) in humans). This makes sense, as due to odour-shock training the odour predicts punishment, whereas shock-odour training turns the odour into a predictor of a period of safety (Sutton and Barto 1990; Chang et al. 2003) and/ or of relief from shock (Solomon and Corbit 1974; Wagner 1981). We therefore refer to these behaviourally opposing associative shock effects as punishment-learning and pain relief-learning.

In a follow-up study (Yarali et al. 2008), we have shown that (i) relief-learning reflects genuine associative increases in attractiveness of the odour, and that (ii) it is likely not mediated by context associations. Parametrically, relief-learning (iii) reaches asymptote after six training trials, and (iv) is optimal at relatively mild shock intensities. Further, (v) out of five odour-pairs, two supported relief-learning at all concentrations tested; for one odour-pair, we observed optimal relief-learning at an intermediate odour concentration; for two odour-pairs, relief-learning could not be demonstrated. Finally, (vi) relief-learning occurs in both genders, and (vii) memory after relief-learning is stable for at least 2 hours after training.

In this study, we compare the molecular underpinnings of punishment- and relief-learning. This seems timely, as despite the rich literature on punishment-learning in flies (Tully and Quinn 1985; Heisenberg 2003; Gerber et al. 2004; Keene and Waddell 2007), *Aplysia* (Lechner and Byrne 1998) and vertebrates (Christian and Thompson 2003; Fanselow and Poulos 2005; Maren 2008), little is known about the genetic or molecular determinants of relief-learning. Such a comparison seems important, however, as the more common the underlying processes are, the more difficult it may be to selectively interfere with either of them. In this regard, we previously found an altered balance between punishment-learning and pain relief-learning in the *white*^{1118} mutant (Yarali et al. 2009). Specifically, punishment-learning is enhanced, whereas pain relief-learning is weakened. In other words, the take-home message of the shock episode is over-all more negative in the *white*^{1118} mutant; whether this genetic effect is related molecularly to altered levels of biogenic amines, in particular serotonin, is a matter of controversy (Sitaraman et al. 2008; Yarali et al. 2009).

Here, we focus on the *synapsin* gene, which codes for an evolutionarily conserved presynaptic phosphoprotein expressed throughout the flies' nervous system (Klagges et al. 1996; Godenschwege et al. 2004; Michels et al. 2005; Knapek et al. 2010). The working

hypothesis of Synapsin function is that it tethers reserve-pool vesicles to the cytoskeleton and regulates their recruitment to the readily-releasable pool in a phosphorylation-dependent way (Hilfiker et al. 1999), conceivably as a target of the cAMP-PKA pathway (see discussion). Thus, Synapsin contributes to the regulation of synaptic output (Hilfiker et al. 1999; Gitler et al. 2004; Sudhof 2004). Such regulation is a prerequisite for synaptic plasticity and associative learning: Synapsin null mutants (*syn*^{97CS}; Fig. 1a) are impaired in punishment-learning (Godenschwege et al. 2004; Knapek et al. 2010; see also Fig. 1b), spatial learning in the heat-box paradigm (Godenschwege et al. 2004) and, as larva, in odour-sugar learning (Michels et al. 2005) (for learning and plasticity phenotypes in vertebrates see Silva et al. 1996; Garcia et al. 2004; Gitler et al. 2004). Therefore, we reasoned that Synapsin is a reasonable candidate for comparing the genetic and molecular bases of punishment- *versus* relief-learning in the fly. Notably, by using an RNAi approach to phenocopy the defects of the *synapsin* null mutant and by rescuing the mutant defect by transgenically restoring the Synapsin protein, we can for the first time attribute punishment- and relief-learning defects of the Synapsin null mutant to the actual absence of the Synapsin protein.

Results

The syn^{97CS} *mutant has reduced punishment- and abolished relief-learning*

We first confirm the defect of flies lacking Synapsin (*syn*^{97CS}; Fig. 1a) in punishment-learning (Godenschwege et al. 2004), which tests for conditioned odour avoidance after odour-shock training. Such confirmation seems mandatory given the parametric differences in the training regimen between our current study and the ones used by Godenschwege et al. (2004) and Knapek et al. (2010). We find that indeed *syn*^{97CS} flies show less negative learning indices than CS wild-type flies (Fig. 1b; U-test: U= 3.0, $P< 0.05$; N= 12, 12 for CS wild-type and *syn*^{97CS}, respectively). Both genotypes show significantly negative learning indices (Fig. 1b; one-sample sign tests: $P< 0.05/2$ in both cases; sample sizes as above). Thus, punishment-learning is impaired, but not abolished, in Synapsin null mutant flies.

When the sequence of odour and shock during training is reversed (i.e. flies receive shock-odour training), learning indices for the CS wild-type strain are more negative than for *syn*^{97CS}, indicating stronger conditioned approach in the CS wild-type than in the *syn*^{97CS} mutant (Fig. 1c; U-test: U= 72.0, $P< 0.05$; N= 16, 16 for CS wild-type and *syn*^{97CS}, respectively). CS wild-type flies show small, yet significantly positive learning indices (Fig. 1c; one-sample sign test: $P< 0.05/2$; sample size as above) indicating conditioned approach towards the trained odour. Parametrically, these scores of the CS wild-type match our

previous findings (Tanimoto et al. 2004; Yarali et al. 2008, 2009). In contrast, learning indices for *syn*^{97CS} flies are not different from zero (Fig. 1c; one-sample sign test: $P > 0.05/2$; sample size as above). Thus, relief-learning is intact in CS wild-type flies, but is abolished in Synapsin null mutant flies.

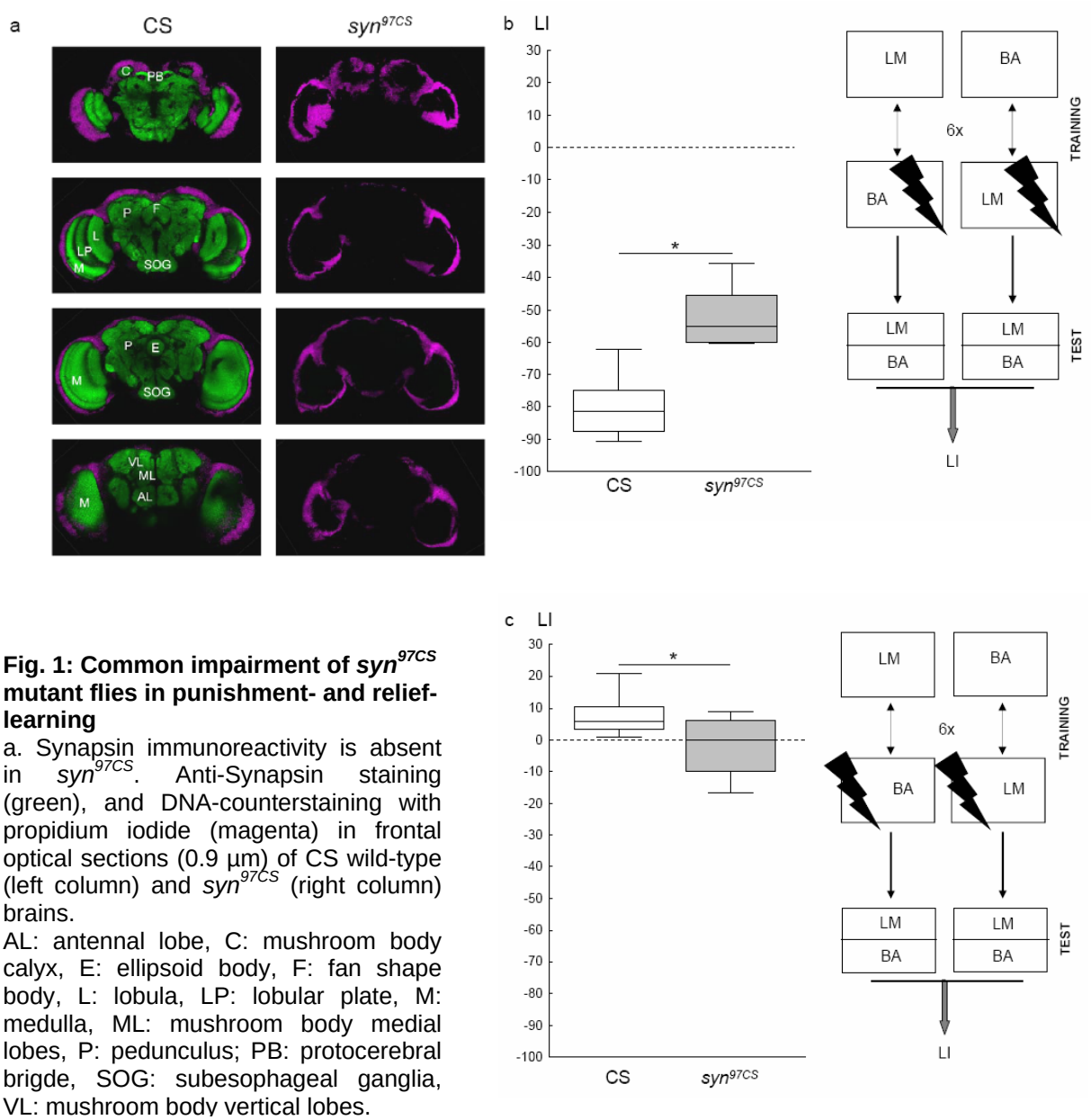

Fig. 1: Common impairment of *syn*^{97CS} mutant flies in punishment- and relief-learning

a. Synapsin immunoreactivity is absent in *syn*^{97CS}. Anti-Synapsin staining (green), and DNA-counterstaining with propidium iodide (magenta) in frontal optical sections (0.9 µm) of CS wild-type (left column) and *syn*^{97CS} (right column) brains.
AL: antennal lobe, C: mushroom body calyx, E: ellipsoid body, F: fan shape body, L: lobula, LP: lobular plate, M: medulla, ML: mushroom body medial lobes, P: pedunculus; PB: protocerebral brigde, SOG: subesophageal ganglia, VL: mushroom body vertical lobes.
b. Punishment-learning: Both CS wild-type and *syn*^{97CS} flies show conditioned aversion towards the trained odour, as is indicated by significantly negative learning indices in both genotypes; this punishment-learning is significantly less strong in *syn*^{97CS} than in CS wild-type.
c. Relief-learning: Only CS wild-type flies show conditioned approach towards the trained odour, as is indicated by significantly positive learning indices. In contrast, such relief-learning cannot be observed in *syn*^{97CS} flies, which show learning indices indistinguishable from zero and significantly smaller than CS wild-type flies.
*: $P < 0.05$; for the comparison of each genotype against zero $P <> 0.05/2$ is used to maintain the experiment-wide error at 5 % (Bonferroni-correction). LI: Learning Index. The middle line represents the median, the boundaries of the box the 25 % and 75 % quartiles, and the whiskers the 10 % and 90 % quantiles, respectively. The white fill indicates presence of Synapsin, the filled shading indicates the absence of Synapsin. The sketch to the right represents the experimental procedure, BA and LM indicate the odours benzaldehyde and limonene, respectively.

To test whether these defects in punishment- and relief-learning of the *syn*^{97CS} mutant are secondary to any sensory or motor impairment, we test whether behaviour towards the to-be-associated stimuli is impaired. This is not the case. There is no between-genotype difference in shock avoidance (Fig. 2a; U-test, U= 104.5, *P*> 0.05; N= 16, 16); obviously, flies do avoid the shock as demonstrated by significantly negative scores (Fig. 2a; one-sample sign test, *P*< 0.05; N= 32 for the dataset pooled across genotypes). With respect to the odours, CS wild-type and *syn*^{97CS} do not differ in their behaviour towards benzaldehyde (BA) (Fig. 2b; U-test, U= 116.5, *P*> 0.05; N= 19, 16), and both show avoidance behaviour (Fig. 2b; one-sample sign test, *P*< 0.05; N= 35 for the pooled dataset). The same pattern of results is found for limonene (LM), the other odour used (Fig. 2c; U-test: U= 158.5, *P*> 0.05; N= 20, 16; one-sample sign test, *P*< 0.05; N= 36 for the pooled dataset). Together, avoidance of the shock as well as of both odours used is indistinguishable between both genotypes.

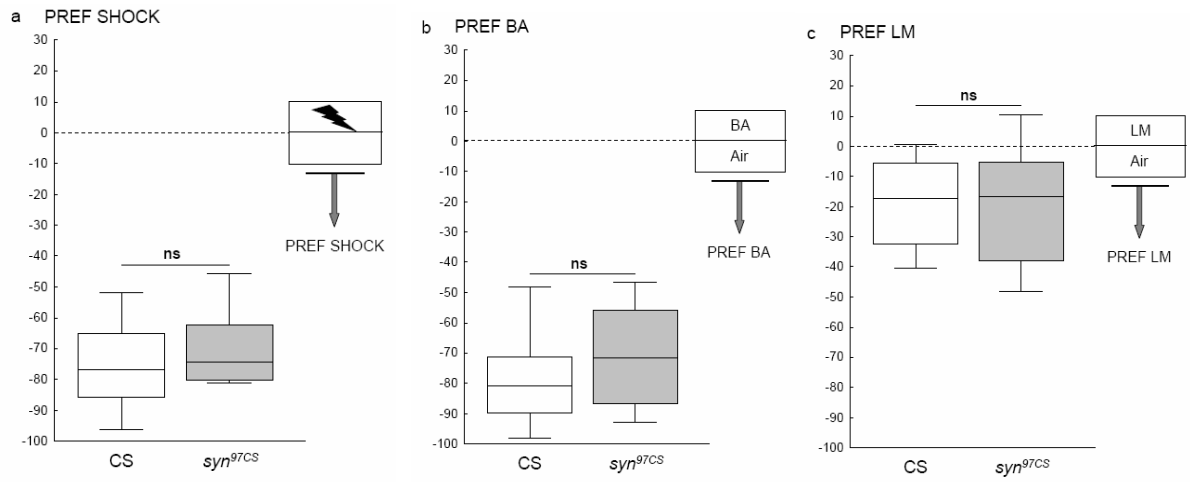

Fig. 2: Naive behaviour towards the to-be-associated stimuli is normal in *syn*^{97CS} mutant flies
Avoidance of the shock (a) and the odours (b: BA; c: LM) is not different between experimentally naive flies of both genotypes. ns: *P*> 0.05. All other details as in figure 1.

Although these kinds of control procedure have been state of the art since the introduction of odour learning in flies (Quinn et al. 1974; Dudai et al. 1976; Tully and Quinn 1985), it has been argued (Preat 1998; Michels et al. 2005; Knapek et al. 2010; see also discussion in Gerber and Stocker 2007) that they may not be sufficient to prove a genuine learning defect. That is, testing odour behaviour in experimentally naïve animals only argues that at the beginning of the experiment the mutants are normal in sensory-motor ability. Whether these faculties are still unaffected at the moment of test remains unclear. For example, the potentially stressful handling during the experiment may disrupt the mutants´ odour behaviour at test; also, exposure to odours during training can alter odour behaviour (Boyle and Cobb 2005; Colomb et al. 2007), as can exposure to shock (Preat 1998). If the

mutant would differ from wild-type in its susceptibility to handling, odour exposure, or shock exposure in such a way that the mutant but not the wild-type is rendered distorted in its odour behaviour at the moment of test, this may feign a learning phenotype. To test for this possibility regarding the odours used here, we run two kinds of sham training control: animals are handled just as in normal training, but either the shock is omitted (odour exposure) or the odours are omitted (shock exposure). After this kind of treatment, we test whether CS wild-type and *syn*^{97CS} flies differ in their behaviour towards BA and LM. We do not find any between-genotype differences in these tests (Fig. 3a: U-test: U= 165.0, *P*> 0.05; N= 21, 20; Fig. 3b: U= 163,5, *P*> 0.05; N= 21, 19; Fig. 3c: U-test: U= 113.0, *P*> 0.05; N= 16, 16; Fig. 3d: U-test: U= 120.0, *P*> 0.05; N= 16, 16). For the dataset pooled across genotypes, we find that flies, after either exposure regimen, still avoid both odours (one-sample sign tests: Fig. 3a: *P*< 0.05; N= 41; Fig. 3b: *P*< 0.05; N= 40; Fig. 3c: *P*< 0.05; N= 32; Fig. 3d: *P*< 0.05; N= 32). We therefore conclude that also at the moment of test, those sensory and motor abilities that are required to measure odour behaviour are not defective in *syn*^{97CS}.

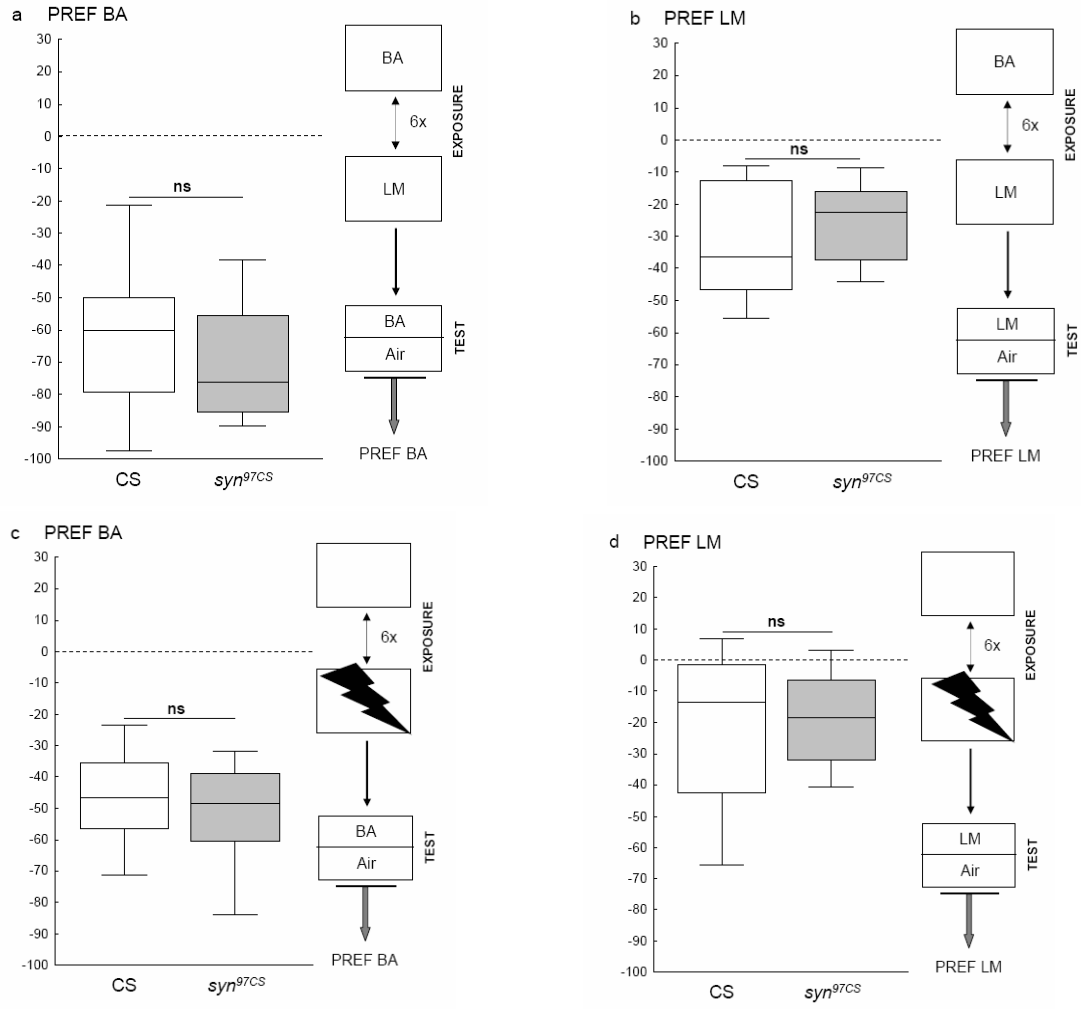

Fig. 3: Olfactory behaviour is normal in *syn*^{97CS} mutant flies also after training-like stimulus exposure
Genotypes do not differ in olfactory behaviour after either odour exposure (a: BA preference, b: LM preference) or shock exposure (c: BA preference, d: LM preference). ns: $P > 0.05$. All other details as in Fig. 1, 2.

RNAi mediated knock-down phenocopies punishment- as well as relief-learning defect

To confirm that the defect of the *syn*^{97CS} mutant in punishment- and relief-learning is indeed due to the lack of the Synapsin protein, and not to side effects of the deletion, we use an RNAi approach. We combine an UAS-RNAi-*syn* strain with the ubiquitous neuronal driver *elav*-Gal4 to obtain F1 progeny with reduced levels of Synapsin (knock-down). Indeed, the reduction of Synapsin in the knock-down flies is obvious relative to the genetic controls in a western blot (Fig. 4a). Testing these genotypes in behaviour, we find that knock-down of Synapsin results in reduced punishment-learning as compared to both effector-control (Fig. 4b; U-test: U= 47.0, $P < 0.05/2$; N= 13, 19), and driver-control (Fig. 4b; U-test: U= 48.0, $P < 0.05/2$; N= 13, 15) (the Kruskal-Wallis test across all genotypes yields: $P < 0.05$, H= 11.77, df= 2, sample sizes as above). All three genotypes show significant learning indices (Fig. 4b; one-sample sign tests: $P < 0.05/3$ in all three cases; sample sizes as above). Hence, punishment-learning is reduced, but not abolished in flies with reduced Synapsin levels.

Also in relief-learning we find that the reduction in Synapsin protein levels by knock-down leads to reduced learning compared to both genetic controls (Fig. 4c; U-test: U= 19.0, $P < 0.05/2$; N= 12, 19 for the comparison to the effector-control; U= 38.0, $P < 0.05/2$; N= 12, 14 as compared to the driver-control) (the Kruskal-Wallis test across all genotypes yields: $P < 0.05$, H= 9.53, df= 2, sample sizes as above). Both genetic controls show small but significant learning indices (Fig. 4c; one-sample sign tests: $P < 0.05/3$ in both cases; sample size as above), but learning indices of experimental flies are not significant different from zero (Fig. 4c; one-sample sign test: $P > 0.05/3$; sample size as above). Thus, relief-learning is intact in control flies but fully abolished in knock-down flies. The fact that two independent methods of reducing Synapsin levels (i.e. the deletion mutant and the RNAi-mediated knock-down) yield concordant learning defects suggest that it is the common effect upon Synapsin levels, rather than their respectively different potential off-target effects, which is responsible for the defects in punishment and relief-learning.

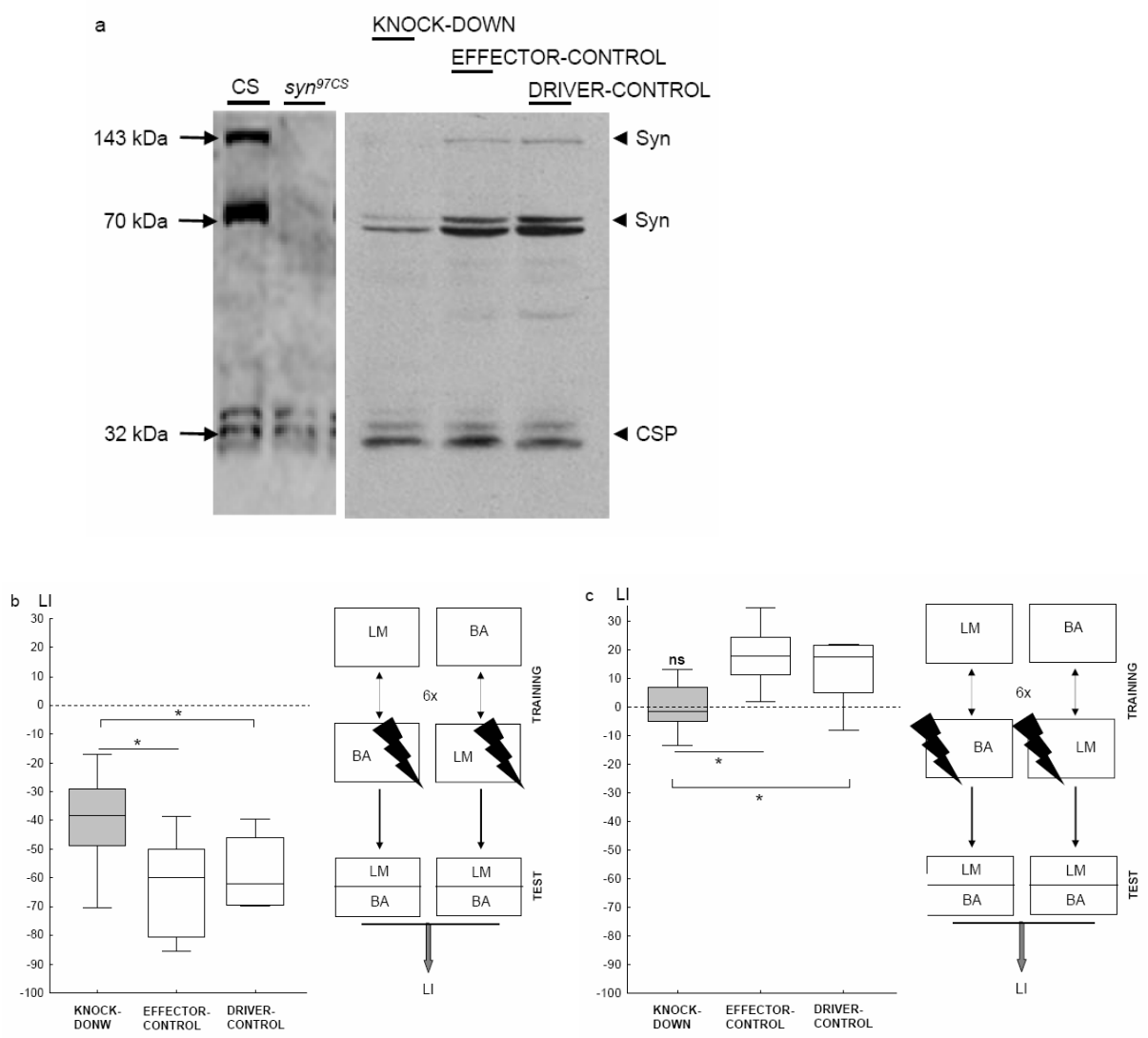

Fig. 4: RNAi-mediated knock-down of Synapsin impairs punishment- and relief-learning
a. Western blots from heads stained for Synapsin and for CSP as a loading control. The left blot, loaded with CS wild-type and *syn*^{97CS}, is shown for reference. The single band at 143 kDa and the double band at 70 kDa, where Synapsin isoforms are expected (Klagges et al. 1996), are absent in *syn*^{97CS}. The right blot is loaded from heads of double heterozygous *elav*-Gal4; UAS-RNAi-*syn* flies to the left (knock-down), UAS-RNAi-*syn* heterozygous flies in the middle (effector-control), and *elav*-Gal4 heterozygous flies to the right (driver-control). In the knock-down flies, a reduction of all Synapsin isoforms is apparent.
b. Punishment-learning is reduced in knock-down as compared to effector-control and driver-control.
c. Relief-learning is abolished in knock-down flies but remains intact in effector- and driver-controls.
*: $P< 0.05/2$ and ns: $P> 0.05/2$ is used for pair-wise comparisons. For comparisons of each genotype against zero $P<> 0.05/3$ is used to maintain the experiment-wide error at 5 % (Bonferroni-correction)

Transgenically restoring Synapsin in the mushroom body restores both punishment- and relief-learning

We test whether transgenically restoring Synapsin expression can rescue the mutant defect in punishment- and/ or relief-learning. We use the mushroom-body specific driver *mb247*-Gal4. The expression of Synapsin in the experimental flies and the lack of expression of Synapsin in

the control flies is observable in a western blot (Fig. 5a) and, in terms of site of expression, is obvious in wholemount brain preparations (Fig. 5b). Given this local expression in the mushroom bodies, we find that both punishment- and relief-learning are fully rescued: in punishment-learning, rescue flies perform better than both effector-control (Fig. 5c; U-test: U= 150.0, $P< 0.05/3$; N= 26, 26), and driver-control (Fig. 5c; U-test: U= 185.5, $P< 0.05/3$; N= 26, 26). Learning indices of rescue flies are as high as CS wild-type flies (Fig. 5c; U-test: U= 290.5, $P> 0.05/3$; N= 26, 26) (the Kruskal-Wallis test across all genotypes yields: $P< 0.05$, H= 29.70, df= 3, sample sizes as above). Also in relief-learning, rescue flies perform better than both effector-control (Fig. 5d; U-test: U= 26.0, $P< 0.05/3$; N= 15, 15), and driver-control (Fig. 5d; U-test: U= 48.0, $P< 0.05/3$; N= 15, 15), and actually do as well as CS wild-type flies (Fig. 5d; U-test: U= 115.0, $P> 0.05/3$; N= 15, 17) (the Kruskal-Wallis test across all genotypes yields: $P< 0.05$, H= 22.25, df= 3, sample sizes as above).

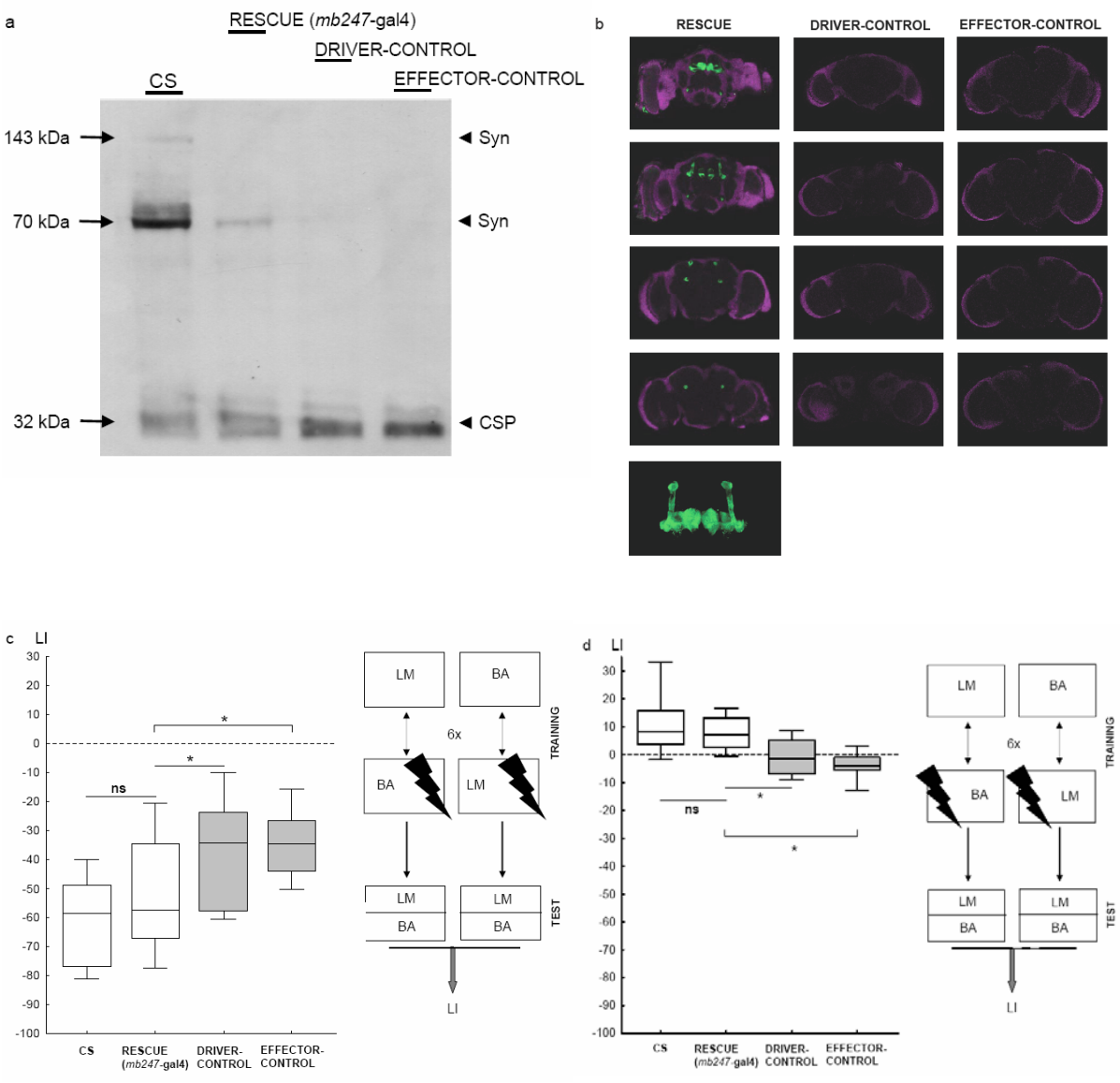

Fig. 5: Restoring the Synapsin protein in the mushroom bodies restores punishment- as well as relief-learning

a. Western blot from heads stained for Synapsin and for CSP as loading control. The lanes are loaded from left to right with CS wild-type, resuce, driver-control, and effector-controls. At 143 kda and 70 kda, the predicted Synapsin isoforms are found in only CS wild-type and rescue flies. Note that the used driver strain expresses in only the mushroom bodies (see b), meaning that the signal in a western blot is expectedly weak.

b. Spatial expression pattern of Synapsin in the experimental flies. Anti-Synapsin staining (green), and DNA-counterstaining with propidium iodide (magenta) in sections of rescue (left column) and control flies (middle and right column). The mushroom body region of the rescue flies is shown at higher magnification at the bottom.

c. Punishment-learning of syn^{97} flies is fully restored upon locally expressing Synapsin (using *mb247*-Gal4 as driver).

d. Relief-learning also is fully restored upon expressing Synapsin with *mb247*-Gal4 as driver.

*: $P< 0.05/3$ and ns: $P> 0.05/3$ is used for pair-wise comparisons. For comparisons of each genotype against zero $P<> 0.05/4$ is used to maintain the experiment-wide error at 5 % (Bonferroni-correction).

Discussion

We report common defects of the Synapsin null mutant syn^{97CS} in both punishment- and relief-learning (Fig. 1); these defects do not reflect any task-relevant sensory-motor impairment (Figs. 2, 3). The appearance of this effect is distinct from what we found for the w^{1118} mutation, which shifts the over-all balance between punishment- and relief-learning to generally more negative values (i.e. towards stronger punishment-learning and absent relief-learning) (Yarali et al. 2009). Notably, both these studies suggest common molecular and genetic determinants for punishment- and relief-learning in flies. If corresponding commonalities between these forms of learning exist in humans, pharmacological attempts to selectively interfere with e.g. excessive punishment-memories after trauma may be critically constrained.

We find that punishment-learning is partially abolished in syn^{97CS} flies, whereas relief-learning is apparently absent in these mutants; both effects of the syn^{97CS} deletion mutant are phenocopied by an RNAi-mediated knock-down of Synapsin. Notably, the partial defect in both the syn^{97CS} flies and in the RNAi-approach with regard to punishment-learning is in agreement with Godenschwege et al. (2004) and Knapek et al. (2010), who reported a 25- 30 % decrement in punishment-learning; a somewhat stronger yet still partial defect of the syn^{97CS} mutant (50 %) was also seen in larval odour-sugar learning (Michels et al. 2005). It remains unresolved whether the apparently full abolishment of relief-learning means that (i) this type of learning is not possible without (or in the case of the RNAi-approach: not even with reduced levels of) Synapsin, or (ii) that a Synapsin-independent residual memory is undetectable because of too low relief-learning scores. In any event, our observation that restoring Synapsin in the syn^{97CS} deletion mutant fully restores both punishment- and relief-learning (Fig. 5c, d) argues that it is indeed the absence of the Synapsin protein, rather than

other effects of the deletion or of genetic background, which is the cause of the defects in associative function. Although the repeated outcrossing of the *syn*^{97CS} deletion mutant to CS wild-type had already made genetic background effects unlikely, we note that the current study is the first to prove a role of the actual Synapsin protein in *Drosophila* associative learning.

Importantly, the rescue of associative function can be obtained by restoring Synapsin locally, in the mushroom body (Fig. 5b). This may mean that (i) punishment- and relief-memory traces are established in different subsets of those appr. 1500 mushroom body cells per hemisphere that are covered by the used *mb247*-Gal4 rescuing strain (Aso et al. 2009). If so, the opposite behavioural effects of punishment- and relief-learning could come about if these subsets of mushroom body neurons were redundant in the sense that although they both sample the complete odour space, they would differ in the kind of reinforcement signal they receive, as well as in terms of their connectivity to different behaviour routines (i.e. towards either conditioned avoidance or conditioned approach). Alternatively, (ii) during punishment- and relief-learning Synapsin may be employed in the same cells, but in different ways and/ or at different subcellular sites. Regarding punishment-learning, Knapek et al. (2010) reported no additive defect of the *syn*^{97CS} mutant and the *rut*^{2080} mutant, which suffers from a disruption of the type I adenylate cyclase. Thus, one may propose that Synapsin acts as a downstream effector of the cAMP pathway: indeed it is very likely that the *rutabaga* adenylate cyclase acts in the mushroom bodies as coincidence detector for the odour-evoked activity of the mushroom body neurons, and a shock-evoked dopaminergic punishment signal (Tomchik and Davis 2009; Gervasi et al. 2010). Thus, upon punishment-learning cAMP would be produced, and PKA be activated. Consequentially, Synapsin can be phosphorylated, and reserve-pool vesicles can be added to the releasable pool (Fiumara et al. 2004; Hilfiker et al. 2005) to enable enhanced transmission onto conditioned avoidance circuitry when the learned odour is encountered again. How could one, within such a framework, accommodate relief-learning within the same cells? As far as we can see, a molecularly independent effect on or an independent effect of Synapsin would need to be proposed that within the same mushroom body cell facilitates a functionally distinct synapse onto conditioned approach circuitry.

Please note that, when using *elav*-Gal4 as driver strain, in preliminary experiments we did not observe a rescue of associative function (Fig. S1); this is in accordance with the lack of rescue observed when using this driver to restore *rutabaga* function (Zars et al. 2000).

Further experiments are necessary to clarify whether the defect in associative learning of the *syn*^{97CS} mutant is due to the requirement of the Synapsin protein during development, or

for associative learning itsself. To answer this question, I use in ongoing experiments *mb247*-Gal4 in conjunction with *tub*-Gal80^{ts}, a method which allows for the temporally controlled expression of Synapsin in adult flies. Wholemount preparations demonstrate the successful rescue of protein expression (Fig. S2b); behavioural experiments will discover if learning can be rescued, too.

The current study prompts analyses of how the fly brain is organized on the cellular and/ or molecular level to establish memories that lead to opposite predictions (i.e. the presence *versus* the absence of shock) and support opposite behaviour (i.e. conditioned avoidance *versus* approach). Given our shared evolutionary heritage, such knowledge may, eventually, help dealing with the behavioural impact of traumatic experience in man (Andreatta et al. 2010).

Materials and Methods

Genotypes and rearing of flies

We compare wild-type CS flies to the deletion mutant *syn*^{97CS}, which had undergone 13 outcrossing steps to ensure effectively identical genetic background (Michels et al. 2005).

To accomplish an RNAi-mediated knock-down of Synapsin, a 497 nt coding fragment of the *syn*-cDNA is amplified by PCR with primers containing unique restriction sites: the primer pair: 5'-GAG CTC TAG AAC GGA TGC AGA ACG TCT G-3' in combination with 5'-GAG CGA ATT CTG CCG CTG CTC GTC TC-3' was used to generate a sense cDNA fragment. In turn, 5'-GAG CGG TAC CAC GGA TGC AGA ACG TCT G-3' in combination with 5'-GAG CGA ATT CGC CCG CTG CCG CTG CTC-3' is used for the anti-sense cDNA fragment. The PCR-amplified fragments are digested with *XbaI/EcoRI* and *EcoRI/KpnI* respectively, subcloned into *XbaI/KpnI* pBluescript KSII (Stratagene, La Jolla, USA) and sequenced. The resulting inverted repeat sequence is excised as a 1kb *NotI/KpnI* fragment, ligated into *NotI/KpnI*-cut pUAST (Brand and Perrimon 1993) and transformed into recombination-deficient SURE2 supercompetent cells (Stratagene, La Jolla, USA). Germ-line transformation then is performed into a *w*^{1118} strain (Bestgene, Chino Hills, USA). The resulting effector strain UAS-RNAi-*syn* [III] is then used for behavioural experiments. To this end, we generate the following genotypes as F1 progeny from the following crosses (in addition to the mentioned status, all flies are homozygous *w*^{1118}):

- Knock-down: Females of the strain *elav*-Gal4 [X] (strain c155 of Lin and Goodman, 1994) are crossed to males of UAS-RNAi-*syn* [III]. In the offspring, this yields double heterozygous *elav*-Gal4/+; UAS-RNAi-*syn*/+ flies.

- Effector-control: Female flies without any transgene are crossed to UAS-RNAi-*syn* males so that all F1 offspring is heterozygous UAS-RNAi-*syn*/+.
- Driver-control: Female *elav*-Gal4 flies are crossed to males without any transgene to yield *elav*-Gal4/+ heterozygous flies in the filial generation.

For attempts to rescue the syn^{97} mutant learning defect, we generate the following strains (in addition to the mentioned status, all fly strains are homozygous w^{1118}):

- *mb247*-Gal4 [III]; syn^{97} is generated by using *mb247*-Gal4 (Schulz et al. 1996), recombined into the syn^{97}- mutant background using classical genetics.
- UAS- *syn* [III]; syn^{97} is generated on the basis of Löhr et al. (2002).
- *mb247*-Gal4 [III]; syn^{97}; *tub*-Gal80^{ts}
- *elav*-Gal4; syn^{97} [X] was generated by classical genetics based on *elav*-Gal4 (Lin and Goodman 1994).

For rescue experiments *(mb247*-Gal4), I use F1 progeny from the following crosses:

- Rescue: Females of the strain *mb247*-Gal4 [III]; syn^{97} are crossed to males of UAS- *syn* [III]; syn^{97}. Thus, *mb247*-Gal4/ UAS-*syn* flies result as offspring which are in the homozygous syn^{97} mutant background.
- Driver-control: Female *mb247*-Gal4; syn^{97} flies are crossed to syn^{97} to yield *mb247*-Gal4/+ heterozygous flies in the homozygous syn^{97} mutant background.
- Effector-control: Female syn^{97} flies are crossed to UAS-*syn*; syn^{97} so that the F1 offspring is UAS-*syn*/+ heterozygous, and in the homozygous syn^{97} mutant background.

For rescue experiments *(elav*-Gal4), I use progeny from the following crosses:

- Rescue: w^{1118}, *elav*-Gal4; syn^{97CS} (female) x w^{1118}; UAS-*syn*, syn^{97CS} (male)
- Driver-control: w^{1118}, *elav*-Gal4; syn^{97CS} (female) x w^{1118} (male)
- Effector-control: w^{1118} (female) x w^{1118}; UAS-*syn*, syn^{97CS} (male)

For induced resuce, I generate the following crosses:

- Rescue: w^{1118}, *mb247*-Gal4; syn^{97CS} (female) x w^{1118}; UAS-*syn*, syn^{97CS}; *tub*-Gal80^{ts} (male)
- Driver-control: w^{1118}, *mb247*-Gal4; syn^{97CS} (female) x w^{1118} (male)
- Effector-control: w^{1118} (female) x w^{1118}; UAS-*syn*, syn^{97CS}; *tub*-Gal80^{ts} (male)

All flies are kept in mass culture at 25 °C, 60-70 % humidity and a 16/8 hour light/ dark cycle. One to five-day old flies are collected and kept at 18°C until the following day. Experiments are performed at 22-25 °C and 75-85 % relative humidity. We use flies in groups of about 150 (learning experiments, shock- and odour-exposure controls) or 50 (shock avoidance and naïve odour avoidance). Training is performed in dim red light to allow sight

for the experimenter (but not for the flies), test in darkness. Electric shock is applied via an electrifiable grid, covering the inner side of the training tubes. A vacuum pump ensures removal of odour-saturated air. As odorants, 80 µl benzaldehyde (BA; Fluka, Steinheim, Germany) and 110 µl limonene (LM; Sigma-Aldrich, Steinheim, Germany) are applied in Teflon containers of 5-mm or 7-mm diameter, respectively.

Learning experiments and behavioural controls

For punishment- and relief-learning, flies receive 6 training trials. At time:= 0 min, flies are loaded to the experimental set-up, which takes appr. 1 min. After an additional accommodation period of 3 min, the control odour is presented for 15 s. Then, for punishment-learning, the to-be-learned odour is presented from 7:15 to 7:30 min. At 7:30 min, the electric shock is delivered. Thus, for punishment-learning, the interstimulus interval (ISI), between the onset of the shock and the onset of the to-be-learned odour is –15 s. The shock consists of 6 pulses of 100 V, each 1.2 s long and followed by the next pulse after on onset-onset interval of 5 s. At 12:00 min, flies are transferred back to food vials for 16 min until the next trial starts. For relief-learning, all parameters are identical, except that the to-be-learned odour is presented from 8:20 to 8:35 min, leading to an onset-of-shock to onset-of-odour interval of 50 s, which corresponds to optimum parameters in this paradigm (Tanimoto et al. 2004; Yarali et al. 2008).

Once training is completed, a 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. After 2 min, the arms of the maze are closed and the number of animals (denoted # in the following) within each arm is counted. A preference index (PI) is calculated as:

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

In half of the cases, flies receive LM as control odour and BA as to-be-learned odour; in the other half of the cases, flies are trained reciprocally. PIs of two reciprocally trained sets of flies are then averaged to obtain a learning index (LI). Positive LIs indicate conditioned approach, negative LIs conditioned avoidance:

$$(2) \qquad LI = (PI\ (BA) + PI\ (LM)) / 2$$

Behavioural controls

To test for shock avoidance, flies are loaded to the experimental set-up and the red light is switched off. After an accommodation period of 2 min flies are transferred to the choice point where they can enter either arm of the maze; ten seconds later, shock is applied in one arm of the maze as specified above. Ten seconds after the onset of the last shock pulse, the maze is closed and flies are counted. A preference index (PI) is calculated to provide negative values for avoidance of the electrified arm:

(3) $PI = (\#_{\text{Electrified arm}} - \#_{\text{Non-electrified arm}})*100 / \#_{\text{Total}}$

To assess olfactory behaviour, flies are loaded to the experimental set-up and the red light is switched off. After an accommodation period of 4 min, flies are brought to the choice point of the T-maze where they can choose between a blank arm with air only and the other arm with odour (either BA or LM); after 2 min, the maze is closed and the flies are counted. A preference index (PI) is calculated as:

(4) $PI = (\#_{\text{Odour}} - \#_{\text{Air}})*100 / \#_{\text{Total}}$

For the odour exposure and shock exposure controls, flies receive the same treatment as if they would be trained, except that either the shock or the odours, respectively, are omitted. Then, behaviour towards BA and LM is measured as described in the preceding paragraph.

Statistical analyses

Non-parametric statistics are used throughout. Kruskal-Wallis or Mann-Whitney U-tests are used to compare multiple or two groups of flies, respectively. To test for differences from zero, we use one-sample-sign-tests. Significance level is $P< 0.05$. For multiple comparisons within a dataset, *P*-levels are adjusted by a Bonferroni correction ($P< 0.05$ divided by the number of comparisons) to maintain the experiment-wide error-rate at 5 %. Data are plotted as box plots, representing the median as the middle line, the 25 % and 75 % quantiles as boundaries of the box and the 10 and 90 % quantiles as whiskers.

Immunohistochemistry and Western Blotting

For wholemount immunohistochemistry, brains are dissected in Ringer's solution and fixed for 2 h in 4 % formaldehyde with PBST as solvent (phosphate-buffered saline containing 0.3

% Triton X-100). Samples are blocked in 3 % normal goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and subsequently incubated overnight with the mouse monoclonal anti-Synapsin antibody SYNORF1 (diluted 1:20 in PBST) (gift of E. Buchner, Universität Würzburg). The sample is then incubated overnight with a Alexa488-coupled goat anti-mouse Ig (diluted 1:250 in PBST) (Invitrogen Molecular Probes, Eugene, OR, USA) to detect the primary antibody. 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. Brains are mounted in Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA), containing propidium iodide for counterstaining of DNA. Preparations are examined under a confocal microscope.

For western blots, three adult heads per lane are homogenized in 10 µl Lämmli-buffer. The sample is heated to 70 °C for 5 min and centrifuged for 2 min before electrophoresis. Proteins are separated by 12.5 % SDS-PAGE in a Multigel chamber (100 mA, 2 h; Peqlab, Erlangen, Germany) and transferred to a nitrocellulose membrane (Kyhse-Andersen 1984). The membrane is blocked overnight (5 % milk powder in 1 x TBST). Immunoreactions are successively performed with two mouse monoclonal antibodies: SYNORF1 for Synapsin detection (Klagges et al. 1996) (dilution 1:100) and ab49 (Zinsmaier et al. 1990, 1994) (dilution 1:133) for detection of the cysteine string proteine (CSP; Arnold et al. 2004) as a loading control (1.5 hours). The membrane is then incubated with the second antibody (goat anti-mouse IgG-HRP coupled, 1:3700; 1 h). Incubation steps are followed by multiple washing procedures (1 x TBST). Visualization is achieved with the ECL Western blot detection reagents (Amersham Bioscience Europe, Freiburg, Germany).

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Supplement

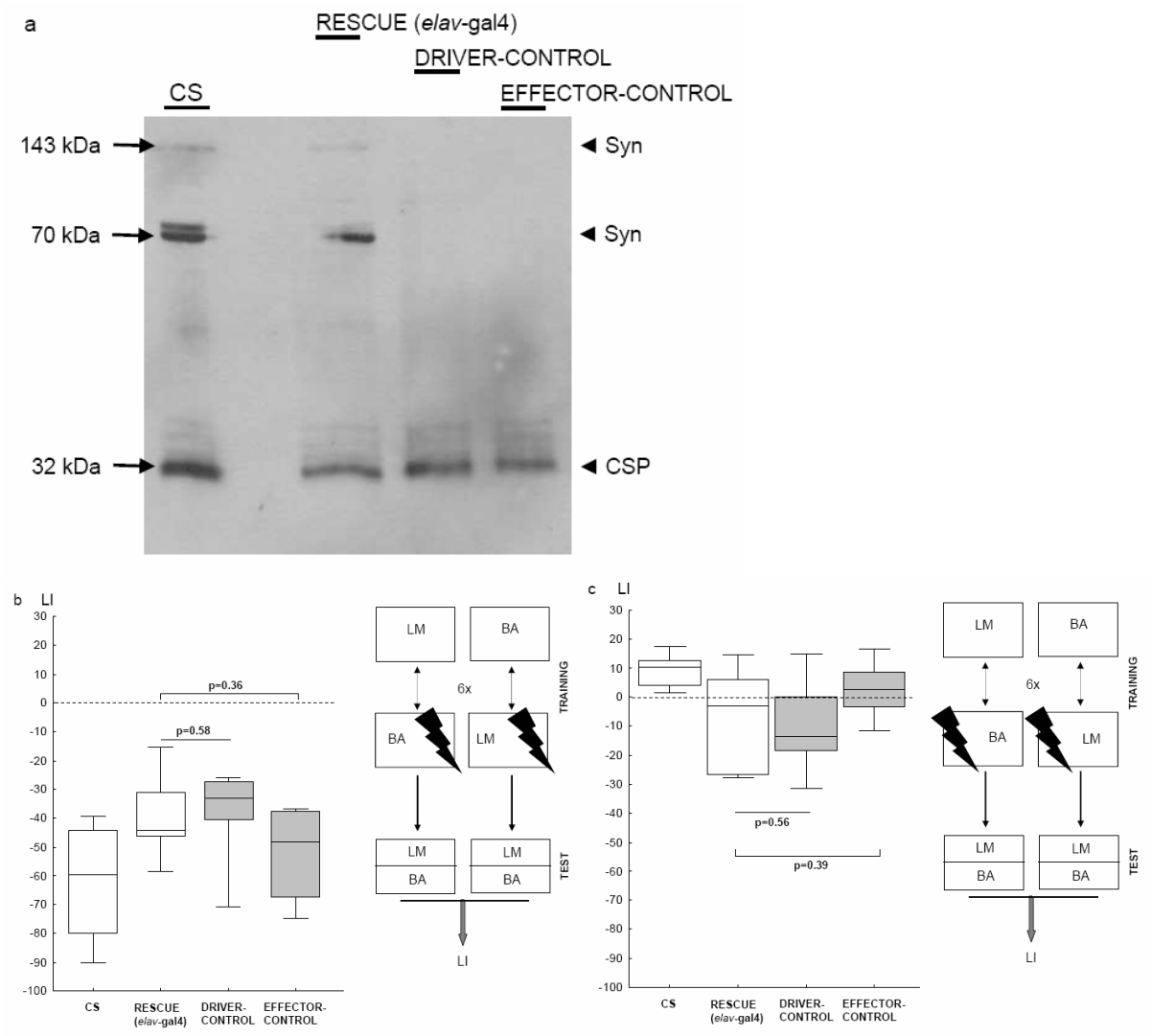

Fig. S1: In the *syn*^{97} mutant background, restoring Synapsin with *elav*-Gal4 [X] does not restore learning; elav-Gal4; *syn*^{97} was generated by classical genetics based on *elav*-Gal4 (Lin and Goodman 1994)

a. Western blots from heads stained for Synapsin and for CSP as loading control. The lanes are loaded from left to right with heads from CS wild-type, rescue, driver-control, and effector-control flies. At 143 kDa and 70 kDa, Synapsin isoforms are found as expected. These bands are absent in control flies, as these are in the *syn*^{97} mutant background.

b. Punishment-learning remains impaired in the *syn*^{97} mutant upon expressing Synapsin with *elav*-Gal4 as driver. CS wild-type, rescue, driver-control, and effector-control flies show punishment-learning (Fig. S1b; one-sample sign tests: *P*> 0.05/4 in all cases; sample sizes are from left to right N = 6, 5, 6, 6). Levels of learning are not significantly different between rescue and either the driver-control or the effector-control flies (Fig. S1b; U-test: U= 12.0, *P*= 0.58 regarding the comparison to the driver-control and U= 10.0, *P*= 0.36 regarding the comparison to the effector-control; sample sizes as above). This is in accordance with the finding of Zars et al. (2000) that *elav*-Gal4 as driver also does not rescue the punishment-learning defect of *rutabaga* mutants; this may be due to either the amount of Synapsin/ rutabaga expression, its cellular site, and/ or its timing being inappropriate with regard to this learning task.

c. Relief-learning also remains impaired in the *syn*^{97} mutant upon expressing Synapsin with *elav*-Gal4 as driver. Only CS wild-type flies show relief-learning (Fig. S1c; one-sample sign test: *P*< 0.05/4; whereas for rescue, driver-control and effector-control: *P*> 0.05/4; sample size from left to right N= 8, 7, 8, 6). Correspondingly, there is no difference between the rescue and either the driver- or the effector-control flies (Fig. S1c; U-tests: U= 23.0, *P*> 0.05/2; regarding the comparison to the driver-control; U= 15.0, *P*> 0.05/2; regarding the comparison to the effector-control; sample sizes as above).

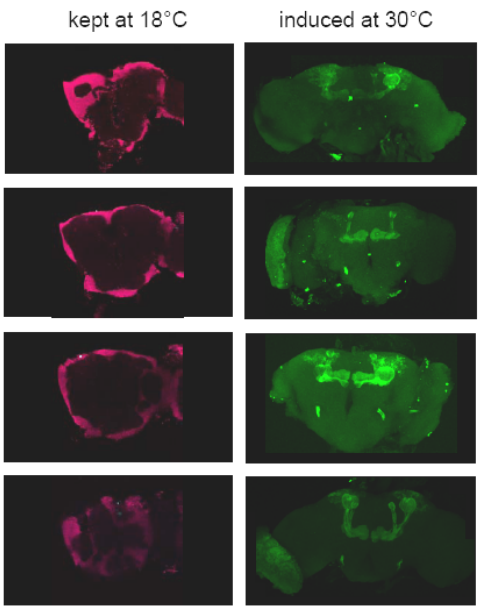

Fig. S2: Induced rescue of Synapsin in the mushroom body. We combine *tub*-Gal80^{ts} with *mb247*-Gal4 and UAS-*syn* for induced expression of Synapsin specifically in the mushroom body, by temperature shift. Immediately after induction in the adult stage (48h at 30°C), wholemounts are prepared.

a. The lack of Synapsin expression in non-induced flies and the successful expression of Synapsin in the mushroom body after incubation is evident in wholemount preparations. Left column: DNA-counterstaining with propidium iodide (magenta) in central brain sections of experimental flies kept at 18°C (Synapsin expression not induced). Right column: Anti-Synapsin staining (green) in z-projections of flies with induced Synapsin expression (48h at 30°C).

Chapter III

Odour perception matches physiological activity patterns in second-order olfactory neurons

Niewalda T, Völler T, Ehmer J, Fiala A, Gerber B

Odour perception matches physiological activity patterns in second-order olfactory neurons

Thomas Niewalda, Thomas Völler, Julia Ehmer, André Fiala, Bertram Gerber

Abstract

How do physiology and perception relate? Given that sensory processing is multi-layered and parallel, the question actually is where along these various processing streams physiological activity patterns and perception correspond. We study the relation between olfactory physiology and perception in *Drosophila*. Perceived-distance between odours is determined by a series of odour recognition experiments. Then, using optical imaging of genetically encoded calcium sensors, odour-induced activity patterns in first- and second-order olfactory neurons are measured to derive, for either site of measurement, physiological-distance scores between odours. We find that physiological distances match perceived distances at the second- rather than the first-order olfactory processing stage. Notably, these distances also match a comprehensive description of the physico-chemical properties of the odours. Our results suggest that the processing step from first- to second-order olfactory neurons categorizes odour representations according to the physical properties of the odour and that these representations can account for the flies´ perception of odours.

Introduction

The discovery of the *Or* gene family in mammals (Buck and Axel 1991), and the subsequent discovery of a functionally corresponding gene family in *Drosophila* (Clyne et al. 1999; Vosshall et al. 1999), was a break-through for research into how olfactory stimuli are translated into physiological activity. This has led to a reasonably detailed picture of how different odours can cause different activity patterns along the olfactory pathway (Stocker 1994; Strausfeld and Hildebrand 1999; Galizia and Menzel 2000; Hallem and Dahanukar 2006; Vosshall and Stocker 2007; Gerber et al. 2009). Odours are detected by sensory neurons housed within hairs on the third antennal segment and maxillary palps; these sensory neurons project to the antennal lobes, the functional equivalent of the olfactory bulb in vertebrates. Each sensory neuron expresses one or few functional *Or* genes, endowing different types of sensory neurons with only partially overlapping ligand profiles (Stortkuhl and Kettler 2001; Hallem and Carlson 2006; Pelz et al. 2006; Kreher et al. 2008); those sensory neurons expressing a common *Or* gene then converge onto one glomerulus within the antennal lobe

(Couto et al. 2005; Fishilevich and Vosshall 2005). For different odours, this entails different combinatorial activity patterns of glomeruli (Fiala et al. 2002; Ng et al. 2002; Wang et al. 2003). Within the antennal lobe, local interneurons shape olfactory signals at the step from sensory neurons to projection neurons (Wilson et al. 2004; Wilson et al. 2005; Bhandawat et al. 2007; Olsen et al. 2007; Root et al. 2007; Olsen et Wilson 2008; Root et al. 2008). From the antennal lobe the projection neurons, corresponding to the mitral cells in vertebrates, relay to the lateral horn, a presumed premotor center, as well as to the Kenyon cells of the mushroom body (Marin et al. 2002; Wong et al. 2002; Masuda-Nakagawa et al. 2005; Murthy et al. 2008), which may be viewed as corresponding functionally to cortical pyramidal neurons in mammals (Davis 2004). Output from the mushroom bodies then projects to presumed premotor areas as well (Ito et al. 1998; Tanaka et al. 2008; Wang et al. 2004). However, whether and at which stage of this pathway activity patterns are relevant for perception is only beginning to be understood (Kreher et al. 2008; Guerrieri et al. 2005). Our results suggest that the processing step from first- to second-order olfactory neurons categorizes odour representations according to the physical properties of the odour and that these second-order representations are the basis of the flies´ perception of odours.

Results

We reason that, within a neuroscience context, perception has to be conceived of behaviourally: if two stimuli are perceived differently, these differences should make (or should allow making) a difference to the animal in terms of its behaviour. We therefore first provide such an behavioural account of perceived difference between odours and then ask at which stage along the fruit fly olfactory pathway a fit is found between physiology and these measures of perception. Finally, we will discuss the relation of both these perceptual and physiological distance measures to the physico-chemical properties of the odours.

Behaviour. We report four independent olfactory recognition experiments to come up with one comprehensive score of perceived distance between six different odour pairs; in all cases, we ask whether flies perceive a test odour *as the same* or *as different* from a previously learned olfactory stimulus.

(i) & (ii) Flies are trained by presenting an odour together with electric shock and then are tested for their avoidance of either that trained odour or for their avoidance of a novel, not previously experienced odour (the dilutions of the four odours are adjusted for equal learnability [Fig. 1A; Fig. S1]). When novel odours are used for testing, learning scores in all cases are symmetrical (Fig. 1B): scores are equal when e.g. the odorant 3-octanol (O) is

trained and *n*-amylacetate (A) is tested as compared to when A is trained and O is tested. We therefore pool these respective subgroups for further analyses. It turns out that in most cases learned responses can hardly be called up by novel odours, reflecting perceived dis-similarity between trained and tested odour (red arrows in Fig. 2A depict a 'Preceptual Distance Score 1'; Fig. 2A´ presents these scores normalized to the highest score thus obtained). An exceptional case are O and A, as training with one of these odours allows the respective other odour to call up a substantial share of the learning score, both when scores are taken (i) immediately (Fig. 2A, 2A´) and (ii) after an additional retention period of 180 min (Fig. 2B, 2B´) (see Fig. S2B concerning the symmetry of these 180-min scores). We interpret such responses as reflecting perceived similarity between these two odours.

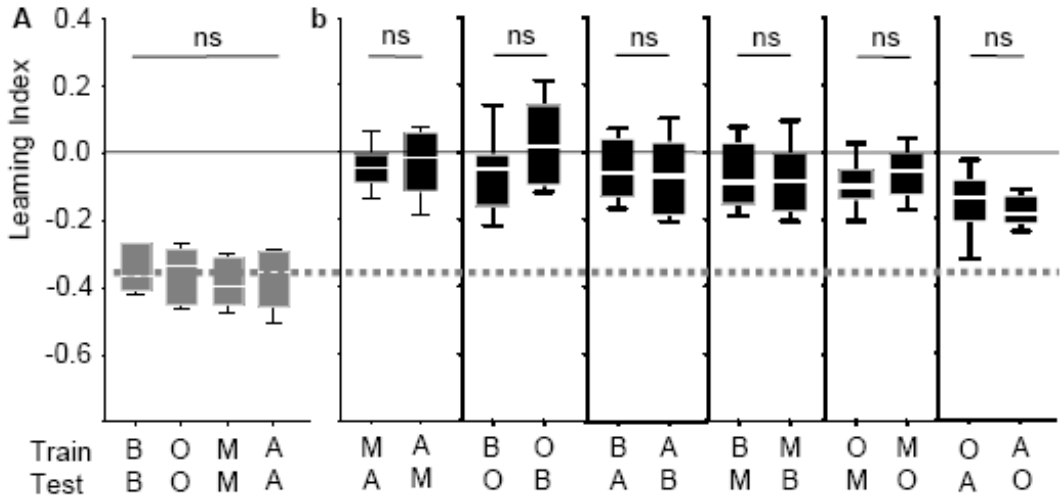

Fig.1: Symmetry of perceived distance
Learning indices (LIs) dependent on the combination of TRAINing *versus* TESTing odour (benzaldehyde: B, 3-octanol: O, 4-methylcyclohexanol: M, *n*-amylacetate: A). In (**A**), flies are tested with the trained odour, whereas in (**B**) they are tested with a not previously trained odour. Odour-intensities were chosen for equal learnability based on Fig. S1. The stippled line in (**B**) represents the median of the pooled data from (**A**).
Learning indices in (**B**) are in all cases symmetrical, in the sense that scores are equal when e.g. O was trained and A is tested as compared to when A was trained and O is tested.
NS: in (**A**) P> 0.05 in a Kruskal-Wallis test, in (**B**) P> 0.05/ 6, Mann-Whitney U-test using a Bonferroni correction as applicable. Data are presented as box plots (middle line: median; box boundaries and whiskers: 25 %/ 75 % and 10 %/ 90 % quantiles). Sample sizes are from left to right: 11, 12, 11, 11, 16, 16, 16,16, 16, 16, 16, 16, 16, 15, 16, 16.

(iii) We train flies with joint presentations of one odour with electric shock and then test the flies for their choice between that trained odour *versus* a novel odour. We reason that to the extent that the flies regard the two odours as different, they should distribute unequally between them; thus in this experiment perceived distance between the choice-odours should show as large learning score (green arrows in Fig. 2C depict a 'Preceptual Distance Score 3'; Fig. 2C´ presents these scores normalized to the highest score thus obtained). We find that

perceived distance is smallest between O and A also in this kind of assay (Fig. 2C, 2C´) (see Fig. S3 concerning the symmetry of scores).

(iv) We 'explicitly' train flies to discriminate between two odours, such that one odour is presented together with an electric shock, whereas the other odour is presented alone. At test we then present both odours in a choice situation. Rationale here is that the more different both odours are regarded by the flies, the easier would it be to make a difference between them; thus, perceived distance should show by easy discrimination and hence high learning scores (blue arrows in Fig. 2D depict a 'Preceptual Distance Score 4'; Fig. 2D´ presents these scores normalized to the highest score thus obtained). We find that again flies regard O and A as least distant (Fig. 2D, D´).

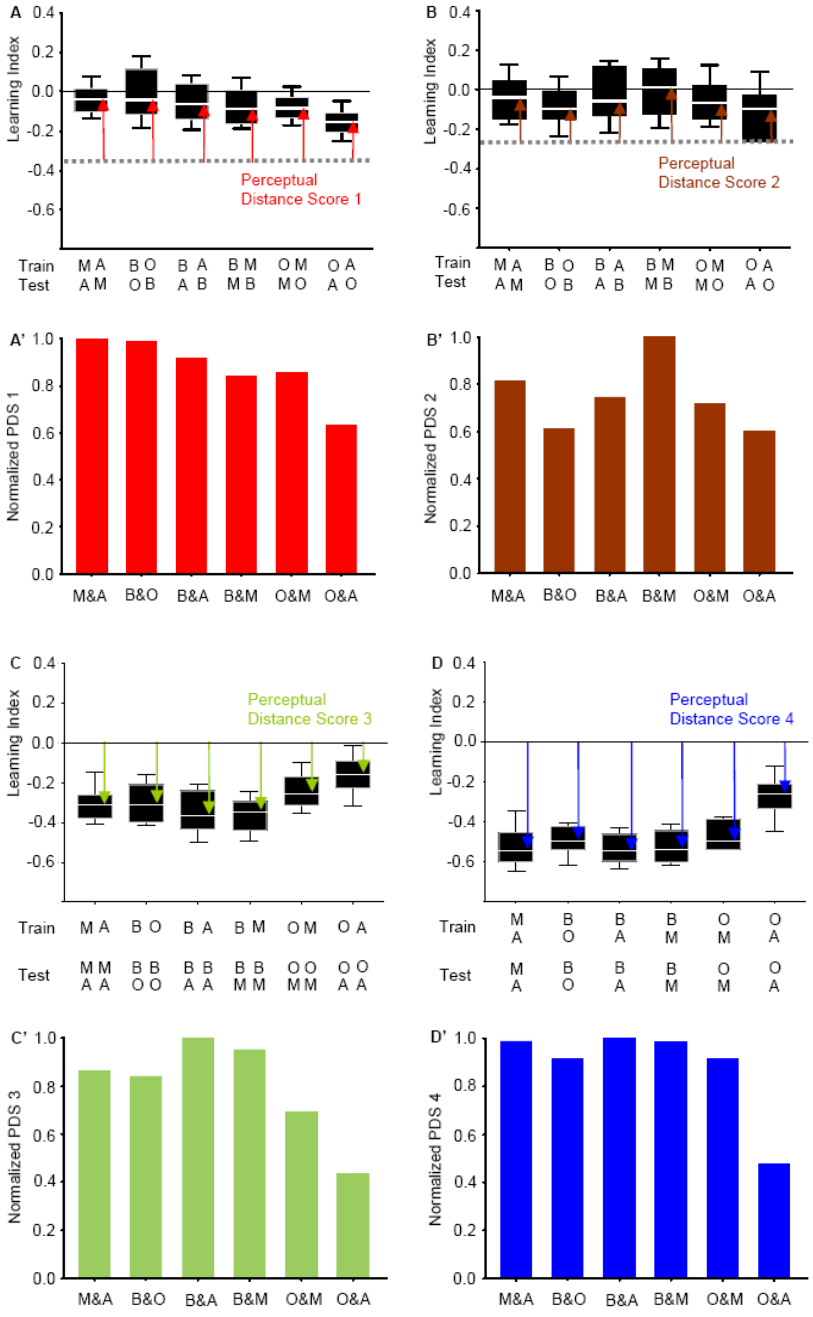

Fig. 2: Concordant perceived distance across four types of behavioural experiment
(**A**) Re-presenting the data from Figure 1, pooled for odour pairs; for abbreviations of odour identity, see legend of Fig. 1. The stippled grey line represents the learning indices that are found when TRAINing and TESTing odour are the same (see Fig. 1A). If, however, flies regard the TESTing odour as different from the TRAINing odour, learning indices should be zero; thus, the degree to which flies regard both odours as different can quantified by the Preceptual Distance Score 1 (red arrows). In (**A´**) these scores are presented normalized to the highest score thus obtained. Sample sizes are from left to right: 32, 32, 32, 32, 31, 32. Other details as in Fig. 1.
(**B**) Same as in (**A**), except that an additional 180-min break is given between training and test. Sample sizes are from left to right: 24, 24, 24, 24, 24, 24.
(**C**) Flies are trained with a given odour, and then are tested for their choice between that trained odour *versus* a novel, not previously trained odour. Thus, if the flies regard the two TESTing odours as the same, scores should be zero. To the extent that both
odours, however, are regarded as different by the flies, learning indices should increase. The level of perceived difference thus can be approximated by the Preceptual Distance Score 3 (green arrows). In (**C´**) these scores are presented normalized to the highest value thus obtained. Sample sizes are from left to right: 24, 24, 20, 23, 24, 24. Other details as in Fig. 1.
(**D**) Flies are trained such that one odour is punished but the other odour is not punished; then, flies are tested for their choice between these two odours. Thus, if the flies cannot tell the two testing odours apart, scores should be zero. To the extent that both odours, however, can be discriminated by the flies, learning indices should increase. The level of perceived difference thus can be approximated by the Preceptual Distance Score 4 (blue arrows). In (**D´**) these scores are presented normalized to the highest value thus obtained. Sample sizes are from left to right: 15, 11, 12, 11, 11, 12. Other details as in Fig. 1.

As perceived distances in these four kinds of assay are fairly concordant (compare Fig.s 2A´, B´, C´, D´), we combine the respective normalized distance scores to yield one comprehensive score of perceived distance for each of the six odour pairs: perceived difference between O and A appears particularly low (Fig. 3A).

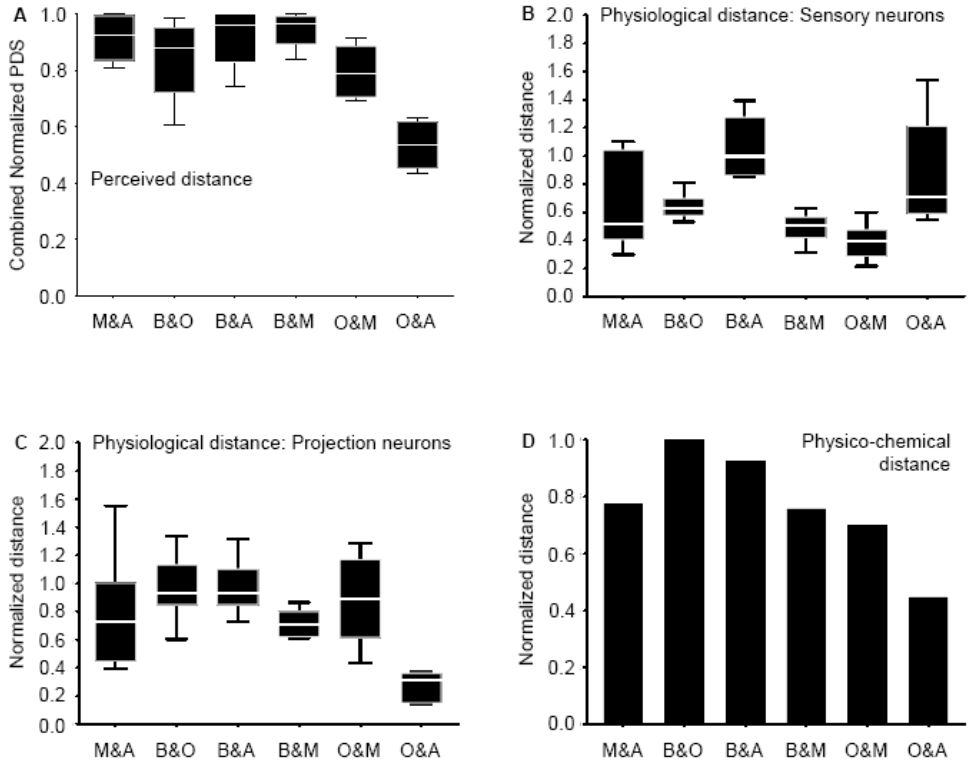

Fig. 3: Concordant distances for perception, projection neuron physiology and physico-chemical properties
(**A**) The normalized Preceptual Distance Scores (Fig.s 2 A´- D') presented combined. Note the small perceived distance between O and A.
(**B**) Physiological 'distances' between the indicated odour pairs as calculated by principal component analysis from calcium imaging data of sensory neurons (see Fig.s 5, 6). O and A do not appear particularly similar from this analysis of sensory neuron activity.
(**C**) Physiological distance as found in projection neurons. Note that O and A turn out as particularly similar.
(**D**) Distances between odour pairs as derived from an exhaustive physico-chemical description (Haddad et al. 2008); O and A appear particularly similar in this kind of analysis.
For abbreviations of odour identity, see legend of Fig. 1

Physiology. To test where along the olfactory pathway differences in neuronal activity match perceived distance between odours, we perform calcium imaging experiments (Fiala et al. 2002; Fiala and Spall 2003). The DNA-encoded fluorescence calcium sensor cameleon 2.1 (Miyawaki et al. 1999) is expressed either in first- or in second order olfactory neurons, i.e. either in sensory neurons or in projection neurons. Odour-evoked calcium increases in these respective populations of cells are measured at the antennal lobes, the site where the sensory neurons relay onto the projection neurons; we use the same odorant dilutions as for the behavioural experiments.

Regarding olfactory sensory neurons, Figure 4A-C exemplifies for benzaldehyde (B) and 3-octanol (O) that calcium signals in the antennal lobe are odour-specific, spatially restricted, bilaterally symmetric, and show remarkably high signal-to-noise ratio. Importantly, the odour-evoked patterns of activity are consistent across individuals, which allows us to compare the activity patterns, averaged across individual flies, between the four odours.

Obviously, the four odours evoke distinct activity patterns at the input stage to the antennal lobe (Fig. 5A) with the activation by O nested within the pattern evoked by A. In order to subject these activity patterns to quantitative analysis, we perform a pixel-wise principal component analysis (PCA), reducing the multidimensional 'odour-space' to two dimensions represented by the first two principal components, covering a total of 66.34 % of the variability in the dataset. In such a PCA, data from the eight experimental flies cluster for each of the four odorants (Fig. 6A); for an internal measure of the fidelity of our method, please note that this PCA yields consistent results for the left and right antennal lobes. Measurements of the distances between pairs of odours in this sensory-neuron 'odour space' (Fig. 3B) do not yield any apparent match to the perceived distances as derived from behavioural analyses (Fig. 3A); specifically, such PCA does not uncover a particularly low distance between O and A. Thus, activity patterns in first-order olfactory neurons do not match perceived distance between odours.

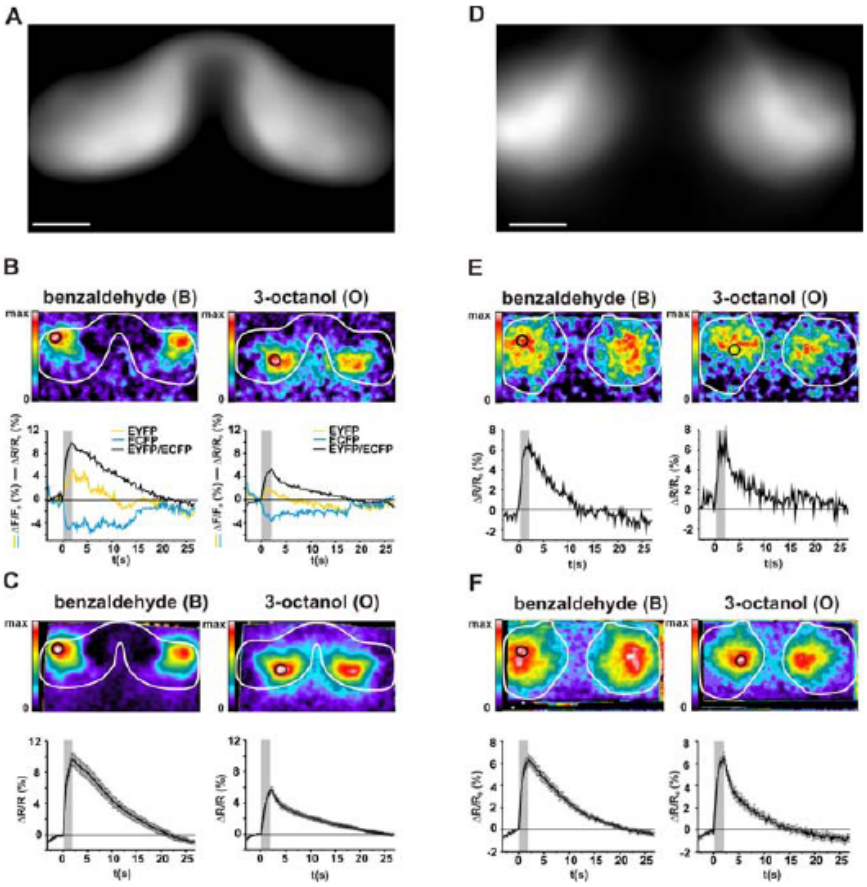

Fig. 4: High signal-to-noise ratio and low inter-individual variability in physiology
(**A**) To illustrate the shape of the antennal lobe as apparent in measurements of the sensory neurons, EYFP emission averaged across 8 individual flies is presented. Scale bar 25 m.
(**B**) Single-fly example of calcium activity in the antennal lobes (white circumfence-line) in sensory neurons after stimulation with benzaldehyde (left) or 3-octanol (right), displayed in false-colour (top). For the encircled region of interest, the time course of the measurements is displayed (bottom) for EYFP (yellow), ECFP (cyan) and the EYFP-to-ECFP ratio (black). The grey bar indicates duration of the odour stimulus.
(**C**) Calcium activity in olfactory sensory neurons averaged across 8 individual animals displayed in false-colour (top). For the encircled region of interest, the time course of calcium activity is displayed for the EYFP-to-ECFP ratio (bottom). Data represent mean ± SEM. The grey bar indicates the duration of the odour stimulus.
(**D, E, F**) Same as **A, B**, **C**, but for antennal lobe-measurements of projection neuron activity. Corresponding analyses regarding the other two odours (4-methylcyclohexanol and *n*-amylacetate) can be found in Fig. S4.

What, then, about the projection neurons? Odour-evoked activity patterns for O, M, and B are more widely distributed across the antennal lobe when compared to the sensory neurons (Fig. 4B *versus* Fig. 4E and Fig. S4B versus Fig. S4E) and appear less consistent between individual flies (see below). Activity patterns, however, still are sufficiently local and conserved across individual flies to allow averaging across animals and comparison of these averaged activity patterns between odours (Fig. 5B). When the calcium signals for the four odours in the projection neurons are subjected to a PCA, data regarding individual odours are rather widely distributed across the two dimensions, reflecting the above-mentioned higher

inter-individual variability (Fig. 6B). Importantly, while five of the odour pairs yield separable clusters in this PCA, the data for O and A form one merged cluster. This is different from what has been mentioned above regarding sensory neurons; it suggests some within-antennal lobe processing which leads to odour classification, in the sense that it leads to a levelling-out of between-odour differences at the step from sensory neurons to projection neurons. Notably, this comes about by a sharpening of the activity pattern evoked by A such that, while at the level of the sensory neurons the signal evoked by O is nested within the one evoked by A, both odours activate practically overlapping areas of the antennal lobe when the projection neurons are considered (Fig. 5). Most importantly, however, this overlap of pattern for O and A in the projection neurons matches to the difficulty the flies have in telling apart these two odours (compare Fig. 3A to C). In other words, differences in activity pattern of second-order olfactory neurons do match perceived distance between odours, such that the processing step from first- to second-order olfactory neurons apparently corresponds to a classification step, making the activity patterns for O and A more similar.

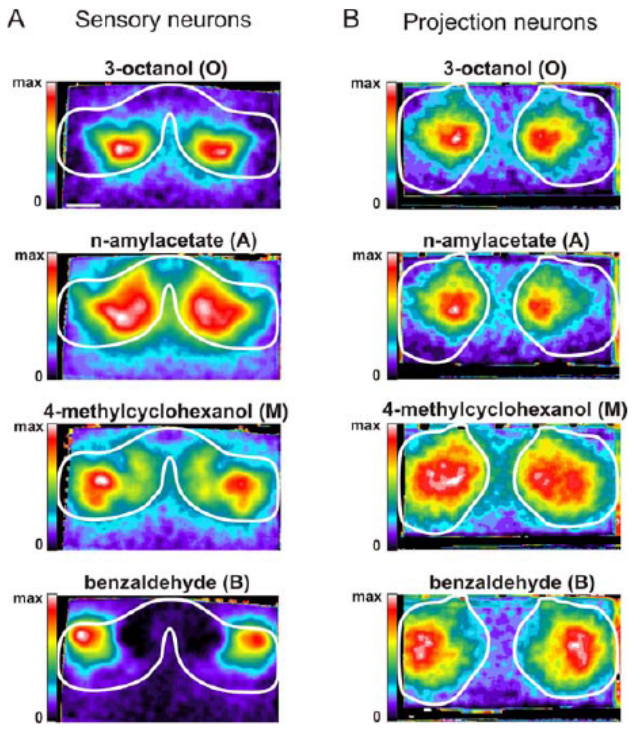

Fig. 5: Activity patterns in first- and second-order olfactory neurons neurons
False-colour coded calcium activity patterns in the antennal lobes evoked by four different odorants, measured from (**A**) sensory neurons or (**B**) projection neurons. Images represent averages of eight individual flies each. Data are normalized to the maximum signal of the averaged image. The white lines indicate the outline of the antennal lobes as labelled by the respective Gal4-line (*Or83b*-Gal4 and *GH146*-Gal4, respectively; see Fig. 4A, D). Note that in the sensory neurons, the activity pattern evoked by O is nested within the one evoked by A; however, in the projection neurons, the pattern evoked by A is sharpened, such that O and A evoke the same pattern of activity.

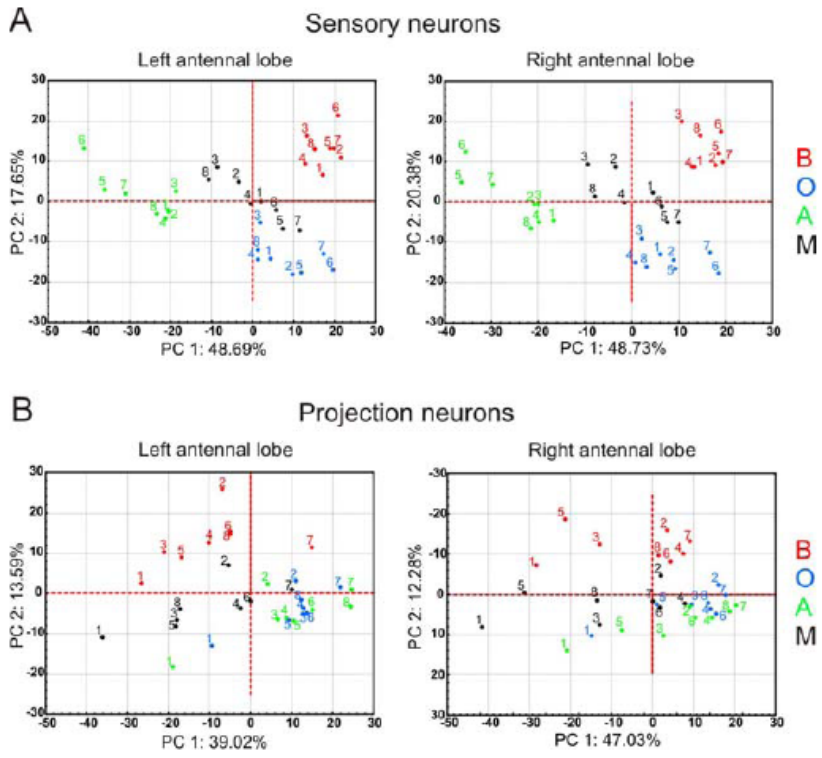

Fig. 6: Quantification of pattern similarity
Pixel-wise principal component analysis across odour-evoked calcium activity within the antennal lobes as measured from (**A**) sensory neurons or (**B**) projection neurons. Different colours indicate different odorants (red: B, blue: O, green: A, black: M; for abbreviations of odour identity, see legend of Fig. 1). Numbers indicate the individual animals tested. Note that in projection neurons, but not in sensory neurons, the representations of O and A overlap.

Discussion

These findings critically extend an earlier study in the honeybee (Guerrieri et al. 2005) that used classical conditioning of the proboscis extension reflex. One out of 16 odours was trained by presenting it together with a sugar reward. Testing of bees was then carried out with a random draw of four from these 16 odours. Such data were then used to generate a 16-dimensional behavioural 'odour space'. Euclidian distances between odour pairs could thus be used for a correlation analysis with odour-pair wise similarities of physiological activity in the antennal lobe which had been obtained ealier (Sachse et al. 1999) using bath-applied calcium dyes. It turned out that behavioural and physiological distances between odour pairs matched fairly well. However, using bath-applied dyes does not allow one to attribute the cellular identity of the measured cells with reasonable certainty. Using genetically encoded calcium sensors, however, leaves little doubt about the cellular source of the signal. Thus, the current approach could specifically pin down the projection neurons as site of correspondence between physiology and perception, and can thus assign a specific, categorizing function to the processing step between sensory- and projection neurons. Also, in the study by Guerrieri et al. (Guerrieri et al. 2005) behavioural scores were in a number of cases asymmetrical:

response levels to aldehydes were generally high after training to odours of other functional class (primary and secondary alcohols, ketones), whereas after training with aldehydes response levels to odours from these other classes were low. Such asymmetries violate common sense notions of similarity, which imply that if X is similar to Y, in turn Y should be similar to X to the same extent. Such asymmetries can result from not adjusting odour intensities for equal learnability, a complication that we took great pains to avoid.

Our findings, however, may appear inconsistent with the recent report of Kreher et al. (Kreher et al. 2008). The authors measured odour-induced electrophysiological activity in adult olfactory sensory neurons which express, rather than their cognate adult *Or* gene, only one of the 21 larval-expressed *Or* genes (see also (Hoare et al. 2008) for *in situ* measurements of larval olfactory sensory neurons which yield fairly, yet not perfectly, corresponding results). This was done for all these 21 larval *Or* genes and a panel of 26 odours to obtain a physiological 'odour space'. Behaviourally, the authors assayed larval *Drosophila* for discrimination ability using a 'masking' experiment: one odour was presented as a point source within the background of another odour ubiquitously present throughout the experimental arena. Rationale was that if a larva does respond to the point source despite the 'mask', it must have the ability to tell both apart. Note that this is a sound argument only when no behavioural responses to the point source are seen if the same odour is used as both point source and mask; that is, when the intensity of the mask indeed saturates the behavioural response. This was shown to be the case for three out of the six odours thus assayed. Also, results were in some cases asymmetric, such that odour X as mask had less effect on responses to odour Y as point source than Y as mask had on X as point source (e.g. ethyl acetate and E2-hexenal as well as ethyl butyrate and 2,3-butanedione). In any event, the authors used these masking data as a measure of perceptual similarity, and found that it correlates fairly well with the distances between odours in the physiological 'odour space' they had described. In other words, a partial correspondence was found between physiology and perception already at the level of first-order olfactory neurons. However, studying only the sensory neurons may overlook potentially better matches between physiology and perception in the projection neurons. Also, it may be that for different kinds of behavioural experiment (masking *versus* recognition), different sites along the olfactory pathway are important for behavioural similarity judgements. Specifically, masking may come about by adaptation within sensory neurons and thus the physiology of these very neurons may be critical for masking-based measures of perception. In turn, more central processing stages may be involved in recognition-type measures of perceived similarity, as in our case.

Finally, when a physico-chemical distance score between odour pairs is calculated which considers a large number of molecular properties (Haddad et al. 2008) and which reasonably predicts physiological distances in several species as well as perceptual distances in humans (Khan et al. 2007), we observe a reasonable match of these physico-chemical scores (Fig. 3D) to the physiological distance scores obtained from the projection neurons (Fig. 3C) as well as to perceived distance (Fig. 3A). In particular, the high perceptual as well as physiological similarity (as measured in the projection neurons) of O and A is found in these physico-chemical scores as well. This may imply that olfactory systems are mapping the physico-chemical properties of odours rather faithfully into physiology and perception, and that, by and large, the same mapping may apply to any behavioural task, memory stage (but compare Fig.s 2A´ to Fig. 2B´ and note the discussion in (Hammer and Menzel 1998; Menzel 1999 regarding the bee), life stage (see discussion in (Kreher et al. 2008 regarding larval *versus* adult *Drosophila*), and species (Stensmyr et al. 2003). We note, however, that well-trained human subjects (N= 75) rarely confuse the six odour pairs used in our study (on average, only 7.3 % of subjects erred in triangulation tests; Dr. Anja Finke, Symrise GmbH & Co. KG, Holzminden, Germany, pers. comm.) [see also Keller and Vosshall (2007)]. Thus, it may not be wise to take the correspondences between perceptual distance, projection-neuron physiological distance, and physico-chemical distance to its logical extreme. Indeed, these correspondences are coarse, within this as well as earlier (Kreher et al. 2008; Guerrieri et al. 2005) studies. To name just the most obvious sources of distortion, this may be due to differences in genotype between behavioural and physiological measurements, imperfections and/ or incompleteness of physiological measurements, the number of odours sampled, and/ or to specific demands imposed by the respective behavioural assays. Also, processing stages downstream of the projection neurons, in particular in the mushroom bodies, may contribute to shape perception, providing the fly with a faithful account of the odours´ physico-chemical properties only from the concerted activity in projection neurons and mushroom bodies (Masuda-Nakagawa et al. 2005; Murthy et al. 2008). These reasonings in mind, a substantially more fine-grained match between perception, local physiology, and physico-chemical odour features may not reasonably be expected.

In summary, our results argue that the processing step from first- to second-order olfactory neurons is acting to categorize odour representations according to the physical properties of the odour, and that these categories are the basis for the flies perception of odours.

Experimental Procedures

Behaviour. Wild-type Canton-S flies are kept in mass culture at 25 °C, 60-70 % humidity and a 14/ 10 hour light/ dark cycle. Flies are collected one to five days after hatching from the pupal case and kept over-night at 18 °C.

Training is performed in dim red light, testing in darkness. As to-be learned stimuli we use benzaldehyde, 3-octanol, 4-methylcyclohexanol, or *n*-amylacetate (B, O, M, A) (CAS: 100-52-7, 589-98-0, 589-91-3, 628-63-7; all from Fluka, Steinheim, Germany, except A, which is from Merck, Darmstadt, Germany), or of ambient air (Θ). A vacuum pump ensures removal of odour-saturated air from the training apparatus. Odorants (130 µl) are applied in Teflon cups of 7-mm diameter either in pure condition or diluted in paraffin oil (B: 1:66; O: 1:1000; M: 1:25; A: 1:1000, unless mentioned otherwise) (paraffin oil from Merck, Darmstadt, Germany). At time:= 0 min, groups of about 100 flies are loaded to the training tubes of the experimental apparatus which allow applying electric shock via an electrifiable grid covering the inner side of the tube. At time:= 2 min, the unconditioned stimulus (either B, O, M, A, or Θ) is presented for 60 s without ensuing punishment. At time:= 4 min, the conditioned stimulus (any of the remaining four stimuli) is presented for 60 s; 15 s after conditioned stimulus onset, an electric shock is applied (90 volts, 12 pulses á 1.2 s within 60 s, using an onset-onset interval of 5 s). At time:= 9:00 min, flies are transferred back to their food vials for 13 min until the next of the in total three such training cycles starts. Across independent measurements, the sequence of stimulus presentation is either as indicated during all three training cycles, or is reversed such that the first stimulus presented is punished during all cycles.

Once training is complete, the regular 13 min break is given (unless mentioned otherwise) until animals are loaded again to the apparatus for testing. After an accommodation period of 4 min, animals are transferred to the choice point of a T-maze, where they choose between two of the five above-mentioned stimuli as indicated below and in the body text for the respective experiment. After 2 min, the arms of the maze are closed and the number of animals (denoted # in the following) within each arm is counted. A preference index (PI) is calculated as:

$$PI = (\#_{\text{Punished stimulus}} - \#_{\text{Non-punished stimulus}}) / \#_{\text{Total}} \qquad (1)$$

After one such score has been obtained, a second set of flies is trained reciprocally: If e.g. in Experiment (iv) (Fig. 2D), one set of flies is punished when receiving M but not when

receiving A, the second set of flies is trained by presenting A with and M without punishment. PIs of these two reciprocally trained sets of flies are then averaged to obtain a learning index (LI). Thus, positive LIs indicate conditioned approach, negative LIs conditioned avoidance. Data are presented as box plots with the middle line showing the median and box boundaries and whiskers the 25%/ 75% and 10%/ 90% quantiles, respectively, and are analyzed with non-parametric statistics (Statistica, Statsoft, Hamburg, Germany), using a Bonferroni correction as applicable. Flies are trained and tested only once.

After adjusting odour dilutions for equal learnability (Fig. S1; Fig. 2a), four experiments of increasing complexity are performed:

(i, ii) In a 4 x 4 experimental design, flies are trained with any one of the four odours *versus* Θ. Then, they are tested either for their avoidance of the trained odour, or of any one of the remaining three non-trained odours, *versus* Θ. This is done either after the regular 13-min break (i), or after an additional 180-min waiting period (ii).

(iii) Flies are trained as in the previous experiment, but then are tested in a two-odour choice between the punished *versus* any of the three non-punished odours.

(iv) Flies are trained differentially between two odours and then are tested for their relative preference between them in a two-odour choice situation.

Physiology. Cameleon 2.1 (Miyawaki et al.1999) is expressed from either *Or83b*-Gal4 (Larsson et al. 2004) or *GH146*-Gal4 (Stocker et al. 1997). All animals are homozygous for both the UAS:*cameleon* insertion (Diegelmann et al. 2002: strain 82) and the respective Gal4 insertion.

5-7 day-old female flies are briefly cooled on ice for immobilization and restrained by inserting them into a truncated pipette tip with the head sticking out. The fly is glued with its head under a transparency foil and then is fixed on a plastic cover slip using dental glue (Protemp II, 3M ESPE, Seefeld, Germany). The third antennal segments and maxillary palps remain dry and untouched. A window is cut into the head capsule and the hole covered by a drop of Ringer's solution (Estes et al. 1996). The preparation is placed under an upright widefield fluorescence microscope (Zeiss Axioscope 2 FS) equipped with a 40 x water immersion objective (Zeiss Achroplan) (Zeiss, Göttingen, Germany) and a cooled CCD camera (CoolSnap HQ, Photometrics, Pleasanton, CA). Excitation light of 436 nm is provided by a xenon lamp and a grid monochromator (Visitron Systems, Puchheim, Germany). Fluorescence emission is guided through a 455 nm DCLP pass filter (Chroma Technologies, Rockingham, VT, USA); the wavelengths of EYFP and ECFP emission (480 nm and 530 nm, respectively) are separated using a beam splitter (Optical Insights, Santa Fe, NM, USA)

equipped with a cameleon filter set (Chroma Technologies, Rockingham, VT, USA). The two half-images of EYFP and ECFP emissions are simultaneously recorded by the two halves of the CCD chip (1392 x 1040 pixel) at a binning of 4. Data acquisition runs at a frame rate of 5 Hz with an exposure time of 100 ms per frame and is controlled by the MetaFluor software (Visitron Systems, Puchheim, Germany).

Odour delivery is achieved using a custom-built olfactometer. A constant air stream supplied by an aquarium pump is directed via a glass pipette to the fly's antennae and maxillary palps. Using computer-controlled electronic valves the constant airstream is shunted to vials that are either blank, contained paraffin oil as solvent-control or either of the four odorants diluted in paraffin oil to the same extent as for the above behavioural experiments. All flies receive cycles of six stimulations each, in the order blank, solvent, O, A, B, and M; specifically, 2-s stimuli are applied 3 s after the onset of the experiment, followed by a 60 s break after which another stimulus is applied until the set of stimulations is complete; this cycle is repeated 3-5 times for each fly.

Image alignment is performed using a modified version of the ImageJ plugin TurboReg (Thevenaz et al. 1998) that allows for the alignment of images without changing the value of any pixel. Data analysis then is performed using a custom-written Java script implemented in ImageJ. Aligned EYFP and ECFP images are used to obtain EYFP/ECFP ratio images. For calculating odour-evoked calcium signals, five frames before odour onset (frame 8-12) are averaged (prestimulus), and five ratio frames beginning 400 ms after odour onset (frames 18-22) are averaged (stimulus). The averaged prestimulus image then is subtracted from the averaged stimulus image to obtain a calcium signal image. To correct for contaminations, images obtained by shunting clean air through the delivery system are subtracted from all signal images.

Time courses of calcium signals within distinct regions of interest are calculated using the MetaMorph software (Visitron Systems, Puchheim, Germany). For time-resolved estimates of calcium activity (e.g. bottom of Fig. 4B, E), fluorescent emission of EYFP and ECFP outside of the labelled structure (the ‘background’ outside the white circumfence line of e.g. top of Fig. 4B) is at each time point subtracted from the value within the chosen region of interest (F-value) (e.g. black circle in Fig. 4B, top). For calculating changes in fluorescence (ΔF), the F value at odour onset (F_0) is subtracted from the F value at the respective time point; ΔF is then divided by $F_0$for normalization ($\Delta F/F_0$). To exploit the sensors´ nature of increasing EYFP fluorescence and decreasing ECFP fluorescence upon increased calcium levels, which largely eliminates movement artefacts, the ratio of F-values for EYFP and ECFP

is calculated (EYFP/ ECFP) (R-value); thus, the normalized change in this ratio ($\Delta R/R_0$) represents calcium activity. Maximum calcium activity is typically found in a time window 3 s after odour onset (e.g. bottom of Fig. 4B); thus, spatially resolved, false-colour coded images (e.g. top of Fig. 4B) represent calcium activity ($\Delta R/R_0$) for each pixel at this time point.

For principal component analysis (PCA), calcium signal images are four-fold reduced in size. Pixel-wise PCA is calculated on the basis of all measured flies for sensory neurons or projection neurons, respectively, and odours using Statistica software (Statsoft, Hamburg, Germany), and the first two components are displayed in Fig. 6. For each fly, the Euclidian distances between the first two principal components for each pair of odours are determined; for each odour pair, these distances then are combined across flies and displayed as box plots in Fig. 3B, C in a manner normalized to the highest median distance thus obtained.

Physico-chemical distances. To estimate physico-chemical distances between the odour pairs, we used the odour metric of Haddad et al. (2008). To generate this metric, odour structures were obtained from PubChem (http://pubchem.ncbi.nlm.nih.gov/) to be input into the Dragon software (http://www.talete.mi.it/products/dragon_description.htm). In the used version 5.4, this metric represents each odorant as vector of 1664 molecular descriptor values and yields, for the respective odour pairs, the following values: M-A: 28.6755; B-O: 37.0393; B-A: 34.1564; B-M: 27.9832; O-M: 25.8083; O-A: 16.5091. In Fig. 3D, these scores are presented normalized to the highest value thus obtained.

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Supplement

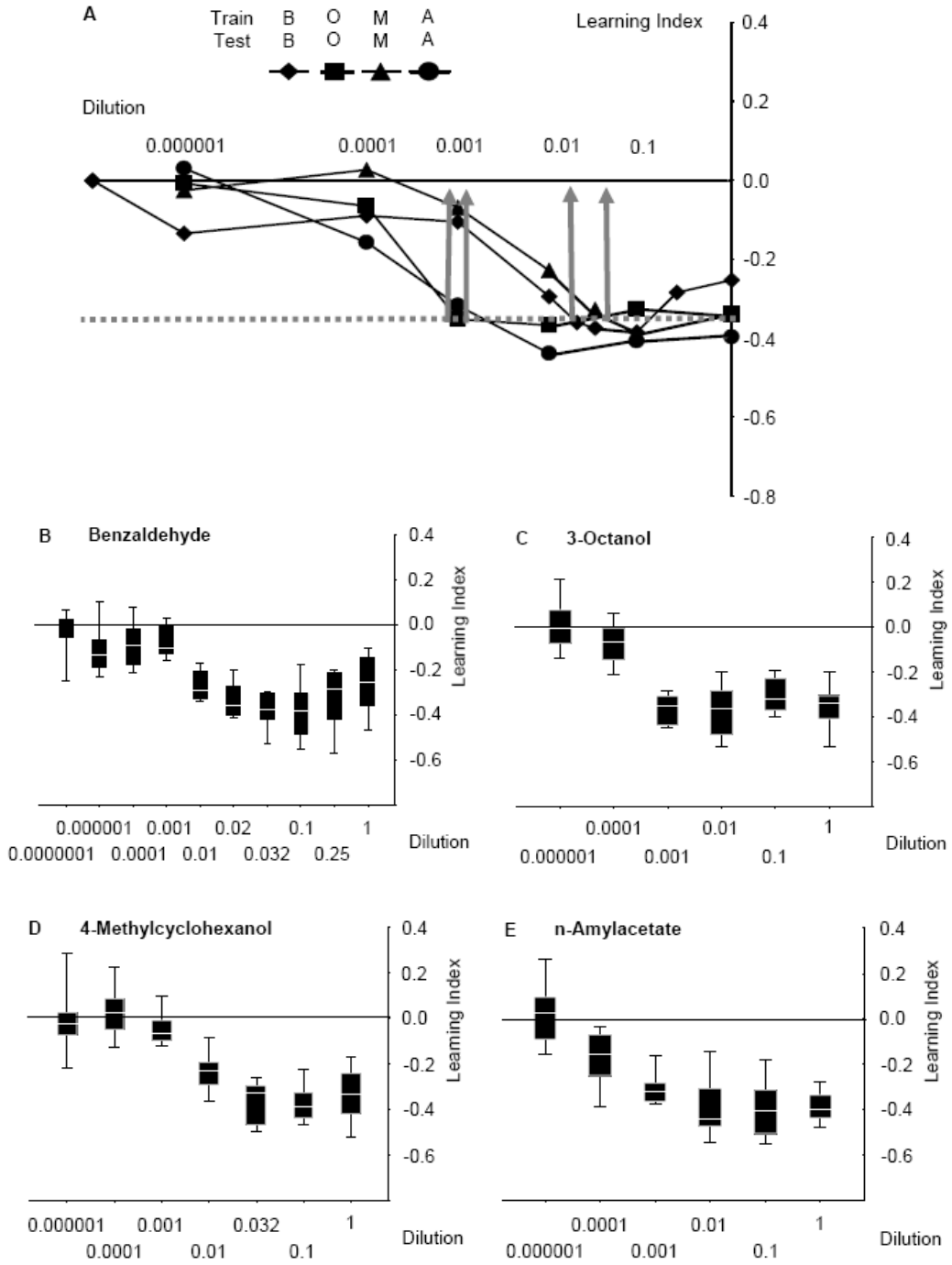

Fig. S1: Adjustment of odour intensity for equal learnability
(**A**) Flies are trained with a given odour at the indicated dilution, and then are tested using that same odour at that same dilution; for abbreviations of odour identity, see legend of Fig. 1. Note that while asymptotic learning scores do not differ between dilutions, the dilutions at which that asymptote is reached differ between odours across almost two orders of magnitude.
Dilutions for further experiments are chosen such that learning indices are the same and, for each kind of odour, have just about reached asymptotic levels (stippled grey line and grey arrows) (B: 1:66; O: 1:1000; M: 1:25; A: 1:1000). Sample sizes are from left to right for B: 12, 12, 12, 12, 12, 12, 12, 8, 8, 8; for O: 11, 12, 12, 12, 8, 8; for M: 12, 8, 12, 12, 12, 8, 8; for A: 12, 12, 12, 12, 8, 8. For details concerning statistics and box plots, see legend of Fig. 1.
(**B, C, D, E**) Data from (**A**) presented as box plots. Sample sizes as in legend of (**A**).

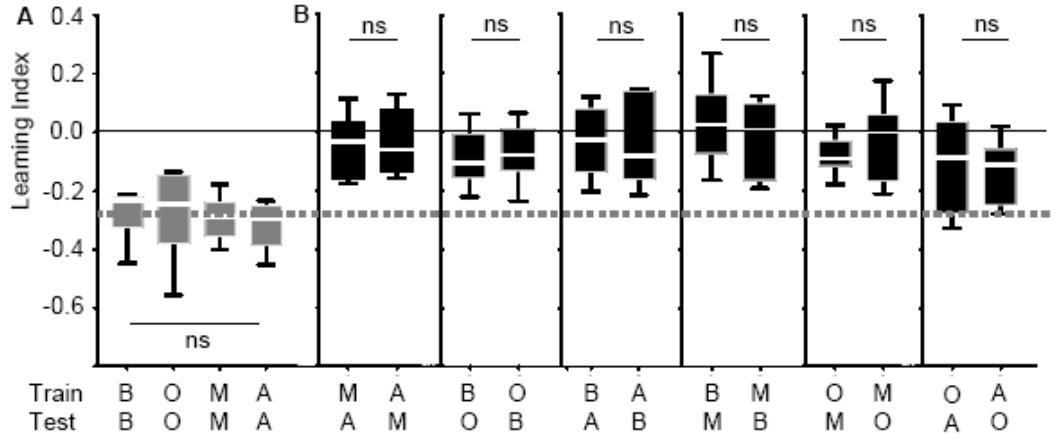

Fig. S2: Symmetry of distance measures
(**A**) Confirming that also after an additional retention period of 180 min learning indices are equal for the chosen dilutions of odour; for abbreviations of odour identity, see legend of Fig. 1. Sample sizes are from left to right 8, 8, 8, 8.
(**B**) Data from Fig. 2B separated by odour; note that learning indices in all cases are symmetrical, in the sense that response levels e.g. to A after training with O are as high as response levels to O after training to A. The stippled line in (**B**) represents the median of the pooled data from (**A**); that stippled line corresponds to the one in Fig. 2B. Sample
sizes are from left to right 12, 12, 12, 12, 12, 12, 12, 12, 12, 12, 12, 12. For details concerning statistics and box plots, see legend of Fig. 1.

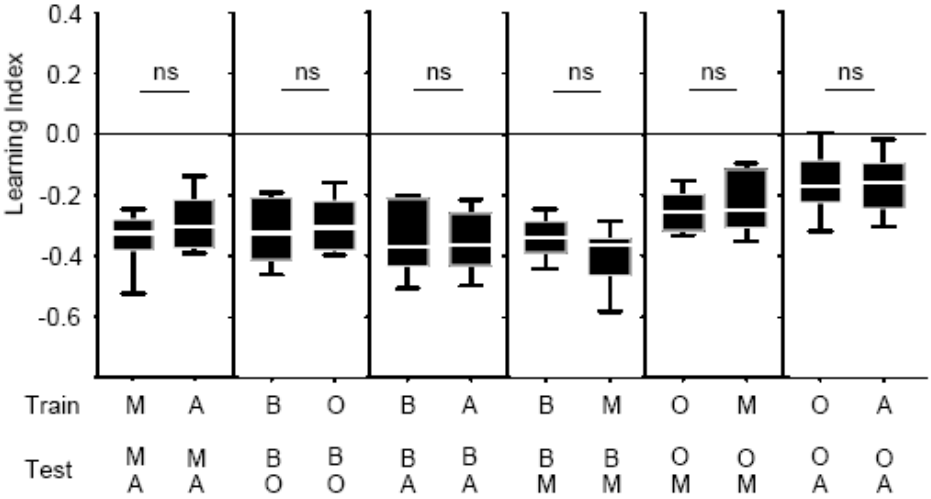

Fig. S3: Symmetry of distance measures
Data from Fig. 2c separated by odour; for abbreviations of odour identity, see legend of Fig. 1. Note that learning indices in all cases are symmetrical, in the sense that learning scores are the same when choice between O and A is assayed after training to O as after training to A. Sample sizes are from left to right 12, 12, 12, 12, 10, 10, 11, 12, 12, 12, 12, 12. For details concerning statistics and box plots, see legend of Fig. 1.

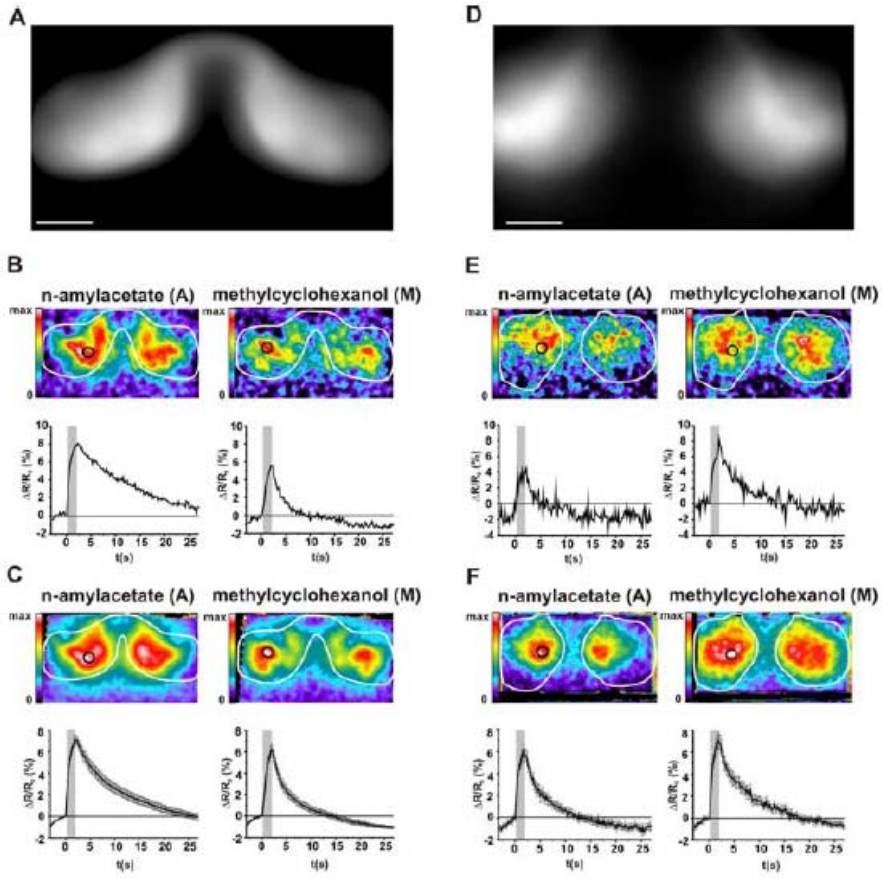

Fig. S4: High signal-to-noise ratio and low inter-individual variability in physiology
(**A**) To illustrate the shape of the antennal lobe as apparent in measurements of the sensory neurons, EYFP emission averaged across 8 individual flies is presented. Scale bar 25 m.
(**B**) Single-fly example of calcium activity in the antennal lobes (white circumfence-line) evoked by *n*-amylacetate (left) or 4-methylcyclohexanol (right) in sensory neurons, displayed in false-colour (top). For the encircled region of interest, the time course of the measurement is displayed as EYFP-to-ECFP ratio (bottom). The grey bar indicates the duration of the odour stimulus.
(**C**) Calcium activity evoked by *n*-amylacetate (left) or 4-methylcyclohexanol (right) in olfactory sensory neurons (white circumfence-line) averaged across 8 individual animals and displayed in false-colour (top). For the encircled region of interest, the time course of the measurement is displayed as EYFP/ECFP ratio (bottom). Data represent mean ± SEM. The grey bar indicates the duration of the odour stimulus.
(**D, E, F**) Same as **A, B**, **C**, but for antennal lobe-measurements of projection neuron activity.

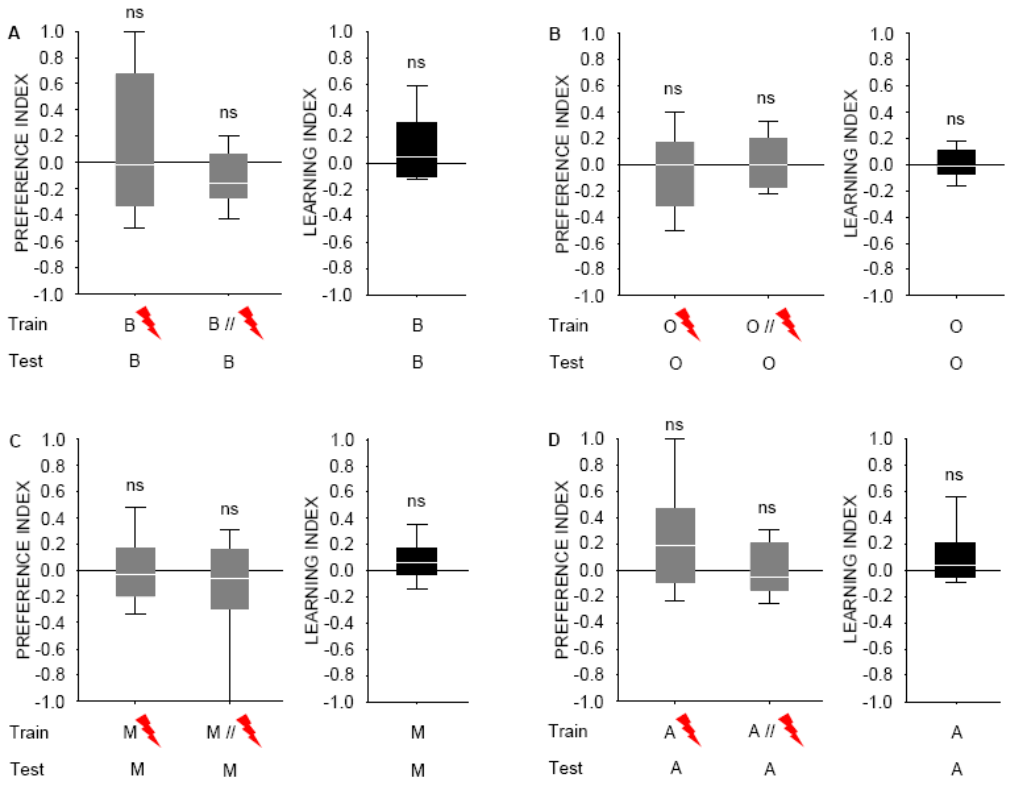

Fig. S5: The Or83b-mutant does not learn odour-shock associations with any of the odours used.
(**A**), (**B**), (**C**), and (**D**) Preference indices for Benzaldehyde, 3-Octanol, 4-Methylcyclohexanol, and n-Amylacetat after odour-shock training, and the learning index calculated herefrom. The learning indices is not significantly different from zero in all cases.

Chapter IV

Salt processing in larval Drosophila: choice, feeding, and learning shift from appetitive to aversive in a concentration-dependent way

Niewalda T, Singhal N, Fiala A, Saumweber T, Wegener S, Gerber B

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Salt Processing in Larval *Drosophila*: Choice, Feeding, and Learning Shift from Appetitive to Aversive in a Concentration-Dependent Way

Thomas Niewalda, Nidhi Singhal, André Fiala, Timo Saumweber, Stephanie Wegener and Bertram Gerber

Department of Genetics and Neurobiology, Universität Würzburg, Biozentrum, Am Hubland, D 970 74 Würzburg, Germany

Correspondence to be sent to: Bertram Gerber, Department of Genetics and Neurobiology, Universität Würzburg, Biozentrum, Am Hubland, D 970 74 Würzburg, Germany. e-mail: bertram.gerber@biozentrum.uni-wuerzburg.de

Abstract

Sodium and chloride need to be ingested and cannot be stored. Therefore, choice of habitat and diet as related to NaCl needs to be tightly regulated. We thus expect that the behavioral effects of salt are organized according to its concentration. Here, we comparatively "fingerprint" the reflex releasing (in choice and feeding experiments) versus the reinforcing effects of sodium chloride ("salt") in terms of their concentration dependencies, using larval *Drosophila*. Qualitatively, we find that the behavioral effects of salt in all 3 assays are similar: choice, feeding, and reinforcing effect all change from appetitive to aversive as concentration is increased. Quantitatively, however, the appetitive effects for choice and feeding share their optimum at around 0.02 M, whereas the dose–response curve for the reinforcing effect is shifted by more than one order of magnitude toward higher concentrations. Interestingly, a similar shift between these 2 kinds of behavioral effect is also found for sugars (Schipanski et al. 2008). Thus, for salt and for sugar, the sensory-to-motor system is more sensitive regarding immediate, reflexive behavior than regarding reinforcement. We speculate that this may partially be due to a dissociation of the sensory pathways signaling toward either reflexive behavior or internal reinforcement.

Key words: *Drosophila* larva, feeding, learning, taste, olfaction, sodium chloride

Introduction

This study provides a behavioral view of salt processing. We compare the dose–effect functions of sodium chloride regarding choice behavior, feeding, and learning in *Drosophila* larvae, an emerging experimental system to understand chemosensory function and its neurobiological bases (reviews by Gerber and Stocker 2007; Gerber et al. 2008) (Figure 1).

Sodium chloride (NaCl, "salt'') is necessary for a multitude of physiological processes, not the least important being neuronal function. Both sodium and chloride need to be ingested and cannot be stored. Thus, both these elements need to be taken up, and choice of habitat and of diet as related to salt content needs to be a well-regulated process, balanced by excretion of surplus salt, if any. As therefore uptake of just the right amount of salt is required, one may expect the behavioral effects of salt being tightly regulated according to concentration. Indeed, the appetitive responses to low salt gradually turn into aversion as concentration is increased (adult: Arora et al. 1987; larvae: Miyakawa 1981; Liu et al. 2003). These opposing behavioral responses involve discrete molecular and cellular processes. (i) A member of the *pickpocket* (*ppk*) gene family (*ppk11*; the *ppk* gene family is homologous to the *epithelial* Na^+ *channelldegenerin* gene family [EnaC] in vertebrates: Lindemann 2001) is exclusively expressed in 3 pairs of gustatory sensory neurons of the larva. Expression of this gene is necessary for the appetitive behavioral responses to low salt but dispensable for the aversive responses to high salt (Liu et al. 2003). (ii) In adults, the so-called L1 neurons are activated by salt with low threshold (between 0.01 and 0.05 M), whereas the L2 neurons have their threshold at about one order of magnitude higher concentration (Ishimoto and Tanimura 2004). (iii) Also in adults, Marella et al. (2006; fig. 3) report that neurons likely expressing different members of the *Gr* gene family can be activated by salt with low threshold as well as by sugars (*Gr5a*) or by salt with high threshold as well as by bitter substances (*Gr66a*) (for further studies concerning *Gr* function also see Ueno et al. 2001; Wang et al. 2004; Marella et al. 2006; Dahanukar et al. 2007).

Given these dissociations between low- and high-threshold salt processing, we use the concentration dependencies of

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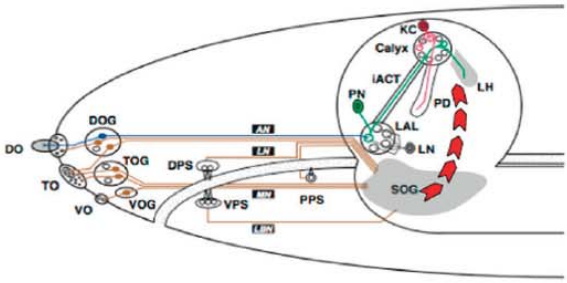

Figure 1 Chemosensory organs and pathways of larval *Drosophila*. Olfactory processing remains supraesophageal. Olfactory sensory neurons (blue) from the dorsal organ project toward the antennal lobe where they form synapses with both local interneurons and antennal lobe output elements, the projection neurons (green). These output neurons bifurcate: one branch directly innervates proposed premotor centers in the lateral horn, whereas the other branch forms a side loop via the mushroom bodies (red). Output from the mushroom bodies then presumably targets supraesophageal premotor centers as well. Taste processing (brown) bypasses the brain proper; rather, gustatory sensory neurons from the various external and internal taste organs project to the subesophageal ganglion. From there, motor centers in the ventral nerve cord and the mouthparts likely are innervated directly. With regard to odor–taste learning, modulatory interneurons are responsible to "short circuit" smell and taste: they receive input in the subesophageal ganglion and provide output toward the brain; the chevrons indicate this proposed pathway. Notably, separate kinds of modulatory interneuron seem to be responsible to carry appetitive (octopaminergic/tyraminergic neurons) and aversive (dopaminergic neurons) reinforcement (Schroll et al. 2006). Note that the actual connectivity toward the motor system is unknown; this, as the general layout of the chemosensory system, by and large corresponds to the situation in adult flies and insects in general. AN: antennal nerve, DO/DOG: dorsal organ/ganglion, DPS: dorsal pharyngeal sense organ, iACT: inner antennocerebral tract, KC: Kenyon cells, LAL: larval antennal lobe, LBN: labial nerve, LH: lateral horn, LN: local interneurons, LN: labral nerve, MN: maxillary nerve, PD: pedunculus, PN: projection neuron, PPS: posterior pharyngeal sense organ, SOG: subesophageal ganglion, TO/TOG: terminal organ/ganglion, VO/VOG: ventral organ/ganglion, VPS: ventral pharyngeal sense organ. Modified from Stocker (2006).

the salt effects as functional "fingerprints" to compare 2 kinds of behavioral function in larval *Drosophila* (see Schipanski et al. [2008] for a similar analysis regarding sugar processing):

- How does salt concentration affect reflexive behavior?
- How does salt concentration affect reinforcement function?

These 2 kinds of effect (i.e., reinforcing vs. reflex releasing) typically are dissociated in terms of the neuromodulators involved: for example, if honeybees are depleted of biogenic amines by injection of reserpine, compensatory injections of octopamine can restore the reinforcing effect of sugar but not its capacity to elicit ingestion reflexes (Menzel et al. 1999). Correspondingly, driving a single, identified octopaminergic neuron can substitute for the reinforcing effect of sugar but does not trigger ingestion reflexes (Hammer and Menzel 1995). In turn, dopamine injections can restore ingestion reflexes in reserpinized bees but not the reinforcing effect of sugar (Menzel et al. 1999) (see also de Araujo et al. 2008 concerning a dissociation of these functions in mice). Within this context, our study aims at parametrically dissociating the reflex releasing (in choice and feeding experiments) versus the reinforcing effects of NaCl in terms of their respective concentration dependencies.

Methods

We use third instar feeding stage larvae aged 5 days (±12 h) after egg laying. Flies of the Canton-S wild-type strain (Michels et al. 2005) are used which are kept in mass culture, maintained at 25 °C, 60–70% relative humidity and a 14/10 h light/dark cycle. Experiments are performed in red light under a fume hood at 20 °C –24 °C room temperature.

Choice behavior

Larvae are offered a choice between 2 substrates, one consisting of pure 1% agarose (electrophoresis grade; Roth, Karlsruhe, Germany) (PURE) and one of agarose with sodium chloride added at the indicated concentration (NaCl, purity 99.5%, Fluka/Sigma-Aldrich, Steinheim, Germany) (see inset of Figure 2).

Petri dishes of 90 mm inner diameter (Sarstedt, Nümbrecht, Germany) are equipped with a vertical barrier in the middle. These barriers are made from overhead transparencies and fixed to the rim of the plates with small stripes of tape. Parafilm is used to tighten the barrier. Then, the respective freshly boiled aqueous agarose solutions are poured into either side of the split petri dish to yield the desired combination of substrates on either side. Before the substances solidify, the barriers are gently torn out yielding a smooth yet sharp border between sides. After 20 min of cooling, plates are covered with their lids and left at room temperature overnight.

Thirty animals are placed to the middle of the plate. Then, animals are allowed to move about the plate for 15 min, until we determine the number of animals (#) located on either the sodium chloride side or the PURE side. Animals that dug into the agarose or crawled up the lids of the plates (approximately 5–15%) are not considered in data analysis. A preference index is calculated as

$$\mathrm{PREF} = (\#_{\mathrm{NaCl}} - \#_{\mathrm{PURE}})/\#_{\mathrm{TOTAL}} \qquad (1)$$

Thus, positive values indicate attraction while negative values indicate repulsion.

Feeding behavior

To measure feeding, 30 larvae are placed on a petri dish filled with 1% agarose containing the chosen concentration of salt (see "Results") and 30% red food dye (RU9805; backfun.de,

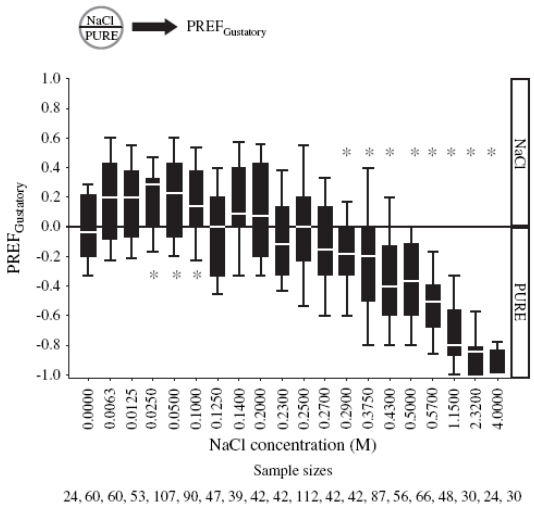

Figure 2 Choice. Preferences between plain agarose (PURE) versus various concentrations of salt; positive values indicate attraction and negative values repulsion. Behavior turns from appetitive to aversive as salt concentration is increased. **P* < .05/20. Data are displayed as box plots, with the bold line indicating the median and box boundaries and whiskers the 25/75% and 10/90% quantiles, respectively.

Uhingen, Germany). On this substrate, the animals are allowed to feed for 15 min and then are washed in tap water and homogenized in 80 µl of distilled water. The homogenate is centrifuged (30 s, 13 200 rpm), and 50 µl of supernatant is loaded into each well of a 96-well plate (Hartenstein, Würzburg, Germany). Using a "Sunrise" spectrophotometer (Tecan AG, Männedorf, Switzerland), absorbance is measured at 500 nm. On each experimental day, we measure the absorbance of homogenate from animals that have been feeding on a plate containing no salt but only dyed agarose. We calculate a median absorbance from 3 to 15 such samples and take this value as baseline to be subtracted from all spectrophotometer readings on that experimental day; this subtraction then yields the feeding scores. Thus, if larvae feed as much in the presence of a given salt concentration as they do in its absence, feeding scores are zero; if they eat more or less than in the absence of salt, respectively, positive and negative feeding scores result. Per experimental day, 3–15 independent samples of 30 larvae each are measured per salt concentration.

Effect as reinforcer

For the learning experiments, larvae are offered a choice between a previously reinforced and a previously nonreinforced odor (see schematics in Figure 3A,C).

We use modified lids for the petri dishes with 15 concentrically arranged holes with 1-mm diameter to improve aeration. All petri dishes are homogeneous in that the complete dish either does or does not contain the reinforcer. Larvae receive either of 2 training regimens: either amyl acetate (AM, 99%; Merck, Hohenbrunn, Germany) is presented with reinforcement and 1-octanol (OCT, 99%; Fluka/Sigma-Aldrich) without reinforcement (AM+/OCT), whereas in the companion group the larvae are trained reciprocally (i.e., AM/OCT+). In half of the cases, we start with the trials involving AM, in the other half with the OCT-containing trials. In the test, we measure the distribution of the larvae between AM versus OCT. For the reinforced trials, we use petri dishes with sodium chloride added to the agarose at the indicated concentration; for the nonreinforced trials, we use petri dishes with only agarose.

Custom-made Teflon containers (diameter 5 mm) with perforated lids (7 concentrically arranged holes with 0.5 mm diameter each) are loaded with 10 µl of odorant (either AM diluted 1:50 in paraffin oil or OCT; Merck, Darmstadt, Germany) and placed onto the assay plate, which either does or does not contain the reinforcer. Thirty larvae are transferred to the assay plate and after 5 min are transferred to a fresh plate with the alternative odorant–substrate combination. This cycle is repeated 3 times. Then, animals are placed in the middle of an assay plate with AM on one side and OCT on the other. This test plate has no reinforcer added, unless noted otherwise.

After 3 min, we determine the number of animals on either side to calculate an odor preference [–1; 1] as the number of animals at the AM side ($\#_{AM}$) minus the ones at the OCT side ($\#_{OCT}$), divided by the total ($\#_{TOTAL}$):

$$\mathrm{PREF} = (\#_{AM} - \#_{OCT})/\#_{TOTAL} \qquad (2)$$

From alternately run, reciprocally trained groups we calculate a learning index [–1; 1]:

$$\mathrm{LI} = \left(\mathrm{PREF}_{AM+/OCT} - \mathrm{PREF}_{AM/OCT+}\right)/2 \qquad (3)$$

Thus, positive LIs indicate appetitive, negative values aversive memory.

Statistical analyzes

Nonparametric statistics (one-sample sign test, Kruskal–Wallis test, Mann–Whitney *U* test) are used throughout (*P* level .05). Where applicable, we divide this significance level by the number of single-group comparisons to maintain an experiment-wide error rate of 5% despite multiple comparisons (Bonferroni correction); if, for example, 20 single-group comparisons are performed (Experiment 1), we present *P* levels as $P < .05/20$ (i.e., .0025). Data are displayed as box plots, with the bold line indicating the median and box boundaries and whiskers the 25/75% and 10/90% quantiles, respectively. In all cases, sample sizes are presented within the figures only.

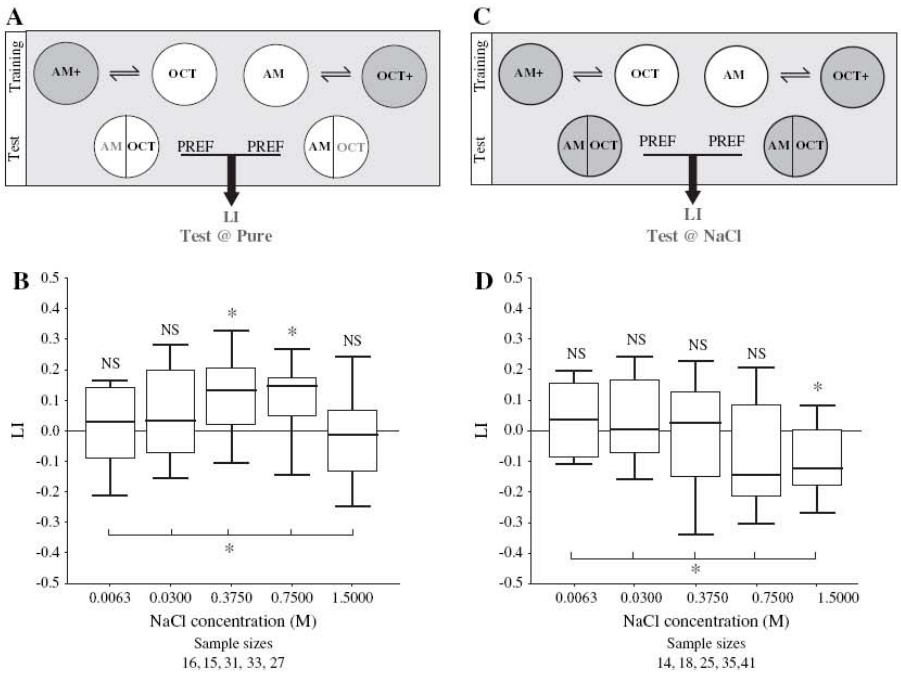

Figure 3 Reinforcement. **(A)** Schematic of the learning experiment. Larvae are trained with 2 odors (AM and OCT) and salt at the indicated concentration as reinforcer (+; indicated by dark gray shading). One group of larvae receives AM while crawling on a reinforcer-containing agarose plate, whereas OCT is presented in the absence of the reinforcer (AM+/OCT). Another group is trained reciprocally (AM/OCT+) (note that for half of the cases the sequence of trials is as indicated; for the other half, sequences are reversed: OCT/AM+ and OCT+/AM). Then, both groups are tested for their preference between AM and OCT. Associative learning shows by differences in preference scores between the groups trained AM+/OCT versus the reciprocally trained AM/OCT+ group. These differences are quantified by the learning index (LI). Positive LI values indicate appetitive learning, negative values aversive learning. **(B)** When testing is carried out in the absence of the reinforcer, low and high training concentrations of salt do not support positive learning scores, whereas intermediate concentrations do. **(C, D)** When testing is carried out in the presence of the reinforcer (indicated by the dark gray shading of the testing situation in C), learning scores are significantly negative only for the highest salt concentration. *$P < .05/5$. Other details as in the legend of Figure 2.

Results

Choice

Choice of NaCl is concentration-dependent when assayed in 20 experimental groups using concentrations between 0.0063 M and 4 M NaCl (Figure 2; Kruskal–Wallis test: $P < .05$, $H = 452.0$, degrees of freedom [df] = 19). Larvae are indifferent toward very low [0–0.0125 M] concentrations and show attractive responses to low concentrations [0.025–0.1 M]; as concentration is further increased, these responses gradually turn into aversion for high concentrations [0.29–4 M]; consequently, there is an intermediate concentration range at which appetitive and aversive properties cancel out [0.125–0.27 M] (all statements refer to one-sample sign tests and a P level of 0.05/20).

For convenience, in Figure 5A,B the results are plotted in terms of a normalized CHOICE score over concentration. Apparently, behavioral responses to NaCl are supported by 2 processes: an appetitive one at low concentrations (below 0.2 M) and an aversive component at high concentrations (above 0.2 M); both processes score even at intermediate (around 0.2 M) concentrations. Notably, the appetitive effect has its optimum at around 0.02 M NaCl.

Reinforcement

We next ask whether a similar concentration dependency is seen with respect to the effect of sodium chloride as a reinforcer. We had shown before that appetitive memories are behaviorally expressed only in the absence of the training reinforcer; arguably, this is because conditioned search behavior is expressed only if there is something to gain from searching, that is, if the sought-for situation is not already present (Gerber and Hendel 2006). Therefore, animals are trained with a given concentration of sodium chloride as reinforcer and then tested for their odor preference between the previously reinforced and the nonreinforced odor in the

absence of the reinforcer, that is, on petri dishes containing pure agarose (see schematic in Figure 3A). Clearly, the concentration of NaCl does influence test performance (Figure 3A, Kruskal–Wallis test: $H = 11.6$, df = 4, $P < .05$). Specifically, larvae do not show appetitive memory scores after training with either high (1.5 M) or low (0.03 M or less) concentrations; however, intermediate concentrations (0.375 and 0.75 M) do support appetitive memory (Figure 3A; all statements refer to one-sample sign tests at a P level of .05/5). Thus, the appetitive reinforcing effect of sodium chloride is concentration-dependent, with an optimum at intermediate concentrations, around 0.5 M NaCl.

In turn, we had shown before that aversive memories are behaviorally expressed only in the presence of the reinforcer; this conceivably is because conditioned escape behavior is expressed only if there is something to gain from that escape, that is, if the situation which the animals are in does indeed call for an escape (Gerber and Hendel 2006). Therefore, animals received the same kind of training as above but were tested on petri dishes containing the respective training reinforcer (see schematic in Figure 3B). Again, the concentration of NaCl obviously influences test performance (Figure 3B, Kruskal–Wallis test: $H = 13.9$, df = 4, $P < .05$). Larvae show aversive memory scores for 1.5 M sodium chloride but not for any lower concentration (Figure 3B; all statements refer to one-sample sign tests at a P level of .05/5); an apparent trend for aversive learning when using 0.75 M sodium chloride remains, due to the large scatter of the data, not significant (i.e., $P = .3$) despite a substantial sample size (i.e., $N = 35$). Thus, the aversive reinforcing effect of sodium chloride is concentration-dependent, being observable only for high concentrations.

For convenience, the results of both learning experiments are plotted as normalized LEARNING score over NaCl concentration in Figure 5. Apparently, the effect of NaCl as reinforcer turns from appetitive to aversive rather abruptly at and above 0.75 M; interestingly, the appetitive effect has its optimum at more than one order of magnitude higher NaCl concentrations as compared with the optimum for choice behavior.

Feeding

We finally ask which NaCl concentrations are "appetizing" (or "disgusting") using a photometer-quantified dye-feeding assay. The interesting question is whether such an "appetizing" effect would show for those concentrations of NaCl for which appetitive choice behavior is seen or for those concentrations which yield appetitive reinforcement.

When NaCl is added to the substrate, the amount eaten differs depending on NaCl concentration (Figure 4, Kruskal–Wallis test: $H = 70.72$, df = 4, $P < .05$). Given that larvae are continuous feeders (Carle 1969), increases in feeding are relatively difficult to detect; in our initial experiment, feeding scores for 0.03 M salt are not statistically significant when using the (rather conservative) Bonferroni correction (Figure 4A, one-sample sign test: $P > .05/5$). When repeating the experiment using this concentration, however, a small yet significantly positive feeding score can be substantiated (Figure 4B, one-sample sign test: $P < .05$).

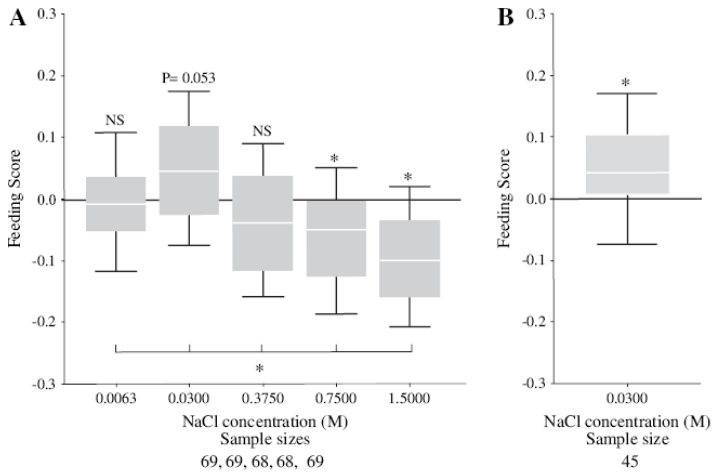

Figure 4 Feeding. Feeding of dyed substrate is assayed in the presence of various concentrations of salt and is quantified photometrically relative to a condition without salt in the substrate; positive values indicate upregulation and negative values downregulation of feeding. **(A)** High salt concentrations downregulate feeding, whereas low salt concentrations tend to upregulate feeding. *$P < .05/5$. **(B)** In a repetition of the experiment for 0.03 M salt, a slight upregulation of feeding can be statistically substantiated. *$P < .05$. Other details as in the legend of Figure 2.

In turn, larvae feed less at 0.75 and 1.5 M NaCl than when no NaCl is present (Figure 4A, one-sample sign tests: $P < .05/5$ for both 0.75 M and 1.5 M, respectively). Thus, feeding is slightly upregulated in the presence of low-concentration NaCl (0.03 M) and strongly downregulated in the presence of higher concentration NaCl (>0.75 M). Both processes score even at around 0.375 M NaCl (Figure 4A, one-sample sign test: $P > .05/5$).

When plotted in terms of a normalized FEEDING score across NaCl concentration (Figure 5), the concentration for which the "appetizing" effect of NaCl is seen fits the range of concentrations for which appetitive choice behavior is apparent but is shifted by about one order of magnitude towards lower concentrations relative to the appetitive learning effect.

Discussion

Qualitatively, the behavioral effects of sodium chloride are similar in all 3 cases tested: choice behavior, feeding behavior, and the reinforcing effect all change from appetitive to aversive as concentration is increased (Figure 5A,B).

The "titration point" of choice behavior as reported here (approximately 0.2 M; Figure 5A,B) is in line with data gathered 25 years ago reporting 0.1–0.2 M as the concentration of equally strong attraction and repulsion (Arora et al. 1987; Miyakawa 1981) as well as with recent data from Liu et al. (2003) who report such a draw at slightly above 0.2 M. Thus, the dose–effect curve for choice behavior of salt in larval *Drosophila* is remarkably reproducible.

Regarding feeding behavior, Hiroi et al. (2004) reported for adult flies that feeding is upregulated by salt at 0.1 M but is downregulated by 0.4 M salt, with the strongest "appetizing" effect between 0.05 and 0.1 M. This fits reasonably well with our results in the larva (Figure 5A) and suggests some functional conservation of salt processing between larva and adult. Based on the observation that most pharyngeal gustatory sensory neurons of the larva are retained into adulthood, such conserved function had already been proposed by Gendre et al. (2004).

Regarding a comparison of choice and feeding, we note that the concentration dependencies for both kinds of behavior match parametrically (Figure 5A): in both cases the effect changes from appetitive to aversive at around 0.2 M. Such shared dose–effect characteristics may suggest that both kinds of behavior rely on common input. Strikingly, the concentration where aversive effects start to unfold in both larva and adult and regarding both choice and feeding (approximately 0.2 M) fits with the electrophysiological threshold of the L2 neurons in adults which start to be activated between 0.1 and 0.4 M (Hiroi et al. 2004; Ishimoto and Tanimura 2004). This not only underscores the functional conservation between larva and adult as well as between the 2 kinds of reflexive behavior examined, but may also suggest a surprisingly straightforward relation between sensory physiology and reflexive behavior.

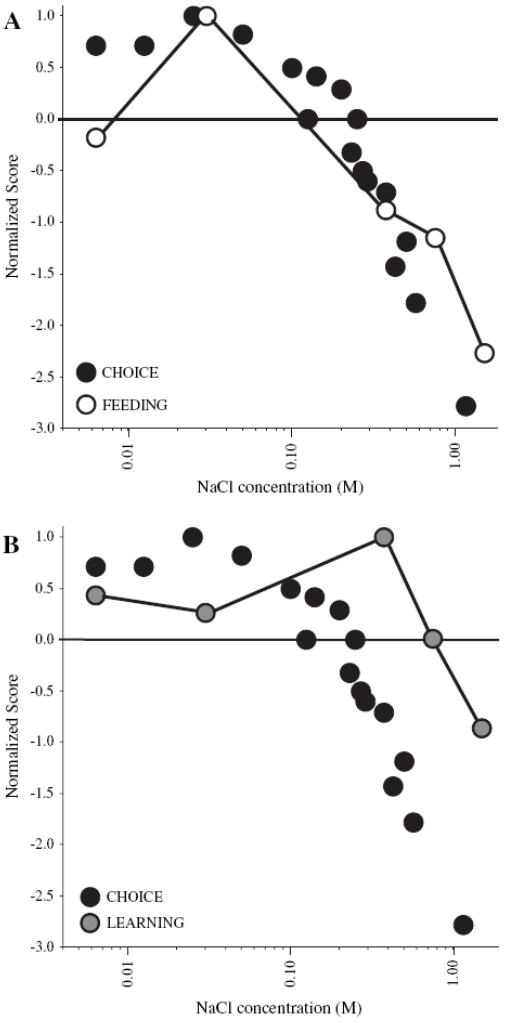

Figure 5 Summary. Semischematic illustration of the relation between choice, feeding, and learning. **(A)** We take the median of the salt preference values for each concentration (Figure 2) and express it relative to the highest score thus obtained; thus, the figure shows the maximum "CHOICE" score as "1." Then, we do accordingly for the median feeding values from Figure 4 and display them as "FEEDING" scores. The dose–effect characteristics between "CHOICE" and "FEEDING" appear similar. **(B)** To deal with the learning values in a similar way, we take the median learning index for a given training concentration as obtained when testing in the absence of the training reinforcer (Figure 3A) as well as the corresponding value for the learning index as obtained when testing in the presence of the training reinforcer (Figure 3B) and average these 2 values. Then, we do the same for all other concentrations and express the respective scores relative to the highest score thus obtained. These "LEARNING" scores then are plotted for comparison with the FEEDING scores. The dose–effect functions appear offset by at least one order of magnitude.

Reflexive behavior versus effect as reinforcer

To compare the dose–effect characteristics of the reflexive versus the reinforcing function of salt, we plot our data in a semischematic way (Figure 5). It is striking that the appetitive effects of salt for reflexive behavior, namely choice and feeding, share their optimum at around 0.02 M (Figure 5A), whereas the strongest effect of salt as appetitive reinforcer is seen for more than one order of magnitude higher concentrations (>0.2 M) (Figure 5B). In other words, the dose–response curve for the reinforcing effect is shifted by one order of magnitude toward higher concentrations. How can such a shift along the concentration axis come about?

One possibility may be that nongustatory processing, for example, via high-osmolarity sensors, selectively impinges upon the reflexive pathway to suppress appetitive tendencies for high salt concentrations. Given, however, that such sensors remain to be characterized in the larva, and given that this would leave the apparent ineffectiveness of relatively low salt concentrations as reinforcer unexplained, an alternative scenario may be warranted.

Suppose one and the same low-threshold salt sensor would be driving appetitive reflex behavior as well as appetitive reinforcement, and a high-threshold salt sensor would drive both aversive reflexes and aversive reinforcement. Could one, within such a scenario, yield the observed shift along the concentration axis? What if the connection of, for example, the low-threshold salt sensor toward reflex behavior would be tuned differently from its connection toward reinforcing neurons?

- A different gain of these connections would correspond to a multiplication step; such multiplication would yield altered amplitudes of attraction and repulsion but would leave the "titration point" between them unaffected. Thus, within such a scenario, the dose–response profile would not shift along the concentration axis.
- Introducing an additive effect also would not do so, as it would rather shift the dose–response profile along the ordinate toward higher or lower behavioral scores for a given concentration.
- Different signal-to-noise ratios would lead to different levels of scatter but would not qualitatively alter the dose–response profile.

Thus, as far as we can see, the assumption that both the reflexive and the reinforcing effects of salt draw upon common input pathways is incompatible with the observed shift of the dose–response curves along the concentration axis regarding these behavioral effects.

We therefore speculate that there may be 4 types of sensors: low-threshold salt sensors hooked up preferentially to appetitive reflex behavior, low-threshold salt sensors preferentially hooked up to appetitive reinforcement, and 2 types of high-threshold salt sensors, preferentially linked to aversive reflex behavior and aversive reinforcement, respectively. The heterogeneity of gustatory sense organs (Figure 1) and the complexity of the projection patterns of the gustatory sensory neurons in the subesophageal ganglion (Colomb et al. 2007) would seem permissive for such functional specialization; in particular, a division of labor between the external sense organs to support reflexive and of the internal sense organs to support the reinforcing effects of salt is conceivable (for a corresponding proposal with regard to mice see de Araujo et al. 2008). The observed shift in the behavioral dose–effect characteristics may then find its explanation either by the expression of differently tuned sets of salt sensors in these respective organs or by a 10-fold dilution of tastant by saliva upstream of the internal sense organs (for a more detailed discussion see Schipanski et al. in press).

To summarize, our study dissociates parametrically the reflex releasing (choice, feeding) from the reinforcing function of salt in terms of their respective dose–effect characteristics: the reinforcing effect is shifted by one order of magnitude toward higher concentrations (Figure 5). Interestingly, a similar shift between these 2 kinds of behavioral effect is also found for sugars (Schipanski et al. 2008), suggesting some degree of generality of such parametric dissociation. Thus, both in the case of salt and for sugar, the input pathways for gustatory behavior appear to be more sensitive than the ones supporting gustatory reinforcement.

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Chapter V

Behavioral analyses of sugar processing in choice, feeding, and learning in larval *Drosophila*

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Behavioral Analyses of Sugar Processing in Choice, Feeding, and Learning in Larval *Drosophila*

Angela Schipanski[1], Ayse Yarali, Thomas Niewalda and Bertram Gerber

Universität Würzburg, Lehrstuhl für Genetik und Neurobiologie, Biozentrum, Am Hubland, 970 74 Würzburg, Germany
[1]Present address: Universitätsklinikum Hamburg-Eppendorf, Institut für Neuropathologie, Martinistraße 52, 20246 Hamburg, Germany

Correspondence to be sent to: Bertram Gerber, Universität Würzburg, Lehrstuhl für Genetik und Neurobiologie, Biozentrum, Am Hubland, 970 74 Würzburg, Germany. e-mail: bertram.gerber@biozentrum.uni-wuerzburg.de

Abstract

Gustatory stimuli have at least 2 kinds of function: They can support immediate, reflexive responses (such as substrate choice and feeding) and they can drive internal reinforcement. We provide behavioral analyses of these functions with respect to sweet taste in larval *Drosophila*. The idea is to use the dose–effect characteristics as behavioral "fingerprints" to dissociate reflexive and reinforcing functions. For glucose and trehalose, we uncover relatively weak preference. In contrast, for fructose and sucrose, preference responses are strong and the effects on feeding pronounced. Specifically, larvae are attracted to, and feeding is stimulated most strongly for, intermediate concentrations of either sugar: Using very high concentrations (4 M) results in weakened preference and suppression of feeding. In contrast to such an optimum function regarding choice and feeding, an asymptotic dose–effect function is found for reinforcement learning: Learning scores reach asymptote at 2 M and remain stable for a 4-M concentration. A similar parametric discrepancy between the reflexive (choice and feeding) and reinforcing function is also seen for sodium chloride (Niewalda T, Singhal S, Fiala A, Saumweber T, Wegener S, Gerber B, in preparation). We discuss whether these discrepancies are based either on inhibition from high-osmolarity sensors upon specifically the reflexive pathways or whether different sensory pathways, with different effective dose–response characteristics, may have preferential access to drive either reflex responses or modulatory neurons mediating internal reinforcement, respectively.

Key words: *Drosophila*, feeding, gustation, learning, olfaction, sugar

Introduction

The sense of taste enables animals to prefer the edible and avoid the non-nutritious or toxic, an unquestionably vital faculty. In addition, gustatory stimuli are effective reinforcers; that is, they can induce memories for those stimuli or actions that repeatedly precede them, such that animals can yield good and avoid bad food, respectively. Gustatory stimuli thus support both immediate, reflexive behavior toward food (such as choice and ingestion) and, by virtue of their association with predictive stimuli or instrumental actions, the search for food. These 2 functions, that is, the reflex releasing and the reinforcing function of tastants, obviously need to be dissociated neuronally. Although at the level of gustatory interneurons such dissociation can clearly be found (e.g., in terms of the sufficiency of octopaminergic signaling for reinforcement, but not for ingestive behavior: Hammer 1997; Hammer and Menzel 1998; Menzel et al. 1999), it is unknown whether different sets of sensory neurons may trigger reflex behavior and instruct reinforcement, respectively (for an interesting study of this issue in mice, see de Araujo et al. 2008). Here, we want to take a first step into such an analysis, by behaviorally "fingerprinting" choice, feeding, and the reinforcing function for their respective dose–effect characteristics. We do so with respect to sweet taste in larval *Drosophila*.

The larva is the feeding and growth stage of the fly life cycle and as such lends itself to studies of gustation. Substrate choice, feeding, and reinforcement learning can be tackled by simple, well-defined behavioral assays; furthermore, the larval gustatory system is relatively simple and reasonably well described at the anatomical, cellular, and to some extent also the molecular level (for a review, see Gerber and Stocker 2007; Gerber et al. 2008). We focus on sweet taste, aiming to relate parametrically the reflex

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releasing and the reinforcing function of various kinds of sugar. Specifically, we ask:

- How does sugar concentration affect choice between sugary and tasteless substrates?
- How do these different sugar concentrations affect feeding behavior?
- How potent are they in inducing learning?
- How do the dose–effect curves for choice, feeding, and learning relate?

We find that the dose–effect curves of the reflexive (choice and feeding) function of both fructose and sucrose are shifted by one order of magnitude relative to the reinforcing function; we discuss whether inhibition from high-osmolarity sensors upon specifically the reflexive pathways is responsible for this parametric dissociation. Alternatively, we suggest that this dissociation is based on a dissociation already at the sensory level, such that different sensory pathways, with different effective dose–response characteristics, may have preferential access to either reflex pathways or to modulatory neurons mediating internal reinforcement.

Materials and methods

Larvae

We use feeding-stage third-instar larvae of the wild-type Canton-S strain, aged 5 days after egg laying. Flies are maintained on standard medium, in mass culture at 25 °C, 60–70% relative humidity and a 14:10 h light:dark cycle. Before each experiment, we remove a spoonful of medium from a food vial, collect the desired number of larvae, briefly rinse them in distilled water, and start the experiment. All experiments are performed under a fume hood in a regularly lit room, at approximately 23 °C ambient temperature.

Choice

The day before experiments, we prepare the petri dishes (55 mm inner diameter; Sarstedt, Nümbrecht, Germany): We split them into 2 halves with a piece of overhead transparency, fill one side with 1% agarose (electrophoresis grade; Roth, Karlsruhe, Germany) (PURE) and then the other side with 1% agarose containing a given sugar (SUGAR). As sugar we use glucose, trehalose, fructose, or sucrose (each with 99% purity; all from Roth) at various concentrations. Once the agarose has solidified, we remove the overhead transparency, cover the dishes with their lids, and leave them at room temperature until the following day.

We place 15 larvae in the middle of the dish and close the lid. The SUGAR side is in half of the cases to the right and in the other half to the left. We record the number of larvae on either side of the dish and calculate a gustatory preference index ($PREF_{Gustatory}$) as

$$PREF_{Gustatory} = (\#SUGAR - \#PURE)/\#Total \quad (1)$$

In this equation, # indicates the number of larvae on the respective half of the dish. Thus, $PREF_{Gustatory}$ values are constrained between 1 and –1, positive values indicating a preference for SUGAR and negative values aversion. These scores are taken at various time points after the animals are placed onto the dish (for details, see Results).

Feeding

To measure feeding behavior on substrates containing sugars at different concentrations, 30 larvae are placed on a petri dish filled with 1% agarose containing the chosen concentration of the respective sugar (either fructose or sucrose, at either 0.02-, 0.2-, 2-, or 4-M concentration) and 30% red food dye (RU9805; backfun.de, Uhingen, Germany). The animals are allowed to feed on this substrate for 15 min; then, they are washed in tap water and, as a group, homogenized in 80 µl of distilled water. The homogenate is centrifuged for 30 s at 13 200 rpm and 50 µl of the supernatant is loaded into single wells of a 96-well plate (Hartenstein, Würzburg, Germany). Then, using a "Sunrise" spectrophotometer (Tecan AG, Männedorf, Switzerland), absorbance at 500 nm is measured. On each experimental day, we measure the absorbance of homogenate from animals that have been feeding on a plate containing no sugar but dye. From 4 to 6 independent samples of this condition, we calculate a median absorbance which we take as baseline. This baseline is subtracted from all spectrophotometer readings on that experimental day to yield the feeding scores. Thus, if larvae feed as much in the presence of a given sugar concentration as they do in its absence, feeding scores are zero; if they eat more or less than in the absence of sugar, respectively positive and negative feeding scores result. Per experimental day, 3 to 12 independent samples of 30 larvae each are measured per sugar concentration.

Learning

Preparation and treatment of petri dishes for the learning experiments are as detailed above, except that we use petri dishes of approximately 90 mm diameter (Sarstedt), filled uniformly either with 1% agarose only or with 1% agarose containing the reinforcer (+). As reinforcer, we use fructose or sucrose at the indicated concentrations.

Prior to the learning experiments, odor containers are prepared: 10 µl of odor substance is filled into each custom-made Teflon odor container (5 mm inner diameter with a lid perforated with seven 0.5-mm diameter holes). As odors, we use *N*-amyl acetate (AM, 99%; Merck, Hohenbrunn, Germany) and 1-octanol (OCT, 99%; Fluka/Sigma-Aldrich, Steinheim, Germany). We dilute AM 1:250 in paraffin oil (Merck).

Immediately before the experiment starts, dishes are covered with modified lids perforated in the center by 15 holes with 1 mm diameter to improve aeration. To start training, 30 larvae are placed in the middle of a reinforcer-added dish with 2 odor containers on opposite sides (7 mm from the edges), both filled with AM. After 5 min, larvae are displaced onto an agarose-only dish with 2 odor containers, this time both filled with OCT, where they also spend 5 min. Three such AM+/OCT training cycles are performed, each using fresh dishes. Along repetitions of the experiment, in half of the cases training starts with a reinforcer-added dish (AM+/OCT for all three training cycles) and in the other half with an agarose-only dish (OCT/AM+ for all three training cycles). Consequently, in half of the cases AM is present in the first trial, whereas in the other half the first trial involves OCT. Once this AM+/OCT training is completed, larvae are transferred to the middle of a fresh agarose-only dish with 2 odor containers, this time filled with OCT on one side and AM on the opposite side to create a choice situation. After 3 min, the number of larvae on each half of the dish is recorded and an olfactory preference (PREF) is calculated as

$$\mathrm{PREF} = (\#\mathrm{AM} - \#\mathrm{OCT})/\#\mathrm{Total} \qquad (2)$$

Again, # indicates the number of larvae observed on the respective half of the dish. PREF values are bound between 1 and –1, positive values indicating preference for and negative values avoidance of AM.

For each group of larvae trained AM+/OCT, a second group is trained reciprocally: AM/OCT+. Associative learning shall result in a stronger preference for AM after AM+/OCT training than after AM/OCT+ training. This difference is quantified by the learning index (LI) as

$$\mathrm{LI} = (\mathrm{PREF}_{\mathrm{AM+/OCT}} - \mathrm{PREF}_{\mathrm{AM/OCT+}})/2 \qquad (3)$$

Here, $\mathrm{PREF}_{\mathrm{AM+/OCT}}$ is the AM preference of the AM+/OCT group and $\mathrm{PREF}_{\mathrm{AM/OCT+}}$ is that of the reciprocally trained AM/OCT+ group. The LI is a pure measure of associative learning because it measures the difference in preference between 2 groups trained reciprocally, but otherwise treated the same (i.e., with respect to handling, exposure to odors, and the reinforcer). LI values are bound between 1 and –1, positive values indicating approach toward the reinforcer-paired odor (appetitive learning) and negative values avoidance from the reinforced odor (aversive learning).

Statistical analysis

All statistical analyses are performed with Statistica on a PC. Preference values, feeding scores, and learning indices from multiple experimental groups are compared with Kruskal–Wallis tests. For subsequent pairwise comparisons, Mann–Whitney *U* tests are used. To test whether values of a given group differ from zero, we use 1-sample sign tests. When multiple 1-sample sign tests or Mann–Whitney *U* tests are performed within one experiment, we adjust significance levels by a Bonferroni correction to keep the experiment-wide error rate at 5%. This is done by dividing the critical *P* value 0.05 by the number of tests; that is, if e.g. four 1-sample sign tests are performed within one experiment, we present statements of significance as $P <> 0.05/4$. We present our data as box plots which represent the median as the middle line and 25/75% and 10/90% as box boundaries and whiskers, respectively. In all cases, sample sizes are presented exclusively within the figures.

Results

Experiment 1: optimizing the duration of the choice assay

First, we want to find an appropriate assay duration for testing the sugar preference of experimentally naive larvae; this seems warranted because here we use assay plates with smaller

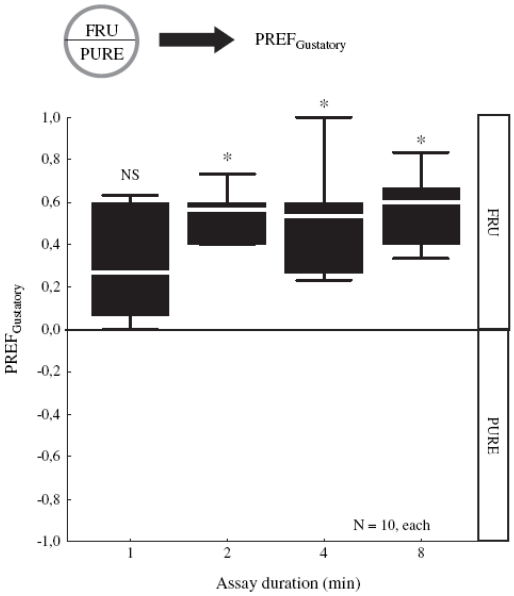

Figure 1 Optimizing the duration of the choice assay. Groups of 15 larvae are allowed to distribute between pure agarose (PURE) on one side and agarose containing 2 M fructose (FRU) on the other. A gustatory preference ($\mathrm{PREF}_{\mathrm{Gustatory}}$) is calculated based on their distribution at different time points after the experiment has started. Positive $\mathrm{PREF}_{\mathrm{Gustatory}}$ values indicate a preference for fructose. At each time point, larvae seem to prefer fructose; this response is statistically significant from 2 min on; it seems to saturate already at 2 min after choice onset. NS, $P > 0.05/4$; *$P < 0.05/4$ in 1-sample sign tests, keeping the experiment-wide error rate at 5% (i.e., Bonferroni correction). Box plots represent median as the middle line and 25/75% and 10/90% as box boundaries and whiskers, respectively.

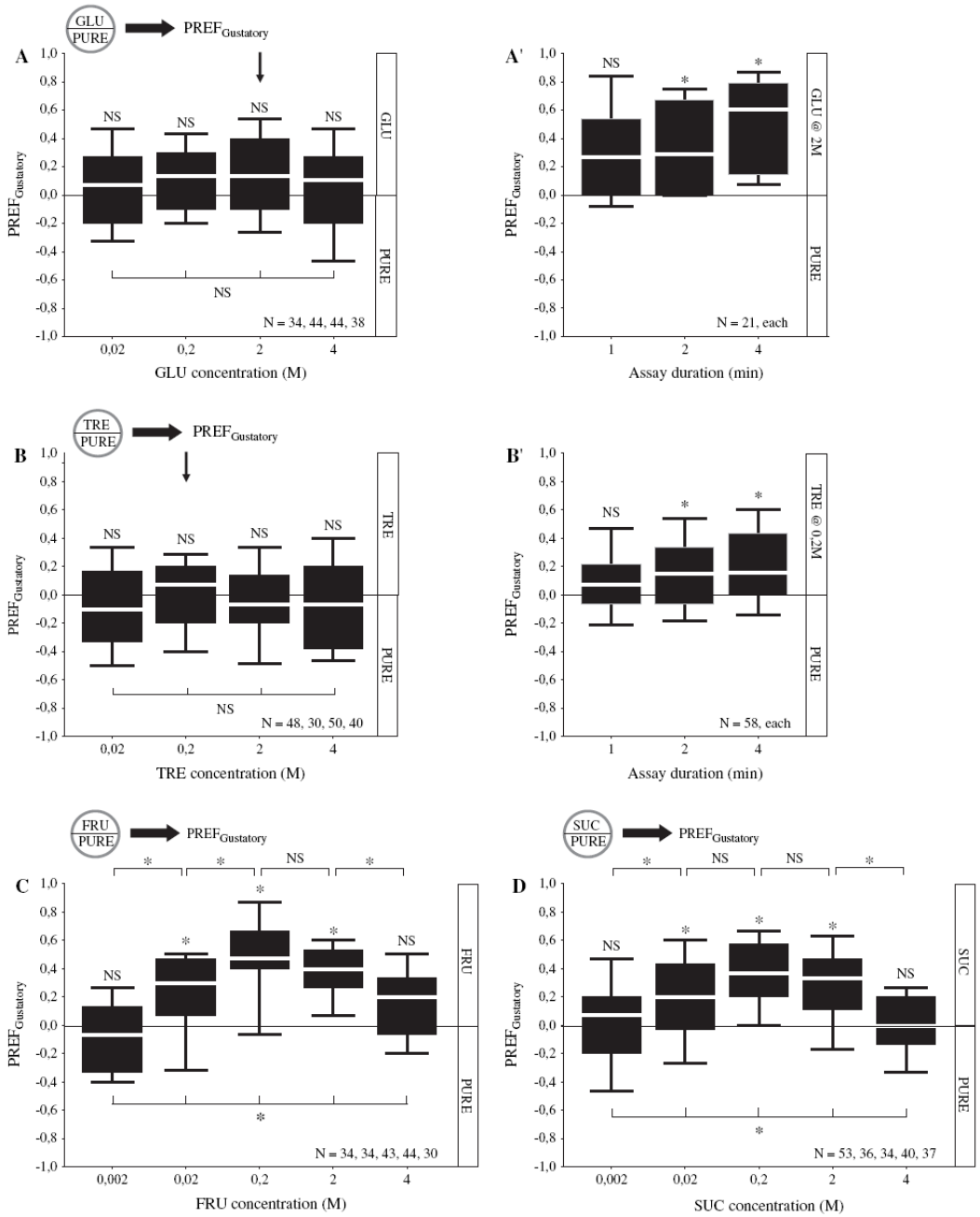

Figure 2 Choice. **(A)** Gustatory preference scores ($PREF_{Gustatory}$) for glucose (GLU) calculated based on the distribution of larvae at 90 s after assay onset. The larvae are indifferent toward glucose at each of the tested concentrations; a comparison across groups reveals no effect of glucose concentration on behavior. The arrow indicates the concentration chosen for the follow-up experiment in A′. **(A′)** Recurrently scoring 1, 2, or 4 min after assay onset reveals

diameter (ca. 55 mm) than in previous studies (ca. 90 mm; see review by Gerber and Stocker 2007). We use fructose at 2-M concentration because in previous work this concentration has been used as gustatory reinforcer in larval learning experiments (reviewed in Gerber and Stocker 2007). We allow the larvae to choose between pure agarose (PURE) and agarose in addition containing 2 M fructose (FRU) and recurrently score for the gustatory preference index ($PREF_{Gustatory}$) at 1, 2, 4, and 8 min. Positive $PREF_{Gustatory}$ values indicate a preference for fructose.

We observe a preference for 2 M fructose over pure agarose beginning from 2 min after assay onset (Figure 1; 1-sample sign tests: $P > 0.05/4$ for 1 min; $P < 0.05/4$ for 2, 4, and 8 min). The larval response to fructose seems to saturate at 2 min. We choose 90 s as assay duration for the following experiments in order to be able to detect both higher and lower preference scores.

Experiment 2: choice

We next test the choice response of experimentally naive larvae between pure agarose (PURE) and agarose containing different types of sugar, at various concentrations. Specifically, we study the preferences for different concentrations of glucose (GLU), trehalose (TRE), fructose (FRU), and sucrose (SUC), scoring the larvae 90 s after the start of the assay.

Scores for glucose (GLU) are indistinguishable from random level for all tested concentrations (Figure 2A; 1-sample sign tests: $P > 0.05/4$ for each concentration) and are uniform across concentrations within the range tested (Figure 2A; Kruskal–Wallis test: $P > 0.05$; $H = 2.16$; degrees of freedom [df] = 3). However, maybe the larvae just need more time to "make up their minds"? Given the trend for highest preference scores for 2 M glucose (arrow in Figure 2A), we repeat the experiment for 2 M glucose, but this time recurrently score at 1, 2, and 4 min after the start of the assay. As expected from the previous experiment, larvae appear indifferent after 1 min, but after 2 min and in particular after 4 min, a substantial preference for glucose is apparent (Figure 2A′; 1-sample sign tests: $P > 0.05/3$ for 1 min, $P < 0.05/3$ for 2 and 4 min).

For trehalose (TRE), we find that preference values scored after 90 s are indistiguishable from random for all tested concentrations (Figure 2B; 1-sample sign tests: $P > 0.05/4$ for each concentration) and are independent of concentration within the range tested (Figure 2B; Kruskal–Wallis test: $P > 0.05$; $H = 2.08$; df = 3). To further probe this apparent lack of behavioral effect of trehalose, we repeat the experiment for 0.2 M trehalose (arrow in Figure 2B), this time, however, scoring recurrently at 1, 2, and 4 min after the start of the assay. Indeed, preferences for trehalose develop over time; we find no preference after 1 min; however, at 2 and 4 min after start of the test, a weak yet significant preference for trehalose is found (Figure 2B′; 1-sample sign tests: $P > 0.05/3$ for 1 min, $P < 0.05/3$ for 2 and 4 min).

Larval preferences for fructose (FRU) are clearly concentration dependent when scored at 90 s (Figure 2C; Kruskal–Wallis test: $P < 0.05$; $H = 61.38$; df = 4). Larvae prefer fructose at intermediate concentrations (Figure 2C; 1-sample sign tests: $P < 0.05/5$ for 0.02 M, 0.2 M, and 2 M) but are indifferent to it at lower and higher concentrations (Figure 2C; 1-sample sign tests: $P > 0.05/5$ for 0.002 M and 4 M). Based on pairwise comparisons, fructose seems to be most attractive to larvae at concentrations between 0.2 M and 2 M (Figure 2C; Mann–Whitney U tests: $P < 0.05/4$; $U = 243.00$ for 0.002 M vs. 0.02 M; $P < 0.05/4$; $U = 390.50$ for 0.02 M vs. 0.2 M; $P > 0.05/4$; $U = 722.00$ for 0.2 M vs. 2 M; $P < 0.05/4$; $U = 350.00$ for 2 M vs. 4 M).

Similarly, sucrose (SUC) is preferred by the larvae depending on its concentration (Figure 2D; Kruskal–Wallis test: $P < 0.05$; $H = 38.72$; df = 4). Larvae find sucrose attractive at intermediate concentrations (Figure 2D; 1-sample sign tests: $P < 0.05/5$ for 0.02 M, 0.2 M, and 2 M), whereas they do not respond to it at lower and at higher concentrations (Figure 2D; 1-sample sign tests: $P > 0.05/5$ for 0.002 M and 4 M). Sucrose has a relatively broad peak of attractiveness, spanning 2 orders of magnitude (between 0.02 M and 2 M), as is revealed by pairwise comparisons (Figure 2D; Mann–Whitney U tests: $P < 0.05/4$; $U = 628.50$ for 0.002 M vs. 0.02 M; $P > 0.05/4$; $U = 448.00$ for 0.02 M vs. 0.2 M; $P > 0.05/4$; $U = 573.50$ for 0.2 M vs. 2 M; $P < 0.05/4$; $U = 361.00$ for 2 M vs. 4 M).

To summarize, all sugars tested are preferred by the larvae when offered against a pure agarose substrate. Preference for glucose and trehalose is weak and/or delayed, whereas fructose and sucrose support fast and strong preference responses in a concentration-dependent way. The fast and strong preference responses toward fructose and sucrose prompt us to choose these 2 for an analysis of their potency as modulators of feeding and as reinforcers. Specifically, we are interested to see whether, concomitant with the loss of preference at very high concentrations of fructose and

a delayed, appetitive response toward glucose. **(B)** Larval preference scores for trehalose (TRE) are not different from random for either of the tested concentrations; behavior does not differ between groups. The arrow indicates the concentration chosen for the follow-up experiment in B′. **(B′)** Recurrently scoring 1, 2, or 4 min after assay onset reveals a delayed and weak appetitive response toward trehalose. **(C)** Larvae respond to fructose (FRU) depending on concentration. Intermediate concentrations of fructose are attractive, whereas larvae are indifferent toward low and high concentrations. **(D)** Also to sucrose (SUC), larval responses are concentration dependent. Intermediate concentrations of sucrose are attractive, whereas low and high concentrations remain without apparent effect. We use Kruskal–Wallis tests for all-group comparisons at $P < 0.05$; if applicable, follow-up pairwise comparisons between groups use the Mann–Whitney U test at $P < 0.05/4$; for single-group comparisons against zero, 1-sample sign tests are used at $P < 0.05/3$ (A′,B′), at $P < 0.05/4$ (A,B) or at $P < 0.05/5$ (C,D). For details concerning the box plots, see legend of Figure 1.

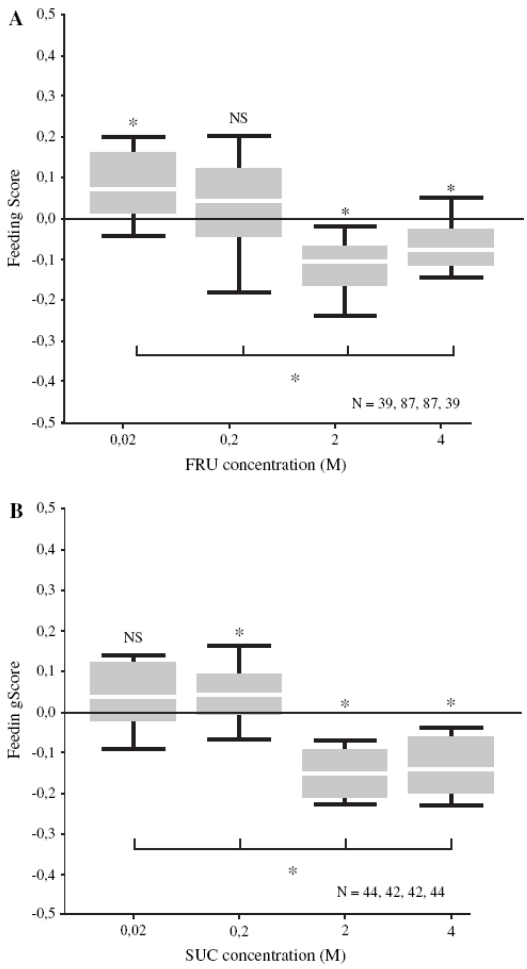

Figure 3 Feeding. Groups of 30 larvae are allowed to feed on petri dishes filled with dyed agarose which contains either fructose (FRU) or sucrose (SUC) at the indicated concentration; the amount fed then is quantified photospectrometrically. Values from a group which is allowed to feed on dyed agarose without any sugar added serves as baseline; absorbance values of this group are subtracted from the spectrometer readings of the experimental groups to yield the feeding score. Therefore, feeding scores greater than zero indicate that the larvae eat more than if sugar were absent, and feeding scores below zero indicate that larvae eat less than in the absence of sugar. **(A)** Fructose enhances feeding at low but suppresses feeding at higher concentration. **(B)** Sucrose also leads to increases in feeding at low but to decreased feeding at higher concentration. We use Kruskal–Wallis tests for all-group comparisons at $P < 0.05$; for single-group comparisons against zero, 1-sample sign tests are used at $P < 0.05/4$. For details concerning the box plots, see the caption of Figure 1.

sucrose (Figure 2C,D), a loss of appetitive effect in feeding or learning assays would be observed.

Experiment 3: feeding

We allow larvae 15-min access to a red-dyed assay plate with sugar added at various concentrations to then estimate photometrically the amount fed. Data are presented as feeding score, expressing the difference in feeding as compared with larvae offered a red-dyed assay plate with no sugar added.

For both fructose and sucrose, the concentration of the added sugar has an effect on feeding behavior (Figure 3A; for fructose: Kruskal–Wallis test: $P < 0.05$; $H = 90.98$; df = 3; Figure 3B; for sucrose: Kruskal–Wallis test: $P < 0.05$; $H = 97.33$; df = 3). Both sugars lead to increases in feeding, relative to the baseline condition with no sugar added, at low but to suppression of feeding at higher concentrations (Figure 3A; for fructose: 1-sample sign tests: $P < 0.05/4$ for 0.02 M, 2 M, and 4 M, $P > 0.05/4$ for 0.2 M; Figure 3B; for sucrose: 1-sample sign tests: $P < 0.05/4$ for 0.2 M, 2 M, and 4 M, $P > 0.05/4$ for 0.02 M). Thus, the dose–effect function concerning feeding is similar to the one for choice in the sense that both sugars lose their appetitive effect at high concentration; maybe surprisingly, both fructose and sucrose even suppress feeding at these concentrations.

Experiment 4: choice revisited

Given that concentrations of fructose and sucrose which suppress feeding (Figure 3A,B) do not seem to induce aversion in a choice assay (Figure 2C,D), we return to the choice assay for both sugars and test whether, if more time is allotted, an aversion response may become apparent. This is not the case: We find for 2 M fructose that responses are appetitive already after 1 min and remain stably appetitive throughout the 16 min of the assay (Figure 4A; 1-sample sign tests: $P < 0.05/5$ for all time points). Concerning 4 M fructose, we find that at short assay duration, there is no significant preference (Figure 4B; 1-sample sign test for 1-min assay duration: $P > 0.05/5$); this is consistent with the results from Experiment 2 (Figure 2C) which had suggested that 2 M but not 4 M fructose supports preference at short (90 s in Figure 2C) assay durations. If 2 min or more time is allowed, however, the larvae eventually express a preference response for 4 M fructose as well (Figure 4B; 1-sample sign tests for 2-, 4-, 8-, and 16-min assay duration: $P < 0.05/5$), with no apparent decrement between 8 and 16 min.

Regarding sucrose, the same pattern of results is found: For a 2-M concentration, larvae express appetitive responses from 1 min on (Figure 4C; 1-sample sign tests: $P < 0.05/5$ for 1-, 2-, 4-, 8-, and 16-min testing times). Using 4 M sucrose, however, larvae remain indifferent for the first 2 min (Figure 4D; 1-sample sign tests: $P > 0.05/5$ for 1 and 2 min); only as time passes, the larvae start to express appetitive responses (Figure 4D; 1-sample sign tests: $P < 0.05/5$

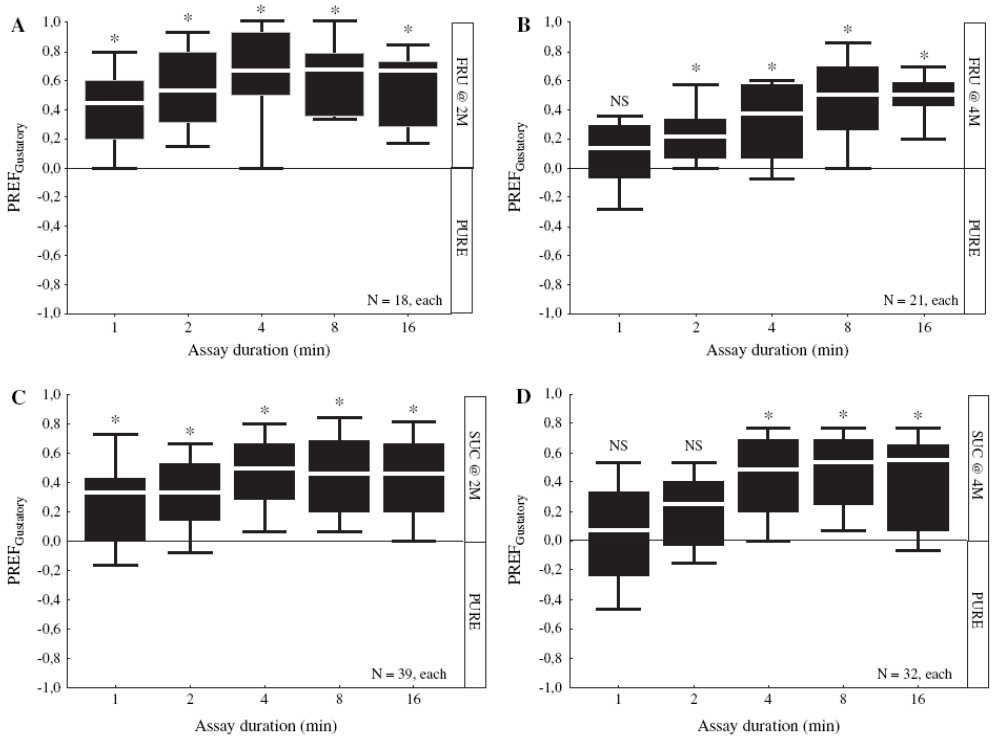

Figure 4 Choice revisited. Gustatory preference scores ($PREF_{Gustatory}$) for high concentrations of fructose (FRU) and sucrose (SUC) scored 1, 2, 4, 8, and 16 min after assay onset. **(A)** For 2 M fructose, preference scores remain stably appetitive throughout the testing period. **(B)** For 4 M fructose, preferences are uncovered for 2 min or longer assay durations. **(C)** Similar to the case of 2 M fructose, also for 2 M sucrose, preference scores are positive throughout the testing period. **(D)** For 4 M sucrose a similar pattern is found as for 4 M fructose; preferences are uncovered only with 4 min or longer assay durations. For single-group comparisons against zero, 1-sample sign tests are used at $P < 0.05/5$. For details concerning the box plots, see the caption of Figure 1.

for 4-, 8-, and 16-min testing times), without any trend for scores turning into aversion over time. The observation that preference responses to 4 M sucrose unfold between 2 and 4 min is consistent with the indifference of the larvae after 90 s as seen in Figure 2D.

Experiment 5: learning

We assess the reinforcing potency of fructose and sucrose in olfactory associative learning (reviewed in Gerber and Stocker 2007): larvae are trained with 2 odors, one of which is presented in the presence of a reinforcer. After such training, larvae are allowed to distribute between the reinforcer-paired odor and the other odor in a choice situation. The LI, which is a measure of associative learning, is based on the comparison between the odor preferences of 2 groups of larvae, trained reciprocally but otherwise handled the same (see Materials and methods and Figure 5A). Based on this experimental design, we train larvae with various concentrations of either fructose (FRU) or sucrose (SUC) as reinforcer. Specifically, we want to compare the strength of these sugars as reinforcers to their ability to govern choice as measured in Experiment 2 and to their effects as modulators of feeding behavior as measured in Experiment 3.

The concentration of fructose (FRU) matters for its reinforcing potency (Figure 5B; Kruskal–Wallis test: $P < 0.05$; $H = 42.38$; df = 4). Low concentrations of fructose apparently do not support learning (Figure 5B; 1-sample sign tests: $P > 0.05/5$ for 0.002 M and 0.02 M), whereas higher concentrations do (Figure 5B; 1-sample sign tests: $P < 0.05/5$ for 0.2 M, 2 M, and 4 M). As revealed by pairwise comparisons between learning indices, the reinforcing potency of fructose seems to saturate at concentrations between 0.2 M and 2 M (Figure 5B; Mann–Whitney U tests: $P > 0.05/4$; $U = 169.00$ for 0.002 M vs. 0.02 M; $P < 0.05/4$; $U = 161.00$ for 0.02 M vs. 0.2 M; $P > 0.05/4$; $U = 165.00$ for 0.2 M vs. 2 M; $P > 0.05/4$; $U = 113.00$ for 2 M vs. 4 M).

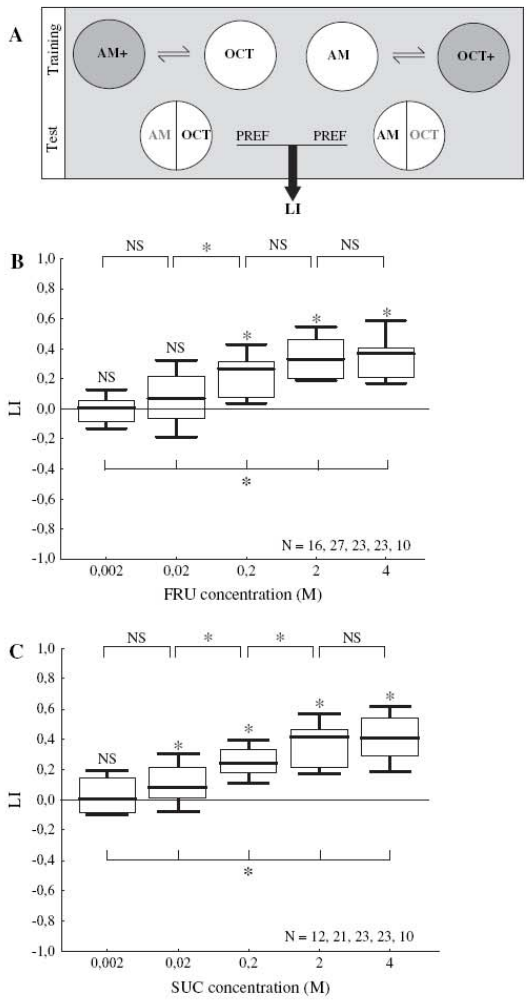

Figure 5 Learning. **(A)** Groups of 30 larvae are trained with 2 odors (i.e., AM and OCT) and a reinforcer (i.e., either fructose: FRU or sucrose: SUC at the indicated concentration). One group of larvae receives AM while crawling on a reinforcer-containing agarose plate, whereas OCT is presented in the absence of the reinforcer (i.e., AM+/OCT training). Another group is trained reciprocally (AM/OCT+) (note that for half of the cases the sequence of trials is as indicated; in the other half, sequences are reversed: OCT/AM+ and OCT+/AM). After repeated training, both groups are tested for their preference between AM and OCT in a choice situation. Associative learning shows by higher preference scores for AM in the group trained AM+/OCT than in the reciprocally trained AM/OCT+ group. This difference is quantified by the LI. Positive LI values thus indicate appetitive learning. **(B)** The strength of fructose as a reinforcer depends on its concentration. Low concentrations of fructose do not support learning, whereas higher concentrations do. Learning gets stronger with increasing fructose concentration until it saturates between 0.2 M and 2 M. **(C)** Sucrose also has a concentration-dependent reinforcing effect. A low sucrose concentration does not support any learning, whereas higher concentrations do. Increasing sucrose concentration strengthens learning until an asymptote is reached at 2 M. For all-group comparisons, Kruskal–Wallis tests are used at $P < 0.05$; for follow-up pairwise comparisons, Mann–Whitney U tests are used at $P < 0.05/4$; for single-group comparisons against zero, 1-sample sign tests are used at $P < 0.05/5$. For details concerning the box plots, see the caption of Figure 1.

The reinforcing potency of sucrose (SUC) also depends on its concentration (Figure 5C; Kruskal–Wallis test: $P < 0.05$; $H = 42.04$; df = 4). Similar to fructose, a low concentration of sucrose does not support learning (Figure 5C; 1-sample sign test: $P > 0.05/5$ for 0.002 M), whereas higher concentrations do (Figure 5C; 1-sample sign tests: $P < 0.05/5$ for 0.02 M, 0.2 M, 2 M, and 4 M). The reinforcing ability of sucrose also increases with rising concentration until it reaches an asymptote at 2 M (Figure 5C; Mann–Whitney U tests: $P > 0.05/4$; $U = 85.00$ for 0.002 M vs. 0.02 M; $P < 0.05/4$; $U = 122.00$ for 0.02 M vs. 0.2 M; $P < 0.05/4$; $U = 130.00$ for 0.2 M vs. 2 M; $P > 0.05/4$; $U = 108.00$ for 2 M vs. 4 M).

Thus, the highest concentration of fructose and sucrose, although little potent in governing choice behavior (Figures 2C,D and 4B,D) and acting as suppressor of feeding (Figure 3A,B), nevertheless acts as a strong appetitive reinforcer (Figure 5B,C).

Discussion

We systematically analyze 4 natural sugars concerning choice behavior in experimentally naive *Drosophila* larvae. We then investigate 2 of these sugars in more detail to determine the relation between the dose dependencies of choice of these sugars versus their effect on feeding versus their reinforcing effect. Before discussing the results of these behavioral experiments, we want to briefly sketch the neurobiological organization of the larval taste system.

Neurobiology of taste processing

The neurobiology of taste processing in the larva is resolved partially and in principle conforms to what had been found in adults (see discussions in Python and Stocker 2002; Ishimoto and Tanimura 2004; Gerber and Stocker 2007; Gerber et al. 2008): Candidate gustatory sensory neurons are located in 2 types of sense organ (both of which likely include some nongustatory sensory neurons as well): external sensilla and internal sensilla. The external ones are the terminal (32 sensory neurons) and the ventral organ (7 sensory neurons) plus some gustatory sensory neurons in the bulge of the dorsal organ (9 sensory neurons). The internal sensilla are located along the pharynx and are organized into dorsal, ventral, and posterior sense organ (17, 16, and 6 sensory neurons, respectively). At present, the exact relation between cellular identity, expression of putative gustatory receptor gene of the *Gr* gene family (Clyne et al. 2000), and ligand profile of the neurons is largely unknown, except for the *Gr5a* and *Gr64a* genes (Dahanukar et al. 2007): In

adult flies, both genes are concordantly expressed in a subset of gustatory sensory neurons. Deletions of *Gr5a* abolish electrophysiological responses to only 4 out of 14 tested sugars (trehalose, methyl-a-glucoside, glucose, and melezitose). In turn, deleting the *Gr64a* gene abolishes (maltotriose, stachyose, raffinose, leucrose, and fructose) or partially reduces (sucrose, maltose, turanose, maltitol, and palatinose) the *Gr5a*-independent activations. Behavioral analyses using the proboscis extension response generally conform to the complementary requirement of *Gr5a* and *Gr64a* for detecting different kinds of sugars and acting within the same set of cells. Note, however, that in the larva *Gr5a* and *Gr64a* may not be expressed, as judged from the lack of reporter expression seen in the respective *Gr*-Gal4 driver strains (Colomb et al. 2007; Tanimura T, Kyushu University, personal communication).

Regarding connectivity toward gustatory interneurons, projections of the *Gr*-expressing neurons typically bypass the brain and project to the subesophageal ganglion where multiple distinct areas receive innervations from distinct subsets of these neurons (Colomb et al. 2007). It is from these areas that both premotor commands as well as internal reinforcement signals likely originate. Although the exact connectivity of gustatory receptor neurons to their postsynaptic targets is not resolved in detail, neurons expressing a given *Gr* gene can be found in different sense organs and project to distinct target regions in the subesophageal ganglion (Colomb et al. 2007); this suggests that one and the same tastant can have access to different kinds of downstream effect, dependent on input site.

Choice

We show that glucose and trehalose support relatively weak and/or somewhat sluggish preference responses (Figures 2A,A′ and 2B,B′), whereas those 2 sugars with a ketose unit (fructose and sucrose) support fast and strong preference. This may suggest that those gustatory sensory neurons which support preference responses are particularly sensitive to sugars containing such a ketose unit, whereas the nature of a sugar as mono- versus disaccharide should be of minor importance. As mentioned above, in adult flies processing of glucose and trehalose on the one hand and of fructose and sucrose on the other hand requires the *Gr5a* and *Gr64a* genes, respectively (Dahanukar et al. 2007); neither of these genes, however, is apparently expressed in the larva (see section Neurobiology of taste processing), suggesting that the discrepancies in behavioral effectiveness between these 2 classes of sugar may have different neurobiological bases in either life stage. In any event, the parametrically concordant behavioral effects of fructose and sucrose in the larva would be consistent with both sugars being processed via concordant sets of sensory neurons.

The preference responses for fructose and sucrose show a clear concentration dependency: Larvae prefer fructose and sucrose at intermediate concentrations, whereas they are indifferent to both lower and higher concentrations (Figure 2C,D); for higher concentrations, preferences can be uncovered only by increasing assay duration (Figure 4). Intuitively, the relatively weak appetitive response to very high sugar concentrations makes sense as things also for us can be "too" sweet. Also, very high concentrations may, although energetically in principle attractive, make substrates unattractive for reasons of viscosity, stickiness, and/or because of osmotic properties; these kinds of effect may undergo some adaptation/habituation to allow uncovering an appetitive effect only with some delay (Figure 4B,D). We thus regard it as little surprising (yet to the best of our knowledge not previously reported) that preference responses of larval *Drosophila* toward sugars follow an optimum function.

Feeding

Fructose and sucrose dose-dependently modulate feeding behavior (Figure 3). This dose dependency is similar to the one seen for choice in that the "appetizing" effect exerted by low concentrations of these sugars is lost for higher concentrations. Strikingly, such higher concentrations even suppress feeding. Whether these feeding-suppressant effects are also mediated by gustatory sensory neurons or rather may come about by neurons sensitive to high viscosity, osmolarity, or "stickiness" remains to be investigated. In any event, similar to the case of the preference responses, it seems plausible that >2-M sugar concentrations can appear impalatably high to the larvae.

We also note that both the increases and the decreases in feeding are moderate; given that insect larvae are notorious and continuous feeders to begin with (Carle 1969), it seems plausible that up and downregulations of feeding may be relatively difficult to obtain experimentally.

Learning

Fructose and sucrose act dose dependently as reinforcers (Figure 5). The reinforcing effect of both sugars reaches a stable asymptote at 2-M concentration, a finding matching the previous report of Neuser et al. (2005) who had looked at the dose–effect function of fructose (FRU) reinforcement in a range from 0.25 to 2 M in an individual–animal version of our learning assay. As we show here, there clearly is no decrement in learning scores if sugar concentrations yet higher than 2 M are used, at least not for a 4-M concentration, which is the limit of solubility of fructose (FRU) and sucrose (SUC) in agarose.

It is currently unknown which gustatory sensory neurons drive internal reinforcement; actually, even the sense organ origin of the responsible neurons (i.e., external vs. internal), is unknown. What has been reported, however, is that artificially driving octopaminergic/tyraminergic neurons is sufficient to substitute for appetitive reinforcement in larval

olfactory learning (Schroll et al. 2006). In bees, artificially driving even a single, identified octopaminergic neuron, the so-called VUMmx1 neuron, is sufficient to substitute for appetitive reinforcement but is not sufficient to trigger feeding reflexes (Hammer 1997; see also Hammer and Menzel 1998; Menzel et al. 1999); a homolog of this neuron is found in both adult (Tanimoto H, Universität Würzburg, personal communication) and larval *Drosophila* (Thum A, Université Fribourg, personal communication), as well as in moths (Dacks et al. 2005). Whether output from octopaminergic/tyraminergic neurons is necessary for appetitive learning in the larva, however, remains unknown. Interestingly, the octopaminergic/tyraminergic neurons do not seem to be directly postsynaptic to gustatory sensory neurons, as argued by light microscopical analyses of their branching patterns as well as from the site of expression of pre- and postsynaptic markers (Thum A, Université Fribourg, personal communication).

Relation between reflex responses and reinforcing capacity

Obviously, while in the choice and the feeding assay both sugars lose their appetitive effect at high concentrations (Figures 2C,D and 3A,B), the reinforcing effect shows an asymptotic dose–effect function; notably, robust appetitive reinforcement is retained even at very high concentrations (Figure 5B,C). In Figure 6, we want to illustrate in a semischematic way the parametric relation between choice, feeding, and the reinforcing effect.

We take the median value of the fructose preference response for a given concentration (Figure 2C) as well as the corresponding value for the sucrose response (Figure 2D) and average these 2 values. Next, we do the same for all other concentrations. We then express these scores relative to the highest score thus obtained, such that the semischematic plot in Figure 6 shows the maximum "choice" score as "1." Then, we do the same for the feeding scores and learning indices obtained for the different concentrations of fructose and sucrose (Figure 3A,B and 5B,C) and display them as "learning" and "feeding" values.

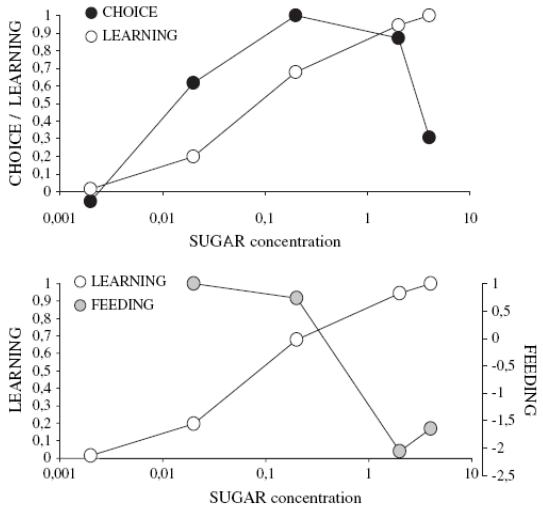

Figure 6 Summary. Semischematic illustration of the relation between preference scores, feeding scores, and reinforcing effect. For details, see the last paragraph of the Discussion.

As can be seen in Figure 6, the dose–effect curve for learning is shifted to the "east," that is toward higher concentrations relative to choice and feeding. Strikingly, a similar east shift is found for salt processing as well: The optimum for the choice responses and for feeding is at around 0.02 M NaCl, whereas the optimum for appetitive learning is shifted by an order of magnitude to around 0.4 M (Niewalda T, Singhal S, Fiala A, Saumweber T, Wegener S, Gerber B, in preparation). Obviously, the discrepancies between the dose–effect functions of tastants with regard to choice and feeding as compared with their reinforcing potency must reflect some dissociation along the respective chemosensory-to-motor pathways. For a start, we note that mere differences in gain between these pathways would leave the "topology" of the dose–effect curve unchanged. Rather, a first possible scenario is that the reduction of the choice and feeding scores for high sugar concentrations is caused by an inhibition from, for example, high-osmolarity sensors specifically upon the reflexive pathways. Such high-osmolarity sensors, however, remain to be characterized in the larva. Alternatively, these parametric dissociations may be based on a dissociation already at the sensory level: Different sensory neurons may have preferential access toward premotor neurons that support choice and feeding on one hand and toward neurons which drive internal reinforcement on the other hand (for a similar proposal with regard to mice, see de Araujo et al. 2008). For example, if the reflexive and reinforcing functions were originating from external and internal taste organs, respectively, and if secreted saliva would dilute the tastants 10-fold, one may indeed expect a shift between the reflexive and reinforcing dose–effect functions by one order of magnitude. This second scenario could explain the apparent generality of such shift (for salt, see this paragraph, above) and would be consistent with the huge salivary glands of larval *Drosophila*. However, if feeding was indeed organized according to the sensors within the external sense organs, high concentration tastants would suppress feeding and the tastants would not "reach" the internal sense organs to signal aversive reinforcement to begin with. As a third scenario, we contemplate whether the respective gustatory sensory neurons may be expressing different gustatory receptor genes which endow them with different dose–effect characteristics. This may at first sight seem little parsimonious but may partially explain why there are so many different gustatory receptor genes. In any event, all these 3 scenarios, certainly, now invite further experimentation.

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Summary

All animals learn in order to cope with challenges imposed on them by their environment. This is true also for both larval and adult fruit flies as exemplified in pavlovian conditioning. The focus of this Thesis is on various aspects of the fruit flies` learning ability.

My main project deals with two types of learning which we call punishment-learning and pain-relief learning. Punishment learning happens when fruit flies are exposed to an odour which is followed by electric shock. After such training, flies have learned that that odour signals pain and consequently will avoid it in the future. If the sequence of the two stimuli is reversed such that odour follows shock, flies learn the odour as a signal for relief and will later on approach it. I first report a series of experiments investigating qualitative and parametric features of relief-learning; I find that (i) relief learning does result from true associative conditioning, (ii) it requires a relatively high number of training trials, (iii) context-shock training is ineffective for subsequent shock-odour learning.

A further question is whether punishment-learning and pain-relief learning share genetic determinants. In terms of genetics, I test a *synapsin* mutant strain, which lacks all Synapsin protein, in punishment and relief-learning. Punishment learning is significantly reduced, and relief-learning is abolished. Pan-neuronal RNAi-mediated knock-down of Synapsin results in mutant-like phenotypes, confirming the attribution of the phenotype to lack of Synapsin. Also, a rescue of Synapsin in the mushroom body of syn^{97} mutants restores both punishment- and relief-learning fully, suggesting the sufficiency of Synapsin in the mushroom body for both these kinds of learning.

I also elucidate the relationship between perception and physiology in adult fruit flies. I use odour-shock conditioning experiments to identify degrees of similarity between odours; I find that those similarity measures are consistent across generalization and discrimination tasks of diverse difficulty. Then, as collaborator of T. Völler and A. Fiala, I investigate how such behavioural similarity/dissimilarity is reflected at the physiological level. I combine the behaviour data with calcium imaging data obtained by measuring the activity patterns of those odours in either the sensory neurons or the projection neurons at the antennal lobe. Our interpretation of the results is that the odours` perceptual similarity is organized by antennal lobe interneurons.

In another project I investigate the effect of gustatory stimuli on reflexive behaviour as well as their role as reinforcer in larval learning. *Drosophila* larvae greatly alter their behaviour in presence of sodium chloride. Increasing salt concentration modulates choice behaviour from weakly appetitive to strongly aversive. A similar concentration-behaviour

function is also found for feeding: larval feeding is slightly enhanced in presence of low salt concentrations, and strongly decreased in the presence of high salt concentrations. Regarding learning, relatively weak salt concentrations function as appetitive reinforcer, whereas high salt concentrations function as aversive reinforcer. Interestingly, the behaviour-concentration curves are shifted towards higher concentrations from reflexive behaviour (choice behaviour, feeding) as compared to associative learning. This dissociation may reflect a different sensitivity in the respective sensory-motor circuitry.

Zusammenfassung

Tiere müssen lernen, damit sie sich in ihrer Umwelt zurechtfinden und die Herausforderungen meistern können, die ihre Umwelt ihnen bietet. Dies gilt auch für Taufliegen im larvalen und erwachsenen Stadium, wie man mit der Pavlovschen Konditionierung zeigen kann. Der Schwerpunkt dieser Doktorarbeit liegt auf verschiedenen Aspekten der Lernfähigkeit von Taufliegen.

In meinem Hauptprojekt erforsche ich die Arten von Lernprozessen, die stattfinden, wenn die Fliegen entweder den Beginn oder das Ende eines Elektroschocks mit einem Duft assoziieren. Wenn Taufliegen einen Duft wahrnehmen, der von einem Elektroschock gefolgt wird, lernen sie, dass dieser Duft Schmerz signalisiert, und werden ihn konsequenterweise in Zukunft vermeiden. Man kann die Abfolge dieser beiden Reize so umkehren, dass der Duft auf den Elektroschock folgt. Durch ein solches Training wird der Duft für die Fliegen zu einem Signal für das Ende des schmerzhaften Elektroschocks und sie werden, wenn sie diesen Duft später wieder einmal wahrnehmen, auf ihn zugehen. Ich berichte im ersten Kapitel über Experimente, die qualitative und parametrische Besonderheiten der letzteren Lernform untersuchen. Ich finde heraus, dass (i) das Lernen über das Ende des Elektroschocks echtes assoziatives Lernen ist, (ii) dass es eine relativ hohe Anzahl von Trainingsdurchgängen erfordert, (iii) dass Kontext-Schock-Training unbedeutend für anschließendes Schock-Duft-Lernen ist.

Im zweiten Kapitel gehe ich der Frage nach, ob die genannten beiden Typen von Lernvorgängen gemeinsame genetische Determinanten haben. Was die Genetik anbelangt, teste ich die Lernfähigkeit eines Synapsin-Mutantenstammes, dem das Synapsinprotein fehlt. Lernen über den Beginn des Elektroschocks ist stark reduziert, und Lernen über das Ende des Elektroschocks fehlt gänzlich. Die Reduzierung des Synapsinproteins im Fliegengehirn durch RNAi resultiert in mutantenähnlichen Phänotypen. Dieser Befund bestätigt, dass der Lernphänotyp auf einem Mangel an Synapsin beruht. Die Expression von Synapsin im Pilzkörper der Mutante erlaubt der Fliege, wieder normal zu lernen; dies weist auf die Hinlänglichkeit von Synapsin im Pilzkörper für beide Arten von Lernen hin.

In einem weiteren Projekt untersuche ich den Zusammenhang zwischen Wahrnehmung und Physiologie in erwachsenen Taufliegen. Ich benutze Duft-Schock-Konditionierungsexperimente, um basierend auf dem Verhalten der Tiere Ähnlichkeitsränge von Düften zu ermitteln, und finde eine einheitliche Rangfolge der untersuchten Düfte für verschiedene Generalisierungs- und Diskriminierungs-Aufgaben von unterschiedlichem Schwierigkeitsgrad. Schließlich erforsche ich in Kooperation mit T. Völler and A. Fiala, wie

der Grad der Verhaltensähnlichkeit /-unähnlichkeit von Düften mit der Physiologie der Fliege in Beziehung steht. Ich kombiniere die Verhaltensdaten mit Daten, die mittels funktioneller Bildgebung unter Verwendung genetisch codierter Kalziumsensoren erhalten wurden. Diese Methode erlaubt, Aktivitätsmuster, die von den untersuchten Düften verursacht werden, entweder in den sensorischen Neuronen oder in den Projektionsneuronen des Antennallobus zu messen. Unsere Interpretation der Ergebnisse ist, dass die Verhaltensähnlichkeit der Düfte auf Ebene der Interneuronen im Antennallobus organisiert wird.

Weiterhin erforsche ich die Wirkung von Kochsalz (Natriumchlorid) auf das Reflexverhalten und die Rolle von Natriumchlorid als Belohnung oder Bestrafung im Larvenlernen. Larven der Taufliege verändern ihr Reflexverhalten in Gegenwart von Natriumchlorid in hohem Maße. Larven bevorzugen niedrige Salzkonzentrationen gegenüber einem Substrat ohne Salz; erhöht man die Salzkonzentration jedoch, kehrt sich das Wahlverhalten ins Gegenteil um, bis die Tiere das salzhaltige Substrat stark vermeiden. Ein ähnlicher Zusammenhang zwischen Konzentration und Verhalten wird auch für das Fressverhalten gefunden: Larven fressen von einem Substrat mit niedrigen Salzkonzentrationen geringfügig mehr, von einem Substrat mit hohen Salzkonzentrationen jedoch deutlich weniger als von einem Kontrollsubstrat ganz ohne Salz. Was das Lernen betrifft, wirken relativ schwache Salzkonzentrationen als Belohnung, während hohe Salzkonzentrationen als Bestrafung wirken. Interessanterweise ist die Verhaltens-Konzentrations-Kurve von Reflexverhalten (Wahlverhalten, Fressverhalten) verglichen mit assoziativem Lernen in Richtung höherer Konzentrationen verschoben. Diese Dissoziation könnte eine verschiedenartige Sensitivität der Schaltkreise widerspiegeln.

Anschrift

Thomas Niewalda

Universität Würzburg, Lehrstuhl für Neurobiologie und Genetik

Biozentrum, Am Hubland

97074 Würzburg

thomas.niewalda@biozentrum.uni-wuerzburg.de

Curriculum Vitae

2000	Abitur
2000-2001	Zivildienst
2001-2006	Diplomstudiengang Biologie, Universität Würzburg
2006	Diplom Biologie, Universität Würzburg
2006-2010	(vorraussichtlich) Promotion

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List of Publications

Niewalda T, Singhal N, Fiala A, Saumweber T, Wegener S, Gerber B (2008) Salt processing in larval Drosophila: choice, feeding, and learning shift from appetitive to aversive in a concentration-dependent way. Chemical Senses

Schipanski A, Yarali A, Niewalda T, Gerber B (2008) Behavioral analyses of sugar processing in choice, feeding, and learning in larval Drosophila. Chemical Senses

Yarali A, Niewalda T, Chen Y, Tanimoto H, Duerrnagel S, Gerber B (2008) 'Pain relief' learning in fruit flies. Animal Behaviour

Manuscripts under revision

Niewalda T, Völler T, Ehmer J, Fiala A, Gerber B. Odour perception matches physiological activity patterns in second-order olfactory neurons

Niewalda T, Michels B, Yarali A, Gerber B. Common requirement of Synapsin in punishment- and pain relief-learning

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