Dissecting the mechanisms of learning-by-doing in *Drosophila*

Björn Brembs[1] & Wolfgang Plendl[2]

1 Freie Universität Berlin, Institut für Biologie – Neurobiologie, Königin-Luise Str. 28/30, 14195 Berlin, Germany. Email: bjoern@brembs.net, phone: +49 (0)30 838 55050, fax: +49 (0)30 838 55455

2 Lehrstuhl für Neurobiologie und Genetik, Biozentrum, Universität Würzburg, Germany.

At the heart of learning-by-doing[1] lies a well-known psychological phenomenon: information will be remembered better if it is actively generated rather than passively read or heard[2,3]. First described in humans[2,4], this generation effect can also be observed in various animal models[3,5-7]. However, the neurobiological mechanisms underlying the generation effect are unknown. Here we show that two reciprocal interactions between its active and passive components contribute to the generation effect in flies. One interaction consists of the active (skill-learning) component facilitating the passive (fact-learning) component. Fact-learning, on the other hand, inhibits skill-learning. Experiments with adenylyl cyclase I deficient *rutabaga* mutant flies revealed that the fact- but not the skill-learning component requires this evolutionarily conserved learning gene. Using mushroom-body deficient transgenic flies we observed that the mushroom-bodies mediate the inhibition of skill-learning. This inhibition also enables generalization and prevents premature habit formation. Extended training in wildtype flies produced a phenocopy of mushroom-body impaired flies, such that generalization was abolished and goal-directed actions were transformed into habitual responses. Thus, our results identify various neural processes underlying learning-by-doing,

delineate some of their synergisms and provide a framework for further dissecting them in a genetically tractable model system.

In the 100 years since the term was coined[1], "learning-by-doing" has been recognized as a successful educational and economic strategy[8]. At its core lies a psychological phenomenon which was described only a few years earlier: Active engagement of the brain provides learning capabilities which are difficult or impossible to achieve by passive observation alone[2,3]. This phenomenon is today known as the generation effect[4] and can also be observed in animals such as monkeys[5], cats[6] or fruit flies[7]. Despite the impact learning-by-doing has on society and the ubiquity of the generation effect, the mechanism by which activity enhances passive learning is unknown.

To study its neurobiological basis, we hypothesized that the generation effect may be brought about by an interaction of two components: an active, skill-learning component and a passive, fact-learning component. We tested this hypothesis by combining two simple experimental instances of fact- and skill-learning, respectively, in the fruit-fly *Drosophila melanogaster*. In both tasks, the fly is tethered to a torque meter to accomplish stationary flight. In the passive fact-learning task, the fly is presented with one of two visual cues in alternation, independently of its own behaviour. The presentation of one of these cues is associated with an infrared beam of light providing instantaneous, aversive heat. The animal learns the fact that one cue is punished and prefers the unpunished over the punished cue in a subsequent choice test without heat[9]. In the active skill-learning task, the fly's spontaneous turning maneuvers[10] are divided into two groups (i.e. attempts to turn left or right, respectively) and one of them is punished by heat. During the subsequent choice test without heat, the animal generates manoeuvres preferentially in the previously unpunished direction[11]. There are no external cues guiding the animal during skill-training or testing. The fly has to rely

solely on its own internal representation of its movements in order to solve this task. In the combined paradigm (Fig. 1), attempted turning will lead to either blue or green illumination (i.e. a right turn will cause illumination of one colour while a left turn will cause illumination of the other). During training, one of the two situations is associated with heat. During test, the heat is permanently switched off. Extending previous results[7], the flies showed the generation effect also in this composite paradigm (Supplementary Figures 6, 7). This finding is consistent with our hypothesis that an interaction of fact- and skill-learning components may underlie the generation effect. But of what nature is this interaction? A simple possibility is that the fact-learning component and the skill-learning component are formed in parallel, and that the two components are summed. A straightforward test of this 'summation' hypothesis is to disable one of the two components and then subject the animals to the composite learning task.

The *rutabaga* (*rut*)-mutant flies lack a type I adenylyl cyclase that is required for most learning tasks including the instance of fact-learning tested here (Supplementary Figure 7). If mutant *rut* flies are only deficient in fact-learning, the summation hypothesis predicts two similar learning scores: reduced, but significant composite learning and unaffected skill-learning. If the Rutabaga cyclase is required for both learning components, the mutant flies should perform poorly in both the composite and the skill-learning task. Surprisingly, *rut* mutants performed well (even exceeding wildtype levels; Supplementary Figure 7) in the skill-learning task, but they failed in the composite task (Fig.2a). How can this dominant-negative effect of the colours be explained? One explanation is that the colour changes may interfere with skill-learning in *rut* flies. However, colour changes unrelated to the flies' behaviour did not disrupt performance (i.e., a yoked control; Supplementary Figure 7). Rather, in *rut* mutant flies, colour changes concomitant with turning behaviour somehow inhibit skill-learning during the composite learning task. To investigate whether this inhibition occurs during acquisition or during retrieval of the skill-learning component, we removed the colours

after training and tested for the skill-learning component in isolation. If the inhibition takes place at the level of acquisition, the learning score should be zero, because no skill was ever learned. If the inhibition takes place during retrieval, the *rut* flies should reveal a significant learning score, because the colours are no longer present and thus cannot interfere with the performance of the skill which was learned during composite training. The significant *rut* learning score places the inhibition firmly at the level of retrieval for the mutant flies (Fig. 2a). The same experiment with wildtype flies did not reveal any significant learning score. We conclude that in wildtype flies fact-learning also exerts an inhibitory effect on skill-learning. In contrast to *rut* flies, this inhibition of skill-learning acts during acquisition and not during retrieval.

These results show that the interaction between fact- and skill-learning components is more complex than mere summation. Counter-intuitively, one factor involved in this interaction is inhibition of skill-learning by a dominant fact-learning component. Because the generation effect entails an overall enhancement of learning, there must be a second, facilitating factor which more than compensates for the skill-learning inhibition. One may assume this second factor to be reciprocal to the first, from the skill-learning component back to the fact-learning component (Fig. 3). While on the surface this arrangement may seem implausible, such an enhancement of fact-learning at the expense of skill-learning allows for keeping the learned fact flexible for use with a different behaviour than with which it was acquired. It has been shown previously that flies can perform such a generalization[7] and that the mushroom-bodies (MB), a prominent neuropil in the insect brain, are required for certain generalization tasks[12,13]. Conspicuously, the general MB function has long been thought to be inhibitory in nature[14-16]. We therefore suspected that the inhibition of skill-learning may be mediated by the MB and enable generalization of the learned fact. To test this hypothesis, we genetically blocked output from the MB, trained the transgenic flies in the composite task and subsequently tested them for generalization of colour memory and for the

isolated skill-learning component (as in the *rut* experiments). Flies with impaired MB function can learn visual cues and perform well in skill-learning as well as several other learning tasks[9]. If the MB mediate the inhibition of skill-learning in order to generalize learned facts, removal of this inhibition in the transgenic flies should lead to significant skill-learning and no generalization. Indeed, flies with blocked MB output perform according to these predictions (Fig. 2b). Further experiments indicate that the MB α and β lobes, but not the γ lobes contribute to this inhibition (Supplementary Figure 8). Are the MB also involved in the facilitation of fact-learning? There is a different composite paradigm in which skill-learning can be prevented technically by making the behaviour-heat association non-predictive[7]. In this experiment, a lack of fact-learning facilitation would entail a decrement in composite performance compared to control animals. No such decrement was observed[9]. Thus, current data are consistent with the hypothesis that the MB mediate inhibition of skill-learning in order to generalize learned facts and are not involved in the facilitation of fact-learning.

Encouraged by these results, we developed our hypothesis one step further. When the colours were removed after composite training, flies with impaired MB function stereotypically continued their attempts to turn in the unpunished direction, despite the change in the environment. Accumulating evidence suggests that the inhibitory nature of the MB allows them to serve a gating function[12,13,16], preventing all but the most important events from forming memories. In our case, one may interpret the results as the MB preventing the formation of a motor memory or habit. Skills and habits are important for efficiently carrying out often-repeated behaviours by limiting the amount of behavioural variability. If our simple skill-learning paradigm indeed were an adequate model for studying habit formation in flies, extended composite training should overcome the MB-mediated inhibition and lead to stereotyped turning attempts and abolished generalization, much as in the transgenic flies. Remarkably, wildtype flies

trained for twice the regular amount of time in the composite task indeed perform as phenocopies of the flies with impaired MB function (Fig. 2c).

Our results allow for the first time to establish a mechanistic model of how active and passive learning systems interact in composite learning situations and which biological substrates mediate the processes resulting in the generation effect (Fig. 3). Acquisition of the *rut*-dependent fact-learning component suppresses acquisition of the *rut*-independent skill-learning component via the MB. The skill-learning component facilitates fact-learning via still unknown, non-MB pathways. This interaction leads to efficient learning, enables generalization and prevents premature habit-formation. Habit formation after extended training reveals the gate-keeping role of the MB, allowing only well-rehearsed behaviours to consolidate into habits. Despite these advances, we still do not know what specific mechanisms lead to the enhancement of learning in the generation effect. However, with this new set of behavioural tools and the molecular genetic arsenal of *Drosophila*, it is only a matter of time until we see progress in this area as well. Lacking any direct evidence, an attractive speculation is that the operant behaviour serves to focus the fly's attention, to more quickly detect coincidences between stimuli. Recent work shows that flies modulate their attention, can focus it to different areas of their visual field and that these attention-like processes require short-term memory genes[17-19]. It is also still unknown what molecular cascades mediate skill-learning. There is preliminary but converging evidence from mice and the marine snail *Aplysia* that one critical molecular component of skill-learning is a calcium-independent but dopamine-dependent adenylyl cyclase acting upstream of protein kinases A and C[20,21].

Methods summary:

Wild-type strain *Berlin* (WT) and *rutabaga* mutant strain *rut*^{2080} were used for this study. Experimental transgenic flies were obtained by crossing a MB-specific GAL4 driver strain (mb247) to an effector strain expressing the catalytic subunit of bacterial tetanus toxin (UAS$_{GAL4}$-TNT). This cross results in a block of synaptic output in the MB neurons targeted by the driver strain. The heterozygote offspring from crossing driver and reporter strain, respectively, to Canton-S wildtype flies served as genetic controls for these experiments. As both crosses were tested simultaneously and their results did not differ, both control groups were pooled. After briefly immobilizing 24-48h old female flies by cold-anesthesia, the flies were glued with head and thorax to a triangle-shaped copper hook the day before the experiment. The animals were then kept individually overnight in small moist chambers containing a few grains of sucrose. The apparatus for dissecting learning-by-doing in *Drosophila* is shown in Fig. 1a, b. The tethered fly, suspended at a torque meter, is flying stationarily in the centre of an arena that is illuminated from behind. The torque meter records the attempts of the fly to turn around its vertical body axis (yaw torque). For green and blue illumination of the arena, the light is passed through monochromatic broad band filters. Filters can be exchanged by a fast solenoid within 0.1s. Alternatively, the arena is illuminated with 'daylight' by passing it through a blue-green filter. Yaw torque is recorded every 50 ms and direction preferences are calculated for nine (extended 15) consecutive 2-min periods (performance index (PI) 1–9; Fig. 1c). During training, one yaw torque/colour combination is paired with ambient temperature and the other with heat from an infrared laser diode. If t_A is the time the fly spends in one of the two situations, and t_B the time in the other, the performance index is calculated as PI = $(t_A - t_B)/(t_A + t_B)$. Error bars in the figures are s.e.m.; asterisks indicate levels of significance against zero (one-sample *t*-test; two-sided *P*-value). For details see Supplementary Methods.

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Correspondence and requests for materials should be addressed to B.B, (bjoern@brembs.net).

Fig. 1: Drosophila composite learning at the torque meter.

a-b, Experimental setup. The fly is attached to a torque meter and its yaw torque, generated by attempted left and right turns, controls the colour of the panorama around it as well as a punishing beam of infrared light. The coloration of panorama illumination is accomplished by a fast solenoid moving two colour filters at the light source such that only light of a specific wavelength (either green or blue) illuminates the panorama at any given time. For instance, right turning may lead to green illumination of the panorama and heat off (**a**), while left turning may lead to blue illumination and heat on (**b**). **c**, Course of experiment. Bars show performance indices (PI) of successive 2-min intervals of pre-test (yellow bars; PI_1, PI_2), training (orange bars; PI_3, PI_4, PI_6, PI_7) and memory test (yellow bars; PI_5, PI_8, PI_9). A PI of 1 means the fly spent the entire period in the unpunished situation, whereas a PI of -1 indicates that the fly spent the entire period in the situation associated with heat. Accordingly, a PI of zero indicates that the fly distributed the time evenly between heated and non-heated situations. Therefore, PIs were tested against zero for statistical significance. The following bar graphs all show the first PI after the last training period (hatched bar). Error bars are s.e.m. throughout.
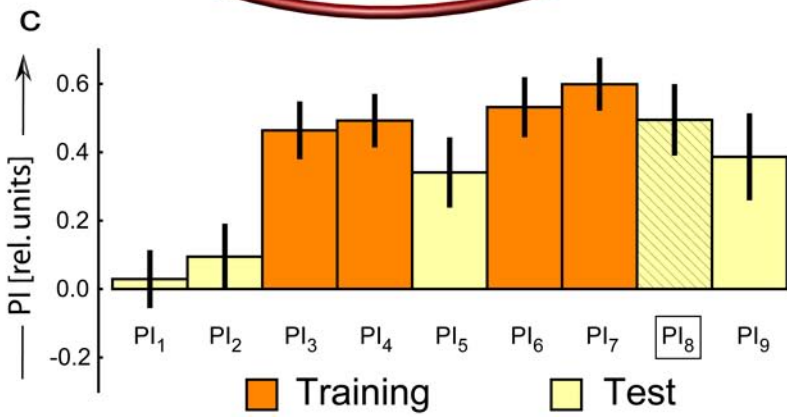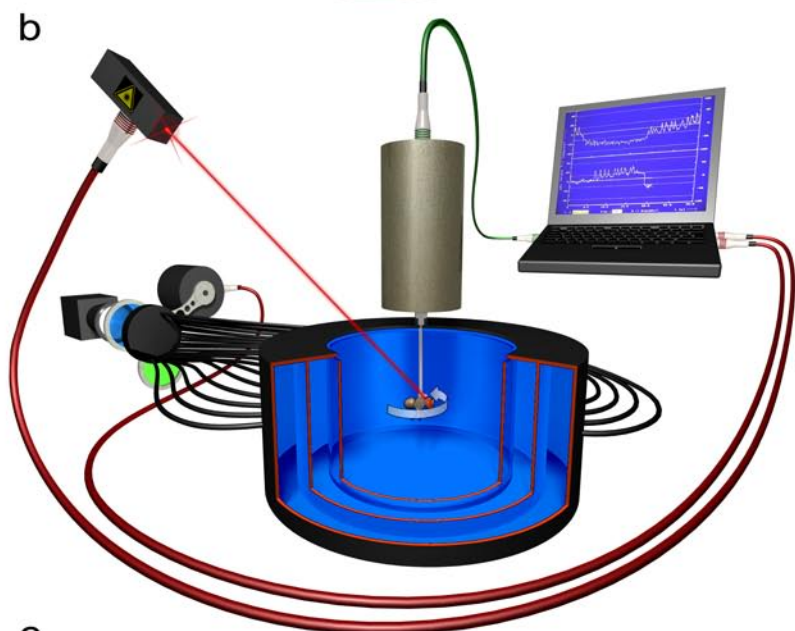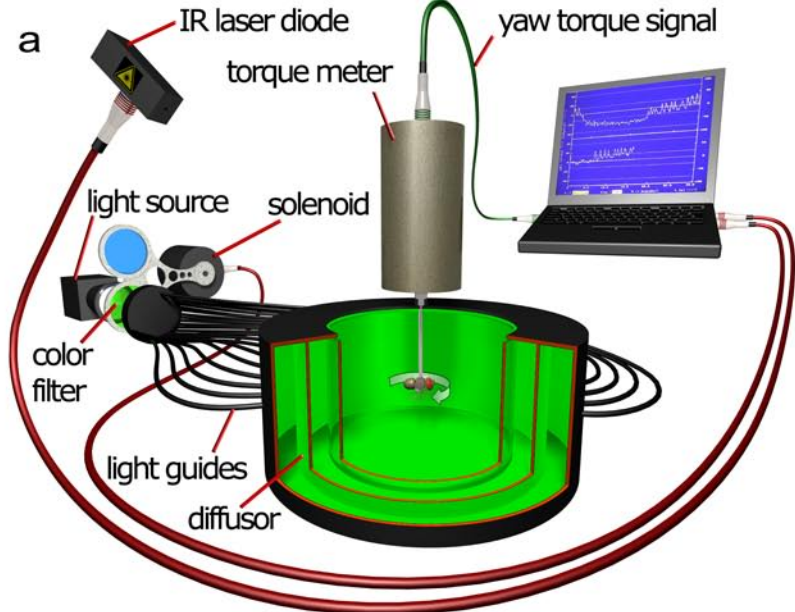
Fig. 2: Experiments with wildtype, mutant and transgenic flies reveal hierarchical interactions between fact- and skill-learning.
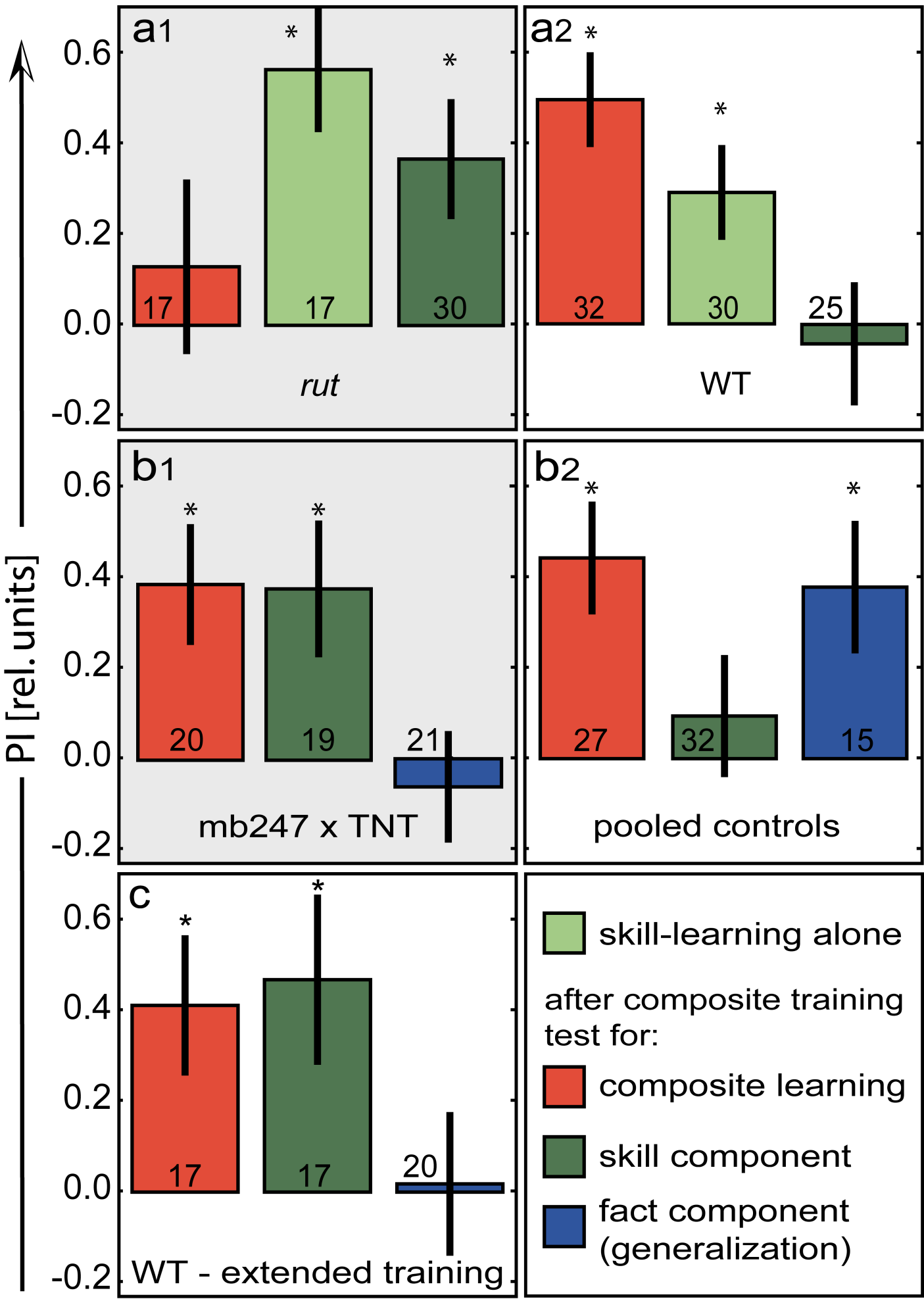
a1, Abolished composite and unaffected skill-learning *rut* mutant flies (red, composite: $t_{16}=0.7$, p<0.5; light-green, skill-learning: $t_{16}=4.3$, p<0.001). After composite training, the skill-learning component is significant (dark green: $t_{29}=2.9$, p<0.007) indicating skill-learning inhibition at the level of retrieval. **a2**, Significant composite and skill-learning in wildtype (WT) flies (composite: $t_{31}=5.1$, p<0.001; skill-learning: $t_{29}=3.0$, p<0.006). After composite training, the skill-learning score is not significant ($t_{24}=-0.3$, p<0.8) indicating inhibition of skill-learning during acquisition. **b1**, Flies expressing the bacterial tetanus toxin light chain in most mushroom-body intrinsic Kenyon cells perform well in composite learning (red: $t_{19}=3.1$, p<0.01), but do not inhibit the skill-learning component during composite training (green: $t_{18}=2.6$, p<0.05). Without inhibition of skill-learning, these transgenic flies are unable to generalize the colour memory (blue: $t_{20}=-0.5$, p<0.6.). **b2**. The genetic control flies (the two heterozygote strains did not differ and were pooled) reproduce the wild-type results: significant composite learning ($t_{26}=3.8$, p<0.001), inhibition of skill-learning ($t_{31}=0.7$, p<0.5) and successful generalization ($t_{14}=2.7$, p<0.05). **c**, Extended training overcomes the inhibition of skill-learning in wildtype flies. The results constitute a phenocopy of the transgenic animals (**b1**). Extended composite learning does not lead to an overtraining decrement ($t_{16}=2.8$, p<0.013). Testing for the skill-learning component after extended composite training shows a release from the inhibition of skill learning ($t_{16}=2.6$, p<0.02). Without inhibition of skill-learning, the flies are unable to generalize ($t_{19}=0.1$, p<0.91).

Grey shading – mutant or experimental animals. No shading – WT or control animals. Numbers at bars – number of animals. * – statistical significance.

Fig. 3: Composite learning consists of two components with reciprocal, hierarchical interactions.

The rut-independent skill-learning component facilitates acquisition of the rut-dependent fact-learning component (generation effect) via unknown, non-mushroom-body pathways. This facilitated fact-learning inhibits acquisition of skill-learning via the mushroom-bodies. These interactions lead to efficient learning, generalisation and prevent premature habit-formation.

a

IR laser diode — yaw torque signal
torque meter
light source — solenoid
color filter
light guides
diffusor

b

c

PI [rel. units]

0.6

0.4

0.2

0.0

-0.2

PI_1 PI_2 PI_3 PI_4 PI_5 PI_6 PI_7 PI_8 PI_9

■ Training   ▨ Test