Report

Double Dissociation of PKC and AC Manipulations on Operant and Classical Learning in *Drosophila*

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Summary

Learning about relationships between stimuli (i.e., classical conditioning [1]) and learning about consequences of one's own behavior (i.e., operant conditioning [2]) constitute the major part of our predictive understanding of the world. Since these forms of learning were recognized as two separate types 80 years ago [3], a recurrent concern has been the issue of whether one biological process can account for both of them [4–9]. Today, we know the anatomical structures required for successful learning in several different paradigms, e.g., operant and classical processes can be localized to different brain regions in rodents [9] and an identified neuron in *Aplysia* shows opposite biophysical changes after operant and classical training, respectively [5]. We also know to some detail the molecular mechanisms underlying some forms of learning and memory consolidation. However, it is not known whether operant and classical learning can be distinguished at the molecular level. Therefore, we investigated whether genetic manipulations could differentiate between operant and classical learning in *Drosophila*. We found a double dissociation of protein kinase C and adenylyl cyclase on operant and classical learning. Moreover, the two learning systems interacted hierarchically such that classical predictors were learned preferentially over operant predictors.

Results

We subjected *rut²⁰⁸⁰* mutants (affecting a type I adenylyl cyclase [AC] that is regulated by Ca^{2+}/Calmodulin and G protein) and transgenic flies expressing an inhibitory pseudosubstrate of protein kinase C (PKCi) under the control of a heat-shock promoter to three experimental procedures: one with only a classical predictor, one with only an operant predictor, and one with both predictors. The Rutabaga type I AC is one of the first learning genes identified and required for various forms of classical learning [10, 11]. It is unknown whether "pure" operant learning (without any classical predictors [4]) also depends on this AC. Flies expressing PKCi have deficits

in modifying their behavior after negative feedback but show intact memory of the stimulus predicting the feedback [12]. Therefore, PKC was considered a likely candidate gene involved in operant learning. In all three experiments, *Drosophila* fruit flies were tethered and suspended at a torque meter measuring the attempts of the flies to turn left or right (yaw torque). An infrared light beam served as an aversive stimulus to train the flies to discriminate between a punished and an unpunished situation. Each fly was trained on one of three different discriminations: (1) only with color as a classical predictor (blue or green; Figures 1A and 1B), (2) only with yaw torque as an operant predictor (left- or right-turning; Figures 2A and 2B), or (3) with a composite of both predictors (Figures 3A and 3B). For details on the experimental procedures, see Supplemental Data (available online) and [13]. Importantly, in all experiments, heat avoidance was normal in all strains (data not shown).

First, we tested the flies for learning the classical color predictor alone (Figure 1C). As expected, *rut* flies were deficient in the paradigm with only a classical predictor ($t_{15} = -0.5$, $p < 0.7$). Wild-type control flies showed normal classical learning ($t_{25} = 2.8$, $p < 0.01$), as did the transgenic flies expressing PKCi ($t_{19} = 2.6$, $p < 0.02$) and the uninduced control flies ($t_{22} = 2.4$, $p < 0.03$). The results were reversed in the strictly operant paradigm (Figure 2C). Despite failing all associative and many nonassociative learning tasks until now, *rut* flies show unimpaired operant behavioral learning ($t_{16} = 4.3$, $p < 0.001$). If anything, learning is slightly enhanced over wild-type control flies ($t_{29} = 3.0$, $p < 0.006$; see also Supplemental Data). In contrast, PKCi-induced flies do not show any behavioral learning ($t_{22} = 0.2$, $p < 0.9$). This deficit is specifically caused by the expression of PKCi because uninduced flies do not show this impairment ($t_{19} = 8.4$, $p < 0.001$) and neither do the heterozygous parental control strains (het. cont. HS: $t_{42} = 4.6$, $p < 0.001$; het. cont. noHS: $t_{40} = 5.7$, $p < 0.001$). With PKC and AC being differentially involved in operant and classical learning, respectively, the final experiment was performed to reveal their relative contributions in an ethologically more relevant, composite learning situation containing both operant and classical predictors (Figure 3C). The failure only of *rut* flies ($t_{16} = 0.7$, $p < 0.5$) and not of PKCi-induced ($t_{26} = 2.1$, $p < 0.05$) or control flies (wild-type: $t_{31} = 5.1$, $p < 0.001$; PKCi noHS: $t_{20} = 3.6$, $p < 0.002$) to master the composite task is evidence that in such learning situations, the classical predictor is learned preferentially over the operant predictor.

Discussion

We found a double dissociation of AC and PKC manipulations on classical and operant learning. Flies devoid of *rut*-AC, despite failing all associative learning tasks until now, perform well in operant learning without predictive stimuli, even outperforming wild-type flies (Figure S1). Conversely, manipulating PKC during training affects operant, but not classical, learning. This is consistent with previous reports showing that PKC manipulations have no effect during training but do have an effect in the maintenance of memory after classical training [14]. Our data clarify and extend another observation [12] in that

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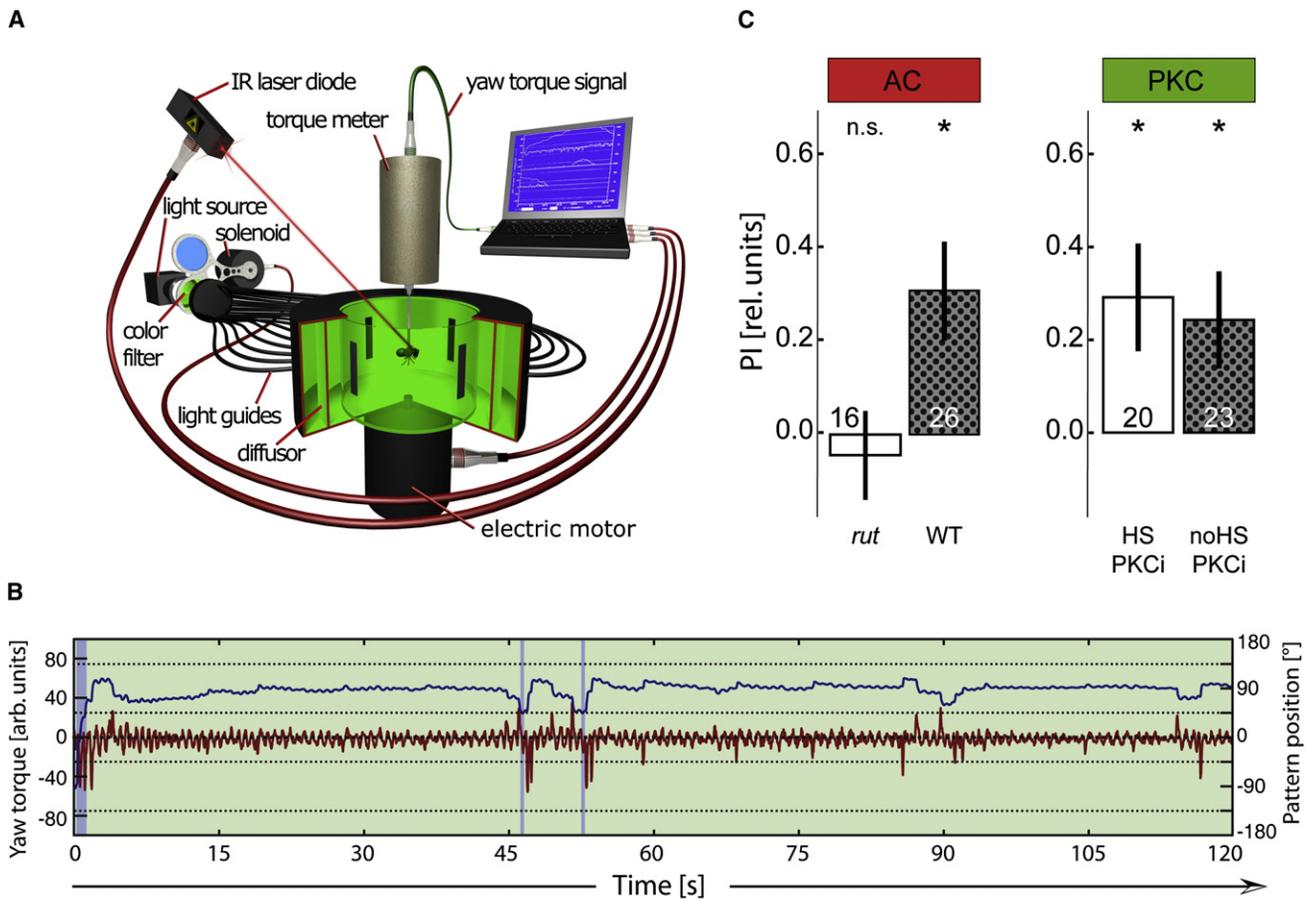
Figure 1. Manipulation of AC, but Not of PKC, Disrupts Learning of a Classical Predictor

(A) Experimental setup. The fly controls the angular position of a drum with four identical vertical bars in a flight-simulator-like situation. The coloration of the arena is switched between bars, such that flying toward one pair of opposing bars leads to green coloration and toward the other pair to blue coloration. During training, heat is made contingent on one color, irrespective of the turning maneuver that changed flight direction.

(B) Sample data from a wild-type fly during the first test period after the final training with heat on blue coloration. The fly uses both left- and right-turning maneuvers (red trace) to change flight direction (blue trace) and hence coloration of the environment (background color of the graph). The fly shows a clear preference for green with only brief excursions into flight directions that lead to blue color, even though the heat is switched off.

(C) Pooled performance indices (PI) from the first test period after training. In this and all subsequent bar graphs: Means are displayed, and error bars represent the standard error of the mean (SEM). Numbers at bars indicate the number of animals. The following abbreviations are used: *rut*, *rut* mutant flies affecting AC; WT, wild-type; HS PKCi, heat-shock-induced expression of the specific PKC inhibitor; noHS PKCi, PKCi expression not induced; and n.s., not significant. *, $p < 0.05$.

expressing PKCi selectively affects the capacity for storing behavioral modifications (operant learning) but leaving both classical learning and the capacity to control external stimuli by ongoing behavioral modifications (operant behavior) intact. Recent evidence from *Aplysia* also implicates PKC in operant learning, suggesting that this is a conserved function of PKC [15]. The discovery of PKC underlying operant learning opens the experimental possibility of localizing the structures where PKC is necessary and sufficient for operant learning in the fly brain, a strategy that was used to map engrams in visual and olfactory learning [10, 11]. Our experiments do not provide any evidence for crosstalk between the AC and PKC pathway, leaving the possibility that operant and classical learning may be based on two largely separate molecular processes, which could occur in the same neuron [5]. The hierarchical interaction between operant and classical components in composite learning situations contrasts with the symmetry in which two equivalent classical predictors are learned in compound conditioning [16]. This hierarchy of multiple memory systems

also suggests how the separate molecular basis for operant learning could be missed despite many years of research: Procedurally operant paradigms are dominated by the formation of a biologically classical memory if environmental predictors are available [4]. For instance, our results predict the deficit of *rut* mutant flies in another procedurally operant paradigm designed to screen for operant mutants (the heatbox [7]), because of the analogy of the spatial cues in the heatbox with the color cues used here. In other words, as soon as predictive stimuli are present in operant-learning situations, not only do these stimuli become equivalent to classical stimuli with respect to their independence from the behavior with which they were learned [4], but these composite experiments also cannot be distinguished genetically from classical experiments any more.

Our data and the current literature are consistent with the hypothesis that operant and classical learning can be distinguished by the differential spatiotemporal requirement of several AC and PKC isoform activities, respectively.

A

B

C
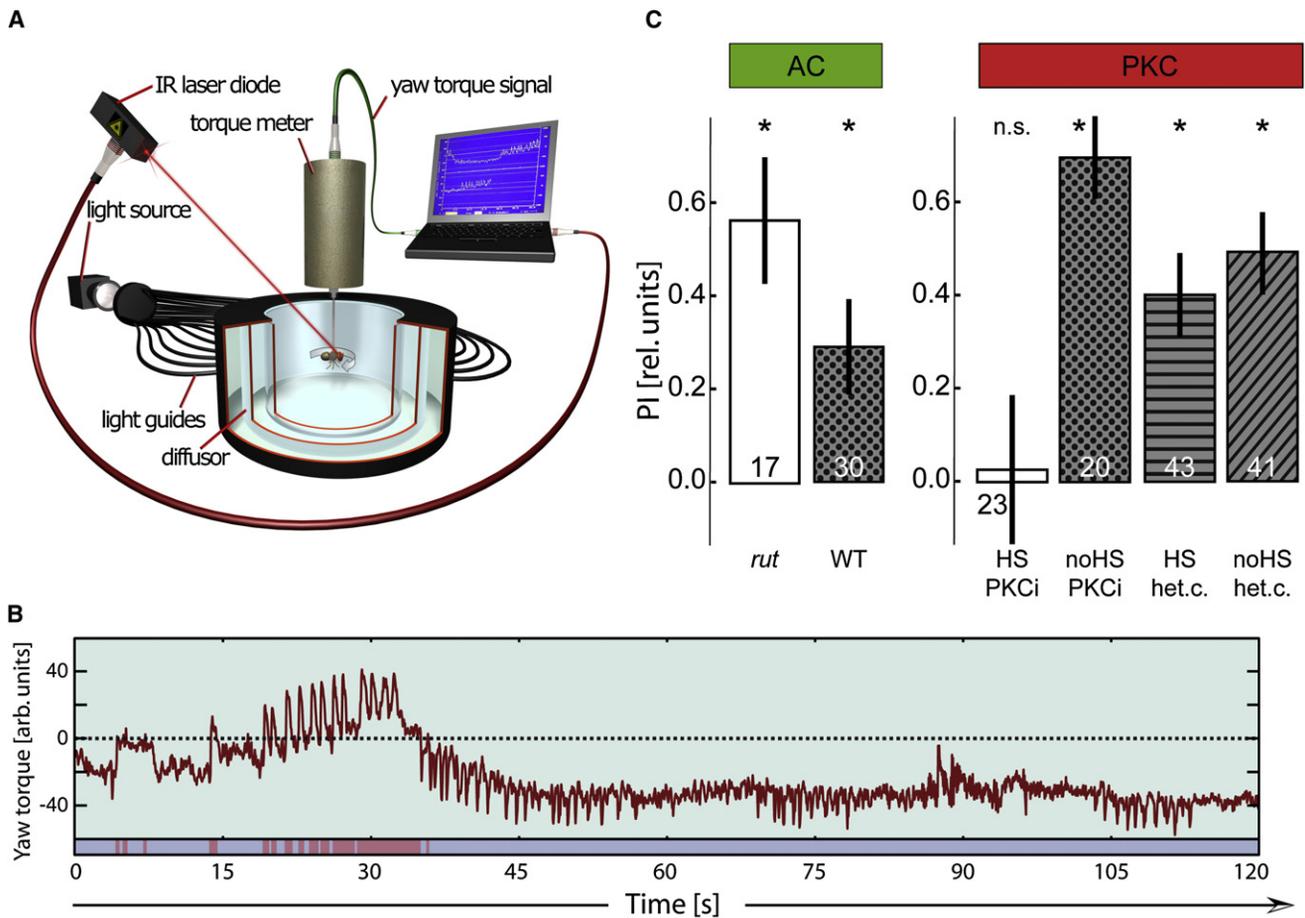
Figure 2. Manipulation of PKC, but Not of AC, Disrupts Learning of an Operant Predictor

(A) Experimental setup. There are no visual cues for the fly. During training, heat is made contingent on either left- or right-turning yaw torque.

(B) Sample data from a wild-type fly during the first test period after the final training with heat on positive (right-turning) yaw torque. The fly only briefly generates right-turning yaw torque during the test phase (unsaturated red/blue bar underneath dark red yaw-torque trace), even though the heat is switched off.

(C) Pooled performance indices (PI) from the first test period after training. The following abbreviations are used: HS het.c., heat-shock-treated heterozygous parental controls strain; noHS het.c., heterozygous parental control strain without heat shock.

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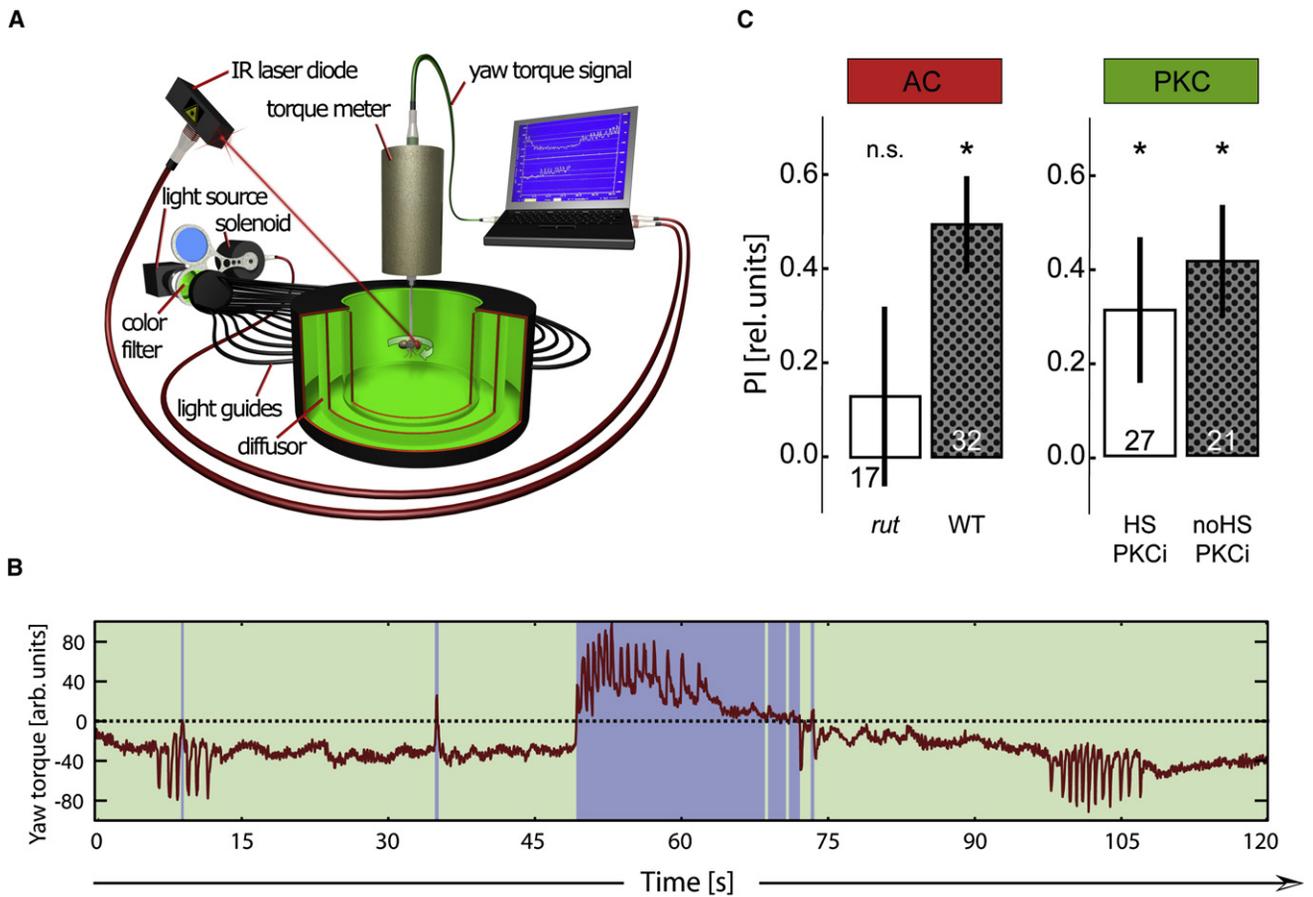
Figure 3. Learning a Classical Predictor Dominates over Learning an Operant Predictor

(A) Experimental design. Throughout the experiment, one yaw-torque domain is coupled to one color and the other to the other color (e.g., right turning causes green illumination and left turning blue illumination of the environment). During training, heat is made contingent on one of the two combinations of yaw torque and color.

(B) Sample data from a wild-type fly during the first test period after the final training with heat on positive (right-turning) yaw torque (red trace) and blue illumination (background coloration). The fly shows the preference for yaw-torque domain or color and only briefly ventures into the previously punished situation, even though the heat is switched off.

(C) Pooled performance indices (PI) from the first test period after training.

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Supplemental Data

Double Dissociation of PKC and AC

Manipulations on Operant

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Supplemental Experimental Procedures

Fly strains. Wild-type strain *Berlin* (WT) and *rutabaga* mutant strain rut^{2080} were used for this study. We used the P[GAL4] technique [1] to express PKCi under the control of a heat-shock promoter as described previously [2]. The system uses two separate components: GAL4 linked to a heat-shock promoter (hsp70–GAL4) to drive expression of the PKC inhibitory peptide linked to the upstream activating sequence (UAS) that responds to GAL4 (UAS–PKCi). "PKCi HS" refers to heat-shock treated progeny of a cross between w; P[w+ hsp70–GAL4], and w; P[w+ UAS–PKCi]. "PKCi noHS" refers to the same strain without heat-shock treatment. The parental heterozygous control strains (het. c.) refer to pooled data from the progeny of two crosses, one between w; P[w+ hsp70–GAL4] and Canton-S and the other between w; P[w+ UAS–PKCi] and Canton S. Both strains showed significant learning scores in the pure operant learning paradigm. Therefore, the two strains were pooled in the heat-shock condition and in the noHS condition, respectively.

Fly preparation. Flies were kept on standard cornmeal/molasses medium [3] at 25°C and 60% humidity with a 12hr light/12hr dark regime [4]. After briefly immobilizing 24-48h old female flies by cold-anaesthesia, the flies were glued (SuperGlue UV glass adhesive, 505127A, Pacer Technology, Cucamonga, Ca., USA) with head and thorax to a triangle-shaped copper hook (diameter 0.05mm) the day before the experiment. The animals were then

kept individually overnight in small moist chambers containing a few grains of sucrose [4]. A

heat-shock of 37°C was applied for 2h with between 0.5-4h recovery time before the

experiment (i.e., the time period of maximal PKCi expression, according to [5]).

Apparatus. The core device of the set-up is the torque compensator (torque meter) [6]. It

measures a fly's angular momentum around its vertical body axis, caused by intended flight

manoeuvres. The fly, glued to the hook as described above, is attached to the torque meter via

a clamp to accomplish stationary flight in the centre of a cylindrical panorama (arena,

diameter 58mm), which is homogeneously illuminated from behind. The light source is a

100W, 12V tungsten-iodine bulb. For green and blue illumination of the arena, the light is

passed through monochromatic broad band Kodak Wratten gelatin filters (#47 and #99,

respectively). 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 (Rosco "surfblue" No.

5433). The transmission spectrum of the Rosco blue-green filter used in this study is

equivalent to that of a BG18 filter (Schott, Mainz) and constitutes an intermediate between the

Kodak blue and green filters [7, 8]. An analogue to digital converter card (PCL812;

Advantech Co.) feeds the yaw torque signal into a computer which stores the trace (sampling

frequency 20Hz) for later analysis. Punishment is achieved by applying heat from an

adjustable infrared laser (825 nm, 150 mW), directed from behind and above onto the fly's

head and thorax. The laser beam is pulsed (approx. 200ms pulse width at ~4Hz) and its

intensity reduced to assure the survival of the fly. The entire experimental procedure is

visualized in ref. [4].

Classical learning. Color learning was performed as described before [8-11]. The arena is

divided into four virtual 90° quadrants, the centers of which are denoted by four identical

vertical bars (width ψ=14°, height θ=40°). A computer controlled electric motor rotates the

arena such that its angular velocity is proportional to, but directed against the fly's yaw torque

(coupling factor K=-11°/s·10^{-10}Nm). This enables the fly to stabilize the panorama and to

control its angular orientation. This virtual 'flight direction' (i.e., arena position) is recorded

continuously via a circular potentiometer (Novotechnik, A4102a306). The analog to digital

converter card feeds arena position with the yaw torque trace into the computer. The color of

the illumination of the whole arena is changed whenever one of the virtual quadrant borders

passes a point in front of the fly. During training, heat punishment is made contingent on one

of the two colors. Thus, color is the only predictor and turning behavior was explicitly non-

contingent with heat, i.e., as a classical predictor color is learned independently of any

concurrently displayed behavior [10]. During test, the heat is permanently switched off and

the fly's color preference recorded.

Operant learning. Yaw torque learning was performed as previously described [10, 12]. The

direction for straight flight in all experiments at the torque meter is determined as the central

value exactly between the maximum left and right turning yaw torque elicited by an

optomotor stimulus. The fly's spontaneous yaw torque range is then divided into a 'left' and

'right' domain at this value. There are no patterns on the arena wall, but the illumination is

spectrally restricted by the blue-green filter. During training, heat is applied whenever the

fly's yaw torque is in one domain and switched off when the torque passes into the other.

Punishment of yaw-torque domains was always counterbalanced. In the test phases, heat is

permanently switched off and the fly's choice of yaw torque domains is recorded.

Composite learning. This is an extension of yaw torque learning, as described before [10].

During training, the fly is heated whenever the fly's yaw torque passes into the domain

associated with punishment. Whenever the fly switches yaw torque domains, not only

temperature but also arena coloration is changed (from green to blue or vice versa). Thus, yaw

torque domain and color serve as equivalent predictors of heat. In contrast to the color

learning paradigm described above, in this paradigm turning behavior is explicitly contingent

with heat. In the test phases, heat is permanently switched off and only the fly's choice of yaw

torque domains/colors is recorded. Punishment of the color/yaw-torque combination was always counterbalanced.

Data evaluation. The color or yaw torque domain preference of individual flies is calculated as the performance index: $PI=(t_a-t_b)/(t_a+t_b)$. During training periods, t_b indicates the time the fly is exposed to the heat and t_a the time without heat. During tests, t_a and t_b refer to the times when the fly chose the formerly (or subsequently) unpunished or punished situation, respectively. Thus, a PI of 1 means the fly spent the entire period in the situation not associated with heat, 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.

Experimental time course. Each fly was used only once. The time-course of the experiment was divided into consecutive periods of 2 minutes duration. Depending on whether heat may be applied during such a period, it is termed a training period (heating possible) or a test period (heat off). Experiments consisted of two pre-test periods (labeled PI_1 and PI_2) 4 training periods (PI_3, PI_4, PI_6 and PI_7) and three memory test periods (PI_5, PI_8 and PI_9). Depicted are always the PI's of the first two minutes after the last training period (PI_8).

Statistics. Individual PI's were tested for significance using a t-Test for single means against zero, following previous studies [7, 8, 11, 13]. All data are expressed as means ± SEM.

Figure S1: *Composite and operant learning with reduced period duration in wildtype and* rut *flies*. For this experiment, period duration was shortened to one minute. To obtain 2-min PIs for comparison with the data in the main text, PI_8 and PI_9 were averaged. Reducing the duration of the experimental periods to 1 minute decreases the total training time to only 4 minutes. **A** – *Composite learning is more effective than pure operant learning in wildtype flies*. With such short training, wildtype flies can solve only the composite learning task ($t_{39}=3.4$, $p<0.002$), while pure operant learning fails to reach a significant value ($t_{44}=0.4$, $p<0.8$). The combination of operant and classical components affords learning capabilities, which are difficult or impossible to achieve by either component alone. **B** – *Mutant* rut *flies show significant operant learning even with reduced training*. As could be suspected from the large learning score with 2 minute periods, experiments with reduced period duration reveal that *rut* mutant flies indeed show enhanced operant learning: they require less training for a significant score than wildtype flies ($t_{22}=3.0$, $p<0.007$). Interestingly, composite learning also shows a significant learning score ($t_{23}=2.8$, $p<0.02$), indicating that the dominant negative effect of the colors shown in the experiments with 2 minute periods develops over the course of training. This failure to perform with increased training is reminiscent of results in a previous study where increased training caused reduced performance when one memory process was eliminated [14].

Displayed are means, error bars are s.e.m. Numbers at bars – number of animals; *rut* – *rut*-mutant flies affecting AC; WT – wildtype; * – $p<0.05$.
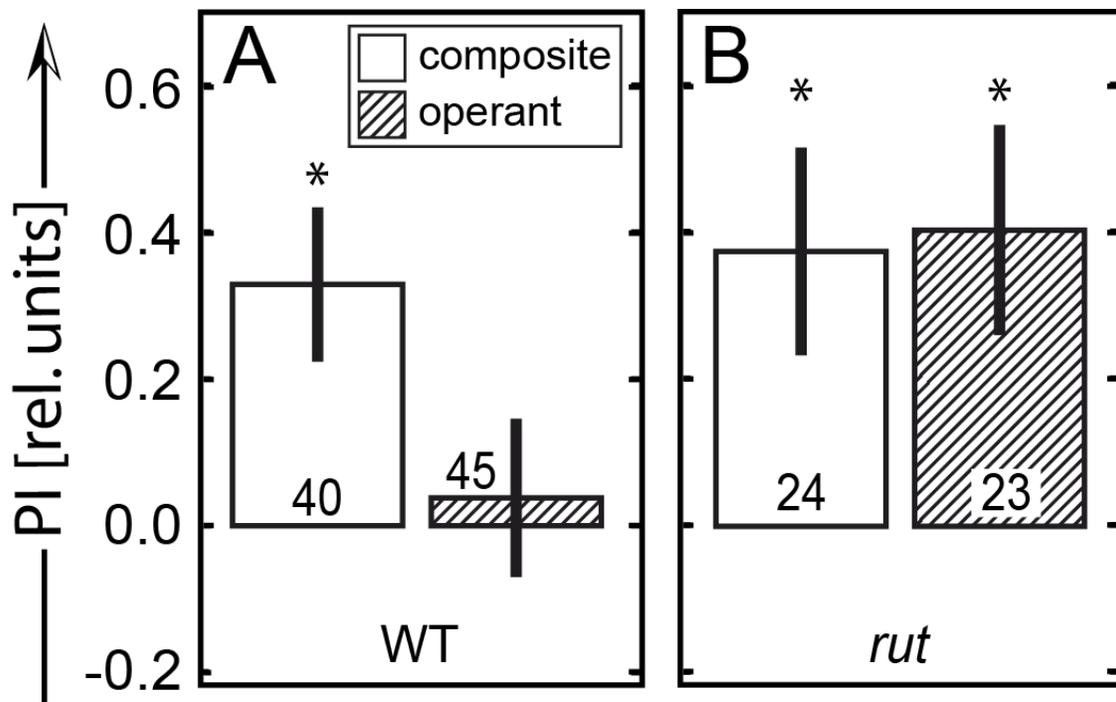
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