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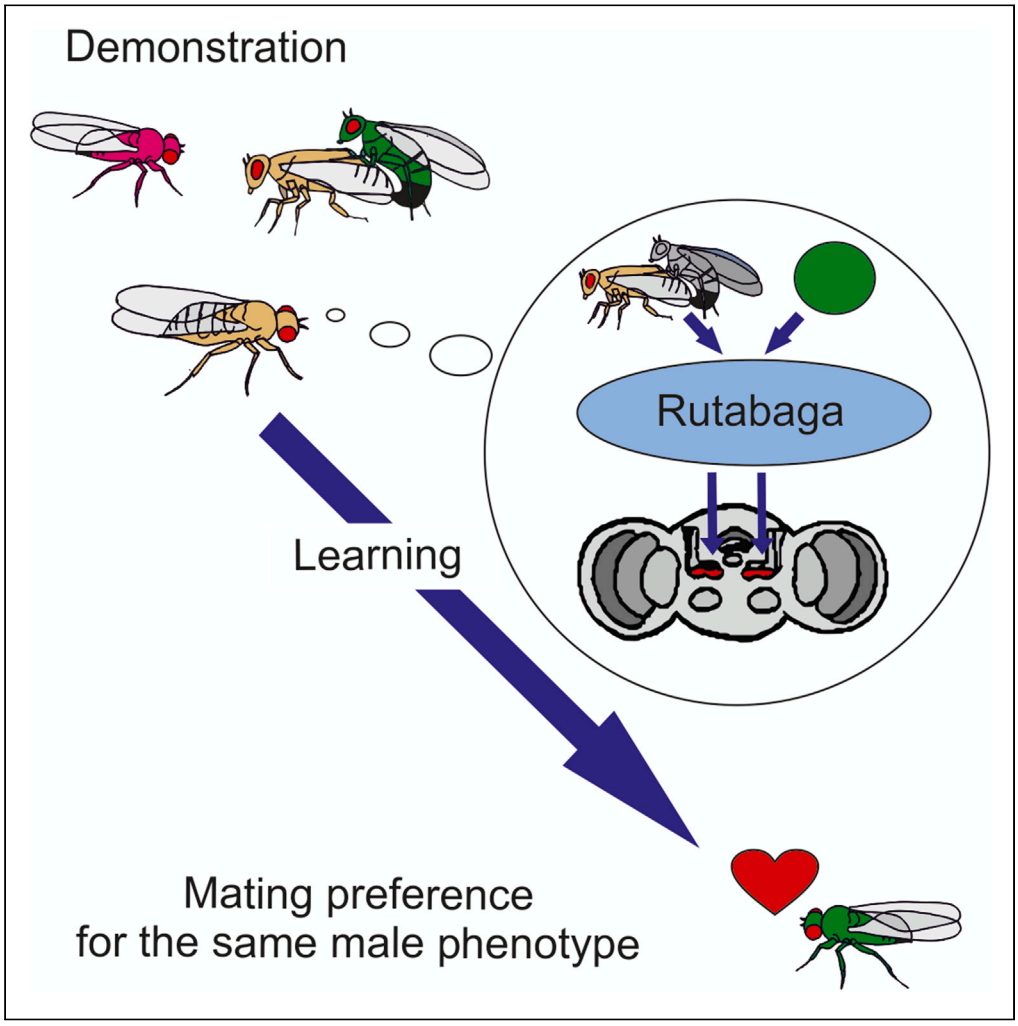

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Highlights

The use of UAS-Gal4 showed that the *rutabaga* gene is required for mate copying

Mate copying occurs only when rutabaga is expressed in mushroom body γ-Kenyon cells

In *D. melanogaster* mate copying requires the same Kenyon cells as asocial learning

Thus, the pathways of social and asocial learning might overlap significantly

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Mate copying requires the coincidence detector Rutabaga in the mushroom bodies of *Drosophila melanogaster*

Sabine Nöbel,[1,2,3,5,*] Etienne Danchin,[3,4] and Guillaume Isabel[4]

SUMMARY

Mate choice constitutes a major fitness-affecting decision often involving social learning leading to copying the preference of other individuals (i.e., mate copying). While mate copying exists in many taxa, its underlying neurobiological mechanisms remain virtually unknown. Here, we show in *Drosophila melanogaster* that the *rutabaga* gene is necessary to support mate copying. *Rutabaga* encodes an adenylyl cyclase (AC-Rut$^+$) acting as a coincidence detector in associative learning. Since the brain localization requirements for AC-Rut$^+$ expression differ in classical and operant learning, we determine the functional localization of AC-Rut$^+$ for mate copying by artificially rescuing the expression of AC-Rut$^+$ in neural subsets of a *rutabaga* mutant. We found that AC-Rut$^+$ has to be expressed in the mushroom bodies' Kenyon cells (KCs), specifically in the γ-KCs subset. Thus, this form of discriminative social learning requires the same KCs as non-social Pavlovian learning, suggesting that pathways of social and asocial learning overlap significantly.

INTRODUCTION

Learning is the act or process of inducing a permanent change in behavior as a result of the sensory and/or behavioral experience of the individual. Associative learning is a simple form of learning that is widely observed across all animal taxa, including humans. Direct associative learning occurs when the animal experiences the association between initially neutral stimulus (which becomes a conditional stimulus (CS) after a successful association) and either an unconditional stimulus (US) or a behavior. On the contrary, indirect associative learning involves a demonstration and no direct experience of the stimuli association. Typically, social learning is supposed to be an indirect form of learning[1] in which a focal individual observes a demonstrator (or teacher) experiencing the association between a cue and a reward. The mechanisms of social learning, in general and more specifically in insects, are now under investigation,[2–6] with valuable recent advances in the social learning model of dialect transmission in *Drosophila* species.[7,8] However, we are still far from understanding these mechanisms thoroughly. In particular, the question of the extent of the overlap between pathways of social learning and the better studied direct associative learning remains poorly explored.[9–11]

Like no other animal, the fruit fly *Drosophila melanogaster* qualifies for studying the underlying mechanisms of social learning. One of the best-descried learning mutants in *Drosophila* is *rutabaga*.[12] *Rutabaga* is an X-linked recessive mutation that codes for a Ca^{2+}/calmodulin-dependent adenylyl cyclase (AC-Rut$^+$) which converts ATP to cAMP through Ca^{2+} and heterotrimeric G-protein signaling.[13,14] *Rutabaga* is considered to act as molecular coincidence detector, capable of integrating information conveyed from separate pathways utilizing receptor/G-protein and Ca^{2+}/calmodulin stimulation.[13,14] It is ubiquitously expressed in the *Drosophila* brain and mediates synaptic plasticity.[14] The mutation affects all learning paradigms tested to date, which include visual, olfactory, and spatial learning, as well as courtship suppression.[15–18]

From these studies, two brain structures, the central complex (CC) and the mushroom bodies (MBs), have emerged as particularly important for learning and memory. The CC spans the sagittal midline and is symmetrically organized.[19] It is composed of four interconnected neuropils: the protocerebral bridge, the fan shaped body, the ellipsoid body, and the noduli,[19] which are all interconnected by sets of columnar interneurons.[20,21] The CC has been shown to be involved in the regulation of various behavioral activities related to walking[21,22] or male courtship,[23] olfactory and visual learning tasks.[24–28] It might act as a higher control center of locomotor behavior[29] and coordinates between brain hemispheres[21] and is required in navigational abilities.[30]

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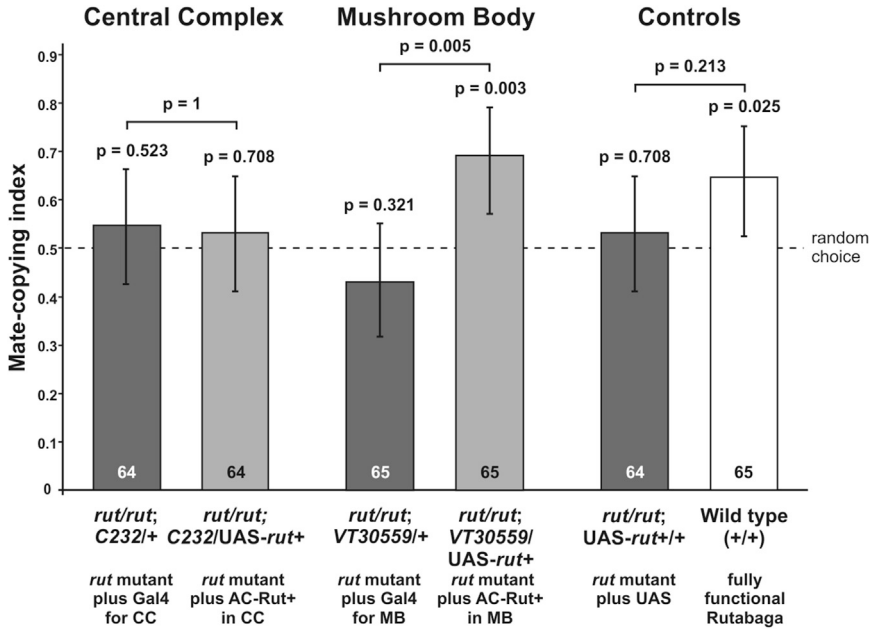

Figure 1. Expression of the Rut-AC^{+} proteins required in mushroom body (MB) Kenyon cells, but not in the central complex (CC) for mate copying

We rescued the expression of the wild type *rut* gene globally in either the CC (two left bars) with the Gal4 driver line *C232* (second bar from left) or in the MB neurons with the driver line *VT30559* (fourth bar from left). The wild type and the line *rut/rut*;UAS-*rut*$^{+}$/+ were used as additional controls to test the specific effect of the UAS-*rut*$^{+}$/+ construct. Number inside bars: number of trials. Statistics: above bars, P-values of the binomial tests of departure from random choice (represented by the dashed line), and that above the horizontal bar is that of the Fisher test. Error bars represent Agresti-Coull 95% confidence intervals.

The MBs consist of 2,000 Kenyon cells in each hemisphere and can be divided into three distinct subtypes by their projection neurons into α/β, α′/β′ and γ lobes.[31] In addition to their morphological distinction, the α/β, α′/β′ and γ neurons are differentiated with respect to their gene expression, neurotransmitter systems, connectivity to extrinsic neurons, and behavioral functions.[32–35] In general, the MBs are seen as sensory integration centers for learning and memory.[31] They are essential for associative appetitive and aversive learning and memory and play a critical role in various learning and memory paradigms such as olfactory and gustatory conditioning, experience-dependent courtship depression, and context generalization in visual learning.[36–43]

Previous studies showed that young fruit flies use social information to choose a mate and develop a preference for male phenotypes that they previously saw being chosen by demonstrator females, i.e., perform mate copying.[44–49] In other words, they copy the mate choice of conspecifics. Mate copying occurs when, after observing another females' mate choice, an observer female tends to preferentially mate with the same male ("individual based" mate copying) or with males of the same phenotype ("trait-based" mate copying) as the one chosen during the demonstration.[50,51] In fruit flies, mate-copying experiments involve a demonstration during which a virgin, naive observer female can watch another female copulating with a male of a given phenotype while a male of a contrasting phenotype stands by, followed by a mate-choice test in which the observer female can mate with one of the two male phenotypes.[45] Mate copying in *Drosophila* is quite sophisticated and has the potential to lead to long-lasting traditions of preferring a certain male phenotype.[46] Although the behavioral patterns are well described,[45–49] the underlying neurobiological mechanisms are unknown.

The powerful genetic tools existing in *Drosophila* make it suitable to study the neurobiological basis of social learning in general. The coincidence detector Rutabaga (AC-Rut^{+}) is required in classical Pavlovian learning to associate a CS and an US[14,16,18,52] and operant Skinnerian learning to associate a behavior and a CS.[24,26] To explore whether AC-Rut^{+} is also required in mate copying and to identify the neural substrates involved, we tested the *rutabaga* mutant (*rut*) in different transgenic contexts. First, we show that this protein is required for mate copying, providing a molecular argument for the fact that this type of learning would be associative. We rescued the wild-type AC-Rut^{+} in a *rutabaga* mutant in the CC and/or the MBs to test whether these neural centers are necessary to perform mate copying. Finally, we test whether this protein was necessary for the proper development of the animal, or only for this type of social learning (as this is the case for Pavlovian or Skinnerian associative learning).

RESULTS

Mate copying requires the expression of the coincidence detector rutabaga specifically in Kenyon cells

Using two Gal4 drivers in a *rut* mutant we expressed the wild type adenylyl-cyclase coincidence detector Rutabaga (AC-Rut^{+}) either in the Central Complex (CC, known to be required in a visual operant learning),[24,26] or in the Kenyon cells (KCs) of the Mushroom Body (MB, known to be required in associative Pavlovian learning[14,16,18,52] (Figure 1)). As in previous studies, we found that wild type females copied the mate

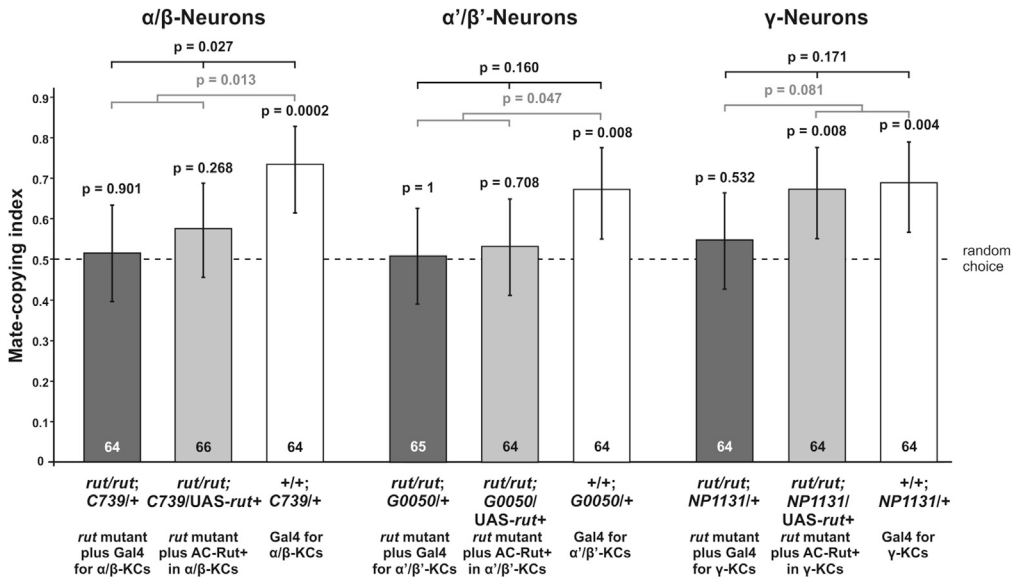

Figure 2. The functional adenylyl-cyclase (Rut-AC$^+$) is required specifically in the γ-Kenyon cells to elicit mate copying

Although the *rut/rut* observer females we used in these experiments could not express a functional adenylyl-cyclase coincidence detector, some of the various construct we built re-establish the expression of the wild type *rutabaga* gene in very specific neuron subsets of the MB, namely the α/β-Kenyon cells (using the Gal4 driver line *C739*), the α′/β′-Kenyon cells (using the Gal4 driver line *G0050*), or the γ-Kenyon cells (using the Gal4 driver line *NP1131*) independently. Only the use of the latter UAS/Gal4 fully rescued mate copying. Number inside bars: number of trials. Statistics: above bars, P-values of the binomial tests of departure from random choice (represented by the dashed line), and that above the horizontal black bar is that of the treatment effect (GLMM). Gray horizontal bars represent post hoc Fisher tests. Error bars represent Agresti-Coull 95% confidence intervals.

choice of conspecifics (binomial test: n = 65, p = 0.025, sixth bar to the right in Figure 1). Females that were homozygote for a non-functional mutation of the *rut* gene and carried the inactive UAS-*rut*$^+$ construct (*rut/rut*;UAS-*rut*$^+$/+; binomial test: n = 64, p = 0.708, fifth bar of Figure 1) did not show any mate copying (binomial test: n = 64, p = 0.708). However, these two groups did not differ significantly, probably due to high variances (Fisher test: n = 129, p = 0.213). Similarly, females lacking a functional AC-Rut$^+$ and carrying only the Gal4-driver like *rut/rut;C232/+* (first bar of Figure 1) or *rut/rut;VT30559/+* (third bar of Figure 1) did not copy (respectively, binomial test: n = 64, p = 0.532; binomial test: n = 65, p = 0.321). Altogether, these results show that the wild type copy of AC-Rut (AC-Rut$^+$), a protein known to be a coincidence detector in non-social associative learning,[53,54] is also required in social learning. Interestingly, females where AC-Rut$^+$ was expressed in the CC only did not copy (*rut/rut;C232/*UAS-*rut*$^+$, binomial test: n = 64, p = 0.708, second bar of Figure 1) while females in which AC-Rut$^+$ was expressed in the whole KCs of the MBs showed mate-copying behavior (*rut/rut;VT30559/*UAS-*rut*$^+$, binomial test: n = 65, p = 0.003, fourth bar of Figure 1). Hence, the functional adenylyl-cyclase AC-Rut$^+$ is required in the MBs but not in the CC for the full expression of this observational social learning.

Mate copying requires the expression of the coincidence detector rutabaga (AC-Rut$^+$) in γ−Kenyon cells only

MBs are constituted of anatomically and functionally distinct groups of neurons named α/β, α′/β′, and γ−KCs.[31,37,55] As the γ−KCs are known to be required in non-social classical associative learning dependent on AC-Rut$^+$[14,18,52] we tested whether mate copying shares the same mechanistic process. Using a *rut* mutant in observer females, we first re-expressed the wild type *rut* gene specifically in the α/β, the α′/β′ or in the γ-KCs (see STAR Methods). We found that rescuing the functional AC-Rut$^+$ protein in these different neurons affected differentially mate-copying scores (Figure 2).

First, using the Gal4 driver line *C739* allowed us to rescue AC-Rut$^+$ expression in the α/β-KCs (three left bars of Figure 2). Such *rut/rut;C739/*UAS-*rut*$^+$ observer females that were able to express a functional Rutabaga protein (AC-Rut$^+$) in the α/β-Kenyon cells, chose randomly (binomial test: n = 66, p = 0.268, second bar Figure 2), as did the control treatment that were homozygote for the *rut* mutation and carried only the Gal4 driver *C739* (*rut/rut;C739/+*; binomial test: n = 64, p = 0.901, first bar Figure 2). Contrastingly, wild type *rut*$^+$ females that carried only a hemizygote copy of *C739* showed normal copying behavior (binomial test: n = 64, p = 0.0002, third bar Figure 2). Hence, AC-Rut$^+$ expression into the α/β-KCs did not rescue this social learning. When comparing these three treatments we found that the treatment effect was significant (GLMM: X^2 = 7.195, df = 1, p = 0.027). The observer females lacking (*rut/rut;C739/+*) or expressing AC-Rut$^+$ (*rut/rut;C739/*UAS-*rut*$^+$) significantly differ from the control group carrying the Gal4 driver *C739* only (post hoc Fisher test: n = 194, p = 0.013).

Second, the specific expression of AC-Rut$^+$ in α′/β′-KCs was addressed with the Gal4 *G0050* driver. Wild type *rut*$^+$ females hemizygote for the Gal4 driver *G0050* showed significant mate copying indexes (binomial test: n = 64, p = 0.008, sixth bar Figure 2). Furthermore, as in

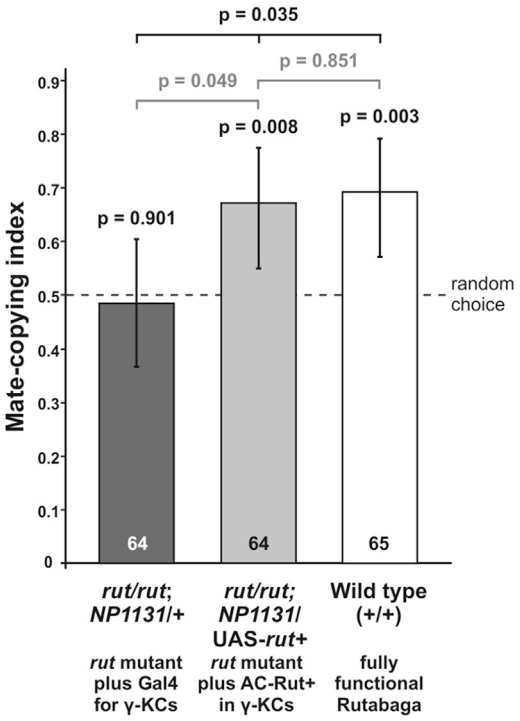

Figure 3. We expressed the wild type rutabaga gene (UAS-*rut*$^+$) in the γ-neurons of the MB with the Gal4 driver line *NP1131* while using pictures of copulating flies instead of real demonstrations

The repeating the experiments of the right block of Figure 2. Each of the pictures we used comprised a female copulating with either a green (or a pink) male while a pink (or a green) male was standing by at some distance from the copulating pair. Number inside bars: number of trials. Statistics: above bars, P-values of the binomial tests of departure from random choice (represented by the dashed line), and that above the horizontal black bar is that of the treatment effect (GLMM). Gray horizontal bars represent post hoc Fisher tests. Error bars represent Agresti-Coull 95% confidence intervals.

previous test, neither the *rut* mutant females carrying only the Gal4 driver (*rut/rut;G0050/+* females, fourth bar Figure 2), nor the *rut* mutant females with a functional AC-Rut$^+$ in the α′/β′-KCs (*rut/rut;G0050*/UAS-*rut*$^+$/, fifth bar Figure 2) were found to perform mate copying (respectively, binomial test: n = 65, p = 1.0, and binomial test: n = 64, p = 0.708). Hence, AC-Rut$^+$ expression into the α′/β′-KCs did not rescue this social learning. These two experiments (with *C739* and *G0050*) suggesting that the α/β and α′/β′ -KCs are not involved in mate copying. When comparing all three treatments we found that the treatment effect was non-significant (GLMM: $X^2 = 3.662$, df = 1, p = 0.160). The observer females lacking (*rut/rut;G0050/+*) or expressing AC-Rut$^+$ (*rut/rut;G0050*/UAS-*rut*$^+$) significantly differ from the control group carrying the Gal4 driver *G0050* only (post hoc Fisher test: n = 193, p = 0.047), corroborating our conclusion.

Third, we then used the Gal4 line *NP1131* to rescue a functional AC-Rut$^+$ protein specifically in the γ-KCs, and found that *NP1131/+* females, i.e., wild type females carrying the Gal4 driver, showed mate-copying indexes that significantly differed from random choice (binomial test: n = 64, p = 0.004, ninth bar of Figure 2). These females mated significantly more often with the male of the color that was seen being chosen during the demonstration than predicted by chance. Contrastingly, *rut* mutant females carrying only the Gal4 driver but no functional copy of the AC-Rut$^+$ (*rut/rut;NP1131/+*) chose randomly (binomial test: n = 64, p = 0.532, seventh bar Figure 2). Finally, we found that mate copying was rescued in *rut/rut; NP1131*/UAS-*rut*$^+$ females, that express a functional AC-Rut$^+$ (binomial test: n = 64, p = 0.008, eighth bar Figure 2), showing the expression of AC-Rut$^+$ in the γ-KCs is necessary and sufficient to rescue mate copying in a *rut* context. When comparing all three treatments we found that the treatment effect was non-significant (GLMM: $X^2 = 3.534$, df = 1, p = 0.171). The observer females expressing AC-Rut$^+$ (*rut/rut;NP1131*/UAS-*rut*$^+$) and the control group of wild type females carrying the Gal4 driver *NP1131* both showed copying, but did not significantly differ from the control group lacking a functional AC-Rut$^+$ (*rut/rut;NP1131/+*; post hoc Fisher test: n = 192, p = 0.081, Figure 2). However, the Fisher test does not account for potential confounding effects such as air-pressure and block (i.e., individuals tested simultaneously). When, instead of a Fisher test, we used a GLMM accounting for these confounding effects, we found a trend (p = 0.060). To sum up, expressing AC-Rut$^+$ in the γ-KCs is required to ensure proper mate copying.

Social learning pathways are similar when using live versus picture demonstrations

In a previous study, we found that mate copying using 2D-images of a sexual intercourse instead of live demonstrations are as effective as showing live copulations in generating social learning.[49] This suggests that flies can extract sex-related information from 2D-images and learn from them. To test whether the KCs are required in a similar way for social learning from 2D-images, we replicated the last part of the previous experiment using 2D-picture of copulating flies as demonstrations instead of the usual live demonstrations.

As with real demonstrations, using photo demonstrations, we found that observer females with the *rut* mutation did not show mate copying (binomial test: n = 64, p = 0.901). Furthermore, the expression of a functional AC-Rut$^+$ in the γ-KC of *rut* mutant observer females rescued this impairment (*rut/rut;NP1131*/UAS-*rut*+, binomial test: n = 64, p = 0.008; middle bar of Figure 3), and led to mate-copying indexes that were very similar to those obtained with wild type observer females carrying the Gal4 driver line *NP1131* (binomial test: n = 65, p = 0.003;

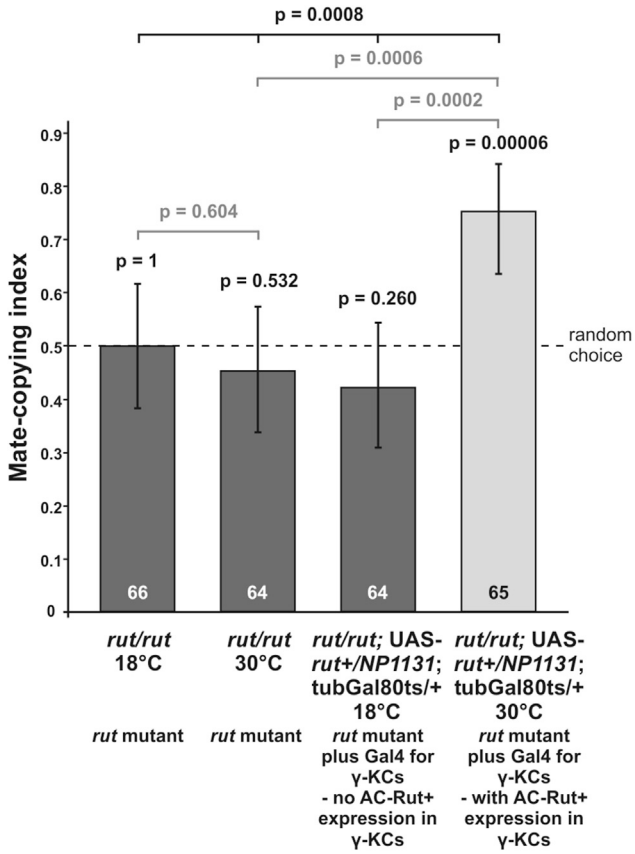

Figure 4. The Rut-AC$^+$ expression is required in γ-Kenyon cells only during the adult stage to ensure mate copying

We expressed the wild type *rutabaga* gene in the γ-neurons of the MB with the Gal4 driver line *NP1131* plus the thermosensitive tubGal80^{ts}. At 18°C, the Gal80^{ts} product blocks the AC-Rut$^+$ expression, while at 30°C AC-Rut$^+$ can be expressed, hence expressing the AC-Rut$^+$ specifically in the γ-KCs during mate-copying experiments. The *rut* null mutant acts as control. Number inside bars: number of trials. Statistics: above bars, P-values of the binomial tests of departure from random choice (represented by the dashed line), and that above the horizontal black bar is that of the treatment effect (GLMM). Gray horizontal bars represent post hoc Fisher tests. Error bars represent Agresti-Coull 95% confidence intervals.

Figure 3; comparison between these last two treatments: Fisher test: n = 129, p = 0.851). These results strongly suggest that the pathway of visual social learning is very similar whether demonstrations involve live copulations or only involve pictures of copulating pairs. In both cases, the AC-Rut$^+$ in γ-KCs plays a key role.

Rescuing the rutabaga protein in the γ-KCs only during adulthood fully restores mate copying

To check whether restoring mate copying requires AC-Rut$^+$ expression throughout development or only during adulthood, we used a combination of the Gal4 driver *NP1131* and the thermosensitive Gal80 (tubGal80^{ts}), which normally functions as a repressor of Gal4.[18] At 30°C, tubGal80^{ts} is inactivated thus allowing the expression of the UAS-*rut*$^+$ construct in the KCs only when exposed to 30°C. Thus, we created *rut/rut*;UAS-*rut*+/*NP1131*;tubGal80^{ts}/+ females that were reared in 18°C (i.e., in the absence of any Rutabaga expression) and then either transferred to 30°C to inactivate tubGal80^{ts} before the experiments or kept at 18°C as control. As an additional control, we did the same with *rut/rut* females.

We found that mate copying was fully restored only in trials where the females were exposed to 30°C before the experiment (GLMM: n = 259, p = 0.0008, Figure 4), implying that the expression of the wild type Rutabaga gene in the γ-KCs is required only adulthood to elicit that specific form of social learning. As expected, *rut/rut* females did not copy at both temperature regimes (18°C binomial test: n = 66, p = 1.0; 30°C binomial test: n = 64, p = 0.532; two left bars of Figure 4). In *rut/rut*;UAS-*rut*+/NP1131; tubGal80^{ts}/+ females reared at 18°C the expression of the AC-Rut$^+$ was blocked permanently during development and mate-copying experiments and those females did not copy the choice of their conspecifics (binomial test: n = 64, p = 0.260). Contrastingly, the group of *rut/rut*;UAS-*rut*+/*NP1131*;tubGal80^{ts}/+ females that developed at 18°C, but were exposed to 30°C before the experiment —which caused the inactivation of Gal80^{ts} allowing the AC-Rut$^+$ expression into the γ-KCs— displayed normal mate copying (binomial test: n = 65, p = 0.00006). Hence, the absence of Rut-AC$^+$ during larval and pupal development did not impair mate copying in adults, provided that Rut-AC$^+$ is expressed at the time of the mate-copying experiment. Hence, the expression of AC-Rut$^+$ in the γ-KCs is necessary and sufficient only in adult females to ensure mate copying.

DISCUSSION

We showed that, both with live and picture demonstrations, the AC-Rut$^+$ protein is involved in mate copying. Expressing AC-Rut$^+$ in the Central Complex did not rescue mate copying, suggesting that the AC-rut$^+$ in the CC is not necessary for that behavioral pattern. This is in contrast to previous studies showing that the CC plays a role in operant visual learning.[24,26,55] Contrastingly, we found that the γ-KCs of the MBs are necessary and sufficient for mate copying, since re-establishing the expression of AC-Rut$^+$ in the γ-KCs fully rescues mate copying in *rut* observer females in the different contexts in which we tested it. This suggests that mate copying shares some mechanisms with classical associative non-social learning. Furthermore, the fact that expressing AC-Rut$^+$ only at the adult stage rescues the full behavioral pattern rules out any developmental issue putatively due to the *rut* mutation.

Interestingly, AC-Rut$^+$ appears as a key protein required in several associative non-social learning paradigm such as classical learning or operant learning. Imaging technique showed previously that AC-Rut$^+$ acts as a coincidence detector in non-social contexts, as MBs AC-Rut$^+$ is activated more strongly when two neurotransmitters conveying information of unconditional and conditional stimuli are both applied simultaneously to a preparation of fly than when the two neurotransmitters are applied independently.[53,54] The fact that AC-Rut$^+$ is required also in this form of social learning strongly suggests the existence of tight links between social and non-social associative learning. In mate copying, the male color can be considered as the CS and the copulation of the demonstrators as the US.[56] This, thus, closely recalls the classical conditioning in *olfactory* learning in which the odor is the CS, and the electric shocks[14] or the sugar[52] the US, and in which the expression of AC-Rut$^+$ is needed in the same γ-KCs of the MB.[55] Furthermore, γ-KCs output are required also in non-social associative *visual* learning.[57] Their similar roles in olfactory and visual learning,[14,16,18,24,52,58] as well as in mate copying (this study), and reacting to courtship conditioning[59,60] show that both, visual and olfactory cues of social or non-social origin elicit the functionality of MB γ-neurons. Thus, these neurons appear to constitute a hub in the neuronal pathways of a large series of types of *Drosophila* associative learning.

Limitations of the study

Inhibiting the expression of a gene like *rutabaga* and restoring its expression in a few neurons in a mutant context is a powerful way to show its involvement in any function, especially as the nature of Rutabaga (usually considered as a coincidence detector) strongly supports our findings. Altogether, our three independent experiments show that γ-KCs are necessary for mate copying and that this pathway involves the rutabaga protein (Figures 2, 3, and 4). Although our first statistical test only reveals a trend for γ-KCs (Figure 2), the fact that we found highly significant results supporting that trend in two independent experiments replicating the same kind of test in different contexts (photo demos and temperature-dependent expression) allows us to conclude that the lack of significance in the first test was probably due to a lack of power because groups that did not copy were slightly, but non-significantly, above 0.5. Remarkably, binomial tests of the individual treatments all support our interpretation. In sum, since we found in three independent experiments (real demos, photo demos, and temperature-dependent expression) evidence that the γ-KCs are required for mate copying, we can trust our conclusions. Finally, the fact that photos are efficient in triggering social learning involving the same mechanistic pathways opens the way to further studies, like calcium imaging, to further decipher the neurobiology of mate copying. Our study opens a new avenue of research to unravel the full pathways of social learning, either upstream or downstream of the γ-KCs.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Experimental protocol – Short-term memory context
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107682.

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AUTHOR CONTRIBUTIONS

S.N. and G.I. designed the study, S.N. performed the experiments under supervision of E.D. and G.I. S.N. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

DECLARATION OF INTERESTS

Authors have nothing to declare.

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REFERENCES

1. Olsson, A., Nearing, K.I., and Phelps, E.A. (2007). Learning fears by observing others: The neural systems of social fear transmission. Soc. Cognit. Affect Neurosci. *2*, 3–11. https://doi.org/10.1093/scan/nsm005.
2. Allsop, S.A., Wichmann, R., Mills, F., Burgos-Robles, A., Chang, C.J., Felix-Ortiz, A.C., Vienne, A., Beyeler, A., Izadmehr, E.M., Glober, G., et al. (2018). Corticoamygdala transfer of socially derived information gates observational learning. Cell *173*, 1329–1342.e18. https://doi.org/10.1016/j.cell.2018.04.004.
3. Debiec, J., and Olsson, A. (2017). Social fear learning: From animal models to human function. Trends Cognit. Sci. *21*, 546–555. https://doi.org/10.1016/j.tics.2017.04.010.
4. Kavaliers, M., and Choleris, E. (2017). Social cognition and the neurobiology of rodent mate choice. Integr. Comp. Biol. *57*, 846–856. https://doi.org/10.1093/icb/icx042.
5. Loureiro, M., Achargui, R., Flakowski, J., Van Zessen, R., Stefanelli, T., Pascoli, V., and Lüscher, C. (2019). Social transmission of food safety depends on synaptic plasticity in the prefrontal cortex. Science *364*, 991–995. https://doi.org/10.1126/science.aaw5842.
6. Segi, Y., Hashimoto, K., and Mizunami, M. (2023). Octopamine neurons mediate reward signals in social learning in an insect. iScience *26*, 106612. https://doi.org/10.1016/j.isci.2023.106612.
7. Kacsoh, B.Z., Bozler, J., and Bosco, G. (2018). *Drosophila* species learn dialects through communal living. PLoS Genet. *14*, e1007430. https://doi.org/10.1371/journal.pgen.1007430.
8. Kacsoh, B.Z., Bozler, J., Hodge, S., and Bosco, G. (2019). Neural circuitry of social learning in *Drosophila* requires multiple inputs to facilitate inter-species communication. Commun. Biol. *2*, 309. https://doi.org/10.1038/s42003-019-0557-5.
9. Heyes, C.M. (1994). Social learning in animals: Categories and mechanisms. Biol. Rev. *69*, 207–231. https://doi.org/10.1111/j.1469-185x.1994.tb01506.x.
10. Heyes, C., and Pearce, J.M. (2015). Not-so-social learning strategies. Proc. R. Soc. *282*, 20141709. https://doi.org/10.1098/rspb.2014.1709.
11. Leadbeater, E., and Dawson, E.H. (2017). A social insect perspective on the evolution of social learning mechanisms. Proc. Natl. Acad. Sci. USA *114*, 7838–7845. https://doi.org/10.1073/pnas.1620744114.
12. Waddell, S., and Quinn, W.G. (2001). Flies, genes, and learning. Annu. Rev. Neurosci. *24*, 1283–1309. https://doi.org/10.1146/annurev.neuro.24.1.1283.
13. Livingstone, M.S., Sziber, P.P., and Quinn, W.G. (1984). Loss of calcium/calmodulin responsiveness in adenylate cyclase of *rutabaga*, a *Drosophila* learning mutant. Cell *37*, 205–215. https://doi.org/10.1016/0092-8674(84)90316-7.
14. Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. (2000). Localization of a short-term memory in *Drosophila*. Science *288*, 672–675. https://doi.org/10.1126/science.288.5466.672.
15. Han, P.L., Levin, L.R., Reed, R.R., and Davis, R.L. (1992). Preferential expression of the *Drosophila rutabaga* gene in mushroom bodies, neural centers for learning in insects. Neuron *9*, 619–627. https://doi.org/10.1016/0896-6273(92)90026-A.
16. McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal rescue of memory dysfunction in *Drosophila*. Science *302*, 1765–1768. https://doi.org/10.1126/science.1089035.
17. Wustmann, G., Rein, K., Wolf, R., and Heisenberg, M. (1996). A new paradigm for operant conditioning of *Drosophila melanogaster*. J. Comp. Physiol. A *179*, 429–436. https://doi.org/10.1007/BF00194996.
18. Zars, T., Wolf, R., Davis, R., and Heisenberg, M. (2000). Tissue-specific expression of a type I adenylyl cyclase rescues the *rutabaga* mutant memory defect: In search of the engram. Learn. Mem. *7*, 18–31. https://doi.org/10.1101/lm.7.1.18.
19. Power, M.E. (1943). The brain of *Drosophila melanogaster*. J. Morphol. *72*, 517–559.
20. Renn, S.C., Armstrong, J.D., Yang, M., Wang, Z., An, X., Kaiser, K., and Taghert, P.H. (1999). Genetic analysis of the *Drosophila* ellipsoid body neuropil: Organization and development of the central complex. J. Neurobiol. *41*, 189–207. https://doi.org/10.1002/(SICI)1097-4695(19991105)41:2%3C189::AID-NEU3%3E3.0.CO;2-Q.
21. Heisenberg, M. (1998). What do the mushroom bodies do for the insect brain? An introduction. Learn. Mem. *5*, 1–10. https://doi.org/10.1101/lm.5.1.1.
22. Martin, J.R., Raabe, T., and Heisenberg, M. (1999). Central complex substructures are required for the maintenance of locomotor activity in *Drosophila melanogaster*. J. Comp. Physiol. A *185*, 277–288. https://doi.org/10.1007/s003590050387.
23. Sakai, T., and Kitamoto, T. (2006). Differential roles of two major brain structures, mushroom bodies and central complex, for *Drosophila* male courtship behavior. J. Neurobiol. *66*, 821–834. https://doi.org/10.1002/neu.20262.
24. Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., Heisenberg, M., and Liu, L. (2006). Distinct memory traces for two visual features in the *Drosophila* brain. Nature *439*, 551–556. https://doi.org/10.1038/nature04381.
25. Neuser, K., Triphan, T., Mronz, M., Poeck, B., and Strauss, R. (2008). Analysis of a spatial orientation memory in *Drosophila*. Nature *453*, 1244–1247. https://doi.org/10.1038/nature07003.
26. Pan, Y., Zhou, Y., Guo, C., Gong, H., Gong, Z., and Liu, L. (2009). Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. Learn. Mem. *16*, 289–295. https://doi.org/10.1101/lm.1331809.
27. Wang, Z., Pan, Y., Li, W., Jiang, H., Chatzimanolis, L., Chang, J., Gong, Z., and Liu, L. (2008). Visual pattern memory requires foraging function in the central complex of *Drosophila*. Learn. Mem. *15*, 133–142. https://doi.org/10.1101/lm.873008.
28. Strauss, R. (2002). The central complex and the genetic dissection of locomotor behaviour. Curr. Opin. Neurobiol. *12*, 633–638. https://doi.org/10.1016/S0959-4388(02)00385-9.
29. Sitnik, N.A., Tokmacheva, E.V., and Savvateeva-Popova, E.V. (2003). The ability of *Drosophila* mutants with defects in the central complex and mushroom bodies to learn and form memories. Neurosci. Behav. Physiol. *33*, 67–71. https://doi.org/10.1023/A:1021131415653.
30. Cheong, H.S., Siwanowicz, I., and Card, G.M. (2020). Multi-regional circuits underlying visually guided decision-making in *Drosophila*. Curr. Opin. Neurobiol. *65*, 77–87. https://doi.org/10.1016/j.conb.2020.10.010.
31. Crittenden, J.R., Skoulakis, E.M., Han, K.A., Kalderon, D., and Davis, R.L. (1998). Tripartite

mushroom body architecture revealed by antigenic markers. Learn. Mem. *5*, 38–51. https://doi.org/10.1101/lm.5.1.38.

32. Keene, A.C., and Waddell, S. (2007). *Drosophila* olfactory memory: Single genes to complex neural circuits. Nat. Rev. Neurosci. *8*, 341–354. https://doi.org/10.1038/nrn2098.
33. Li, F., Lindsey, J.W., Marin, E.C., Otto, N., Dreher, M., Dempsey, G., Stark, I., Bates, A.S., Pleijzier, M.W., Schlegel, P., et al. (2020). The connectome of the adult *Drosophila* mushroom body provides insights into function. Elife *9*, e62576. https://doi.org/10.7554/eLife.62576.
34. Shih, M.F.M., Davis, F.P., Henry, G.L., and Dubnau, J. (2019). Nuclear transcriptomes of the seven neuronal cell types that constitute the *Drosophila* mushroom bodies. G3 (Bethesda) *9*, 81–94. https://doi.org/10.1534/g3.118.200726.
35. Tanaka, N.K., Tanimoto, H., and Ito, K. (2008). Neuronal assemblies of the *Drosophila* mushroom body. J. Comp. Neurol. *508*, 711–755. https://doi.org/10.1002/cne.21692.
36. Akalal, D.B.G., Wilson, C.F., Zong, L., Tanaka, N.K., Ito, K., and Davis, R.L. (2006). Roles for *Drosophila* mushroom body neurons in olfactory learning and memory. Learn. Mem. *13*, 659–668. https://doi.org/10.1101/lm.221206.
37. Bouzaiane, E., Trannoy, S., Scheunemann, L., Plaçais, P.Y., and Preat, T. (2015). Two independent mushroom body output circuits retrieve the six discrete components of *Drosophila* aversive memory. Cell Rep. *11*, 1280–1292. https://doi.org/10.1016/j.celrep.2015.04.044.
38. Cervantes-Sandoval, I., Martin-Peña, A., Berry, J.A., and Davis, R.L. (2013). System-like consolidation of olfactory memories in *Drosophila*. J. Neurosci. *33*, 9846–9854. https://doi.org/10.1523/JNEUROSCI.0451-13.2013.
39. Guven-Ozkan, T., and Davis, R.L. (2014). Functional neuroanatomy of *Drosophila* olfactory memory formation. Learn. Mem. *21*, 519–526. https://doi.org/10.1101/lm.034363.114.
40. Kirkhart, C., and Scott, K. (2015). Gustatory learning and processing in the *Drosophila* mushroom bodies. J. Neurosci. *35*, 5950–5958. https://doi.org/10.1523/JNEUROSCI.3930-14.2015.
41. Masek, P., and Scott, K. (2010). Limited taste discrimination in *Drosophila*. Proc. Natl. Acad. Sci. USA *107*, 14833–14838. https://doi.org/10.1073/pnas.1009318107.
42. McGuire, S.E., Le, P.T., and Davis, R.L. (2001). The role of *Drosophila* mushroom body signaling in olfactory memory. Science *293*, 1330–1333. https://doi.org/10.1126/science.1062622.
43. Qin, H., Cressy, M., Li, W., Coravos, J.S., Izzi, S.A., and Dubnau, J. (2012). Gamma neurons mediate dopaminergic input during aversive olfactory memory formation in *Drosophila*. Curr. Biol. *22*, 608–614. https://doi.org/10.1016/j.cub.2012.02.014.
44. Mery, F., Varela, S.A.M., Danchin, E., Blanchet, S., Parejo, D., Coolen, I., and Wagner, R.H. (2009). Public versus personal information for mate copying in an invertebrate. Curr. Biol. *19*, 730–734. https://doi.org/10.1016/j.cub.2009.02.064.
45. Dagaeff, A.-C., Pocheville, A., Nöbel, S., Loyau, A., Isabel, G., and Danchin, E. (2016). *Drosophila* mate copying correlates with atmospheric pressure in a speed learning situation. Anim. Behav. *121*, 163–174. https://doi.org/10.1016/j.anbehav.2016.08.022.
46. Danchin, E., Nöbel, S., Pocheville, A., Dagaeff, A.-C., Demay, L., Alphand, M., Ranty-Roby, S., van Renssen, L., Monier, M., Gazagne, E., et al. (2018). Cultural flies: Conformist social learning in fruitflies predicts long-lasting mate-choice traditions. Science *362*, 1025–1030. https://doi.org/10.1126/science.aat1590.
47. Monier, M., Nöbel, S., Danchin, E., and Isabel, G. (2019). Dopamine and serotonin are both required for mate-copying in *Drosophila melanogaster*. Front. Behav. Neurosci. *12*, 334. https://doi.org/10.3389/fnbeh.2018.00334.
48. Nöbel, S., Danchin, E., and Isabel, G. (2018). Mate-copying for a costly variant in *Drosophila melanogaster* females. Behav. Ecol. *29*, 1150–1156. https://doi.org/10.1093/beheco/ary095.
49. Nöbel, S., Monier, M., Villa, D., Danchin, É., and Isabel, G. (2022). 2-D sex images elicit mate copying in fruit flies. Sci. Rep. *12*, 22127. https://doi.org/10.1038/s41598-022-26252-5.
50. Pruett-Jones, S. (1992). Independent versus nonindependent mate choice: do females copy each other? Am. Nat. *140*, 1000–1009. https://doi.org/10.1086/285452.
51. Bowers, R.I., Place, S.S., Todd, P.M., Penke, L., and Asendorpf, J.B. (2012). Generalization in mate-choice copying in humans. Behav. Ecol. *23*, 112–124. https://doi.org/10.1093/beheco/arr164.
52. Trannoy, S., Redt-Clouet, C., Dura, J.M., and Preat, T. (2011). Parallel processing of appetitive short-and long-term memories in *Drosophila*. Curr. Biol. *21*, 1647–1653. https://doi.org/10.1016/j.cub.2011.08.032.
53. Gervasi, N., Tchénio, P., and Preat, T. (2010). PKA dynamics in a *Drosophila* learning center: coincidence detection by rutabaga adenylyl cyclase and spatial regulation by dunce phosphodiesterase. Neuron *65*, 516–529. https://doi.org/10.1016/j.neuron.2010.01.014.
54. Tomchik, S.M., and Davis, R.L. (2009). Dynamics of learning-related cAMP signaling and stimulus integration in the Drosophila olfactory pathway. Neuron *64*, 510–521. https://doi.org/10.1016/j.neuron.2009.09.029.
55. Kahsai, L., and Zars, T. (2011). Learning and memory in *Drosophila*: Behavior, genetics, and neural systems. Int. Rev. Neurobiol. *99*, 139–167. https://doi.org/10.1016/B978-0-12-387003-2.00006-9.
56. Avarguès-Weber, A., Lihoreau, M., Isabel, G., and Giurfa, M. (2015). Information transfer beyond the waggle dance: Observational learning in bees and flies. Front. Ecol. Evol. *3*, 24. https://doi.org/10.3389/fevo.2015.00024.
57. Vogt, K., Aso, Y., Hige, T., Knapek, S., Ichinose, T., Friedrich, A.B., Turner, G.C., Rubin, G.M., and Tanimoto, H. (2016). Direct neural pathways convey distinct visual information to *Drosophila* mushroom bodies. Elife *5*, e14009. https://doi.org/10.7554/eLife.14009.
58. Isabel, G., Pascual, A., and Preat, T. (2004). Exclusive consolidated memory phases in Drosophila. Science *304*, 1024–1027. https://doi.org/10.1126/science.1094932.
59. Krüttner, S., Stepien, B., Noordermeer, J.N., Mommaas, M.A., Mechtler, K., Dickson, B.J., and Keleman, K. (2012). *Drosophila* CPEB Orb2A mediates memory independent of Its RNA-binding domain. Neuron *76*, 383–395. https://doi.org/10.1016/j.neuron.2012.08.028.
60. Rouse, J., Watkinson, K., and Bretman, A. (2018). Flexible memory controls sperm competition responses in male *Drosophila melanogaster*. Proc. Biol. Sci. *285*, 20180619. https://doi.org/10.1098/rspb.2018.0619.
61. Loyau, A., Blanchet, S., Van Laere, P., Clobert, J., and Danchin, E. (2012). When not to copy: female fruit flies use sophisticated public information to avoid mated males. Sci. Rep. *2*, 768. https://doi.org/10.1038/srep00768.
62. O'Dell, K.M., Armstrong, J.D., Yang, M.Y., and Kaiser, K. (1995). Functional dissection of the drosophila mushroom bodies by selective feminization of a genetically defined sub compartments. Neuron *15*, 55–61. https://doi.org/10.1016/0896-6273(95)90064-0.
63. Kvon, E.Z., Kazmar, T., Stampfel, G., Yáñez-Cuna, J.O., Pagani, M., Schernhuber, K., Dickson, B.J., and Stark, A. (2014). Genome-scale functional characterization of *Drosophila* developmental enhancers *in vivo*. Nature *512*, 91–95. https://doi.org/10.1038/nature13395.
64. Placais, P.Y., de Tredern, É., Scheunemann, L., Trannoy, S., Goguel, V., Han, K.A., Isabel, G., and Preat, T. (2017). Upregulated energy metabolism in the *Drosophila* mushroom body is the trigger for long-term memory. Nat. Commun. *8*, 1–14. https://doi.org/10.1038/ncomms15510.
65. Aso, Y., Grübel, K., Busch, S., Friedrich, A.B., Siwanowicz, I., and Tanimoto, H. (2009). The mushroom body of adult *Drosophila* characterized by GAL4 drivers. J. Neurogenet. *23*, 156–172. https://doi.org/10.1080/01677060802471718.
66. Blum, A.L., Li, W., Cressy, M., and Dubnau, J. (2009). Short- and long-term memory in *Drosophila* require cAMP signaling in distinct neuron types. Curr. Biol. *19*, 1341–1350. https://doi.org/10.1016/j.cub.2009.07.016.
67. Krashes, M.J., Keene, A.C., Leung, B., Armstrong, J.D., and Waddell, S. (2007). Sequential use of mushroom body neuron subsets during drosophila odor memory processing. Neuron *53*, 103–115. https://doi.org/10.1016/j.neuron.2006.11.021.
68. Lin, H.H., Lai, J.S.Y., Chin, A.L., Chen, Y.C., and Chiang, A.S. (2007). A map of olfactory representation in the *Drosophila* mushroom body. Cell *128*, 1205–1217. https://doi.org/10.1016/j.cell.2007.03.006.
69. Yang, M.Y., Armstrong, J.D., Vilinsky, I., Strausfeld, N.J., and Kaiser, K. (1995). Subdivision of the *Drosophila* mushroom bodies by enhancer-trap expression patterns. Neuron *15*, 45–54. https://doi.org/10.1016/0896-6273(95)90063-2.
70. R Development Core Team. R: A Language and Environment for Statistical Computing (R Development Core Team, Vienna, 2021)
71. Bates, D.W., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using *lme4*. BMJ Qual. Saf. *24*, 1–3. https://doi.org/10.48550/arXiv.1406.5823.
72. Fox, J., and Weisberg, S. (2019). An {R} Companion to Applied Regression (Sage).

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Data is deposited on Mendeley Data	https://data.mendeley.com/datasets/tb6xfd7bh2/2	https://doi.org/10.17632/tb6xfd7bh2.2
Experimental models: Organisms/strains		
Drosophila melanogaster, Canton-S strain	Gift from Dr. Thomas Preat	N/A
Drosophila melanogaster, rut^{2080}	Gift from Dr. Thomas Preat	N/A
Drosophila melanogaster, *rut; UAS-rut/+*	Bloomington Drosophila Stock Center	9405
Drosophila melanogaster, Gal4 *C232*	Gift from Dr. Alberto Ferrus	N/A
Drosophila melanogaster, Gal4 of α α′ββ γ KCs Gal4 *VT30559*	Vienna Drosophila Resource Center	VDRC_206077; FlyBase_FBst0486483
D. melanogaster, Gal4 of αβ KCs: y[1] w[67c23]; P{w[+mW.hs] = GawB}Hr39[c739]	Bloomington Drosophila Stock Center	BDSC_7362; FlyBase_FBti0002926
D. melanogaster: Gal4 of α′ β′ KCs: *P{GAL4} G0050*	Gift from Dr. Thomas Preat	FlyBase_FBti0100740
D. melanogaster, Gal4 of γ KCs: *P{GAL4} NP1131*	Gift from Dr. Alberto Ferrus	N/A
D. melanogaster: Gal4 *NP1131; tub-Gal80^{ts}*	this article	N/A
D. melanogaster, w-	Gift from Dr. Thomas Preat	N/A
Software and algorithms		
R Version 4.0.2 Plus packages *lme4* and *car*	The R Project for Statistical Computing	https://www.r-project.org/
Other		
Green powder #B-731	Shannon Luminous Materials, Inc.	http://www.blacklite.com/
Pink powder #1162R	BioQuip Products, Inc.	https://www.bioquip.com/search/DispProduct.asp?pid=1166A
Pictures of flies	Nöbel et al.[49]	

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sabine Nöbel (sabine.noebel@zoologie.uni-halle.de).

Materials availability

This study did not generate new unique materials.

Data and code availability

- The data generated during this study is available at Mendeley Data: https://doi.org/10.17632/tb6xfd7bh2.2.
- Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Fly maintenance

We used the common laboratory strain Canton-S of *D. melanogaster* (later we refer to it as wild type (WT)) and the mutant lines rut^{2080}, rut^{2080};UAS-*rut*, Gal4 *C232*, Gal4 *VT30559*, Gal4 *739*, Gal4 *NP1131*, Gal4 *G0050*, and tubGal80^{ts}. The Gal4 lines were outcrossed for at least 5 generations to white flies with Canton-S background before experiments started.

All fly lines were raised in 30 ml vials containing 8 ml corn meal-agar-yeast medium (for the recipe see supplemental information) at 25°C ± 1°C and 59% ± 4% humidity with a 12:12 h light:dark cycle. Flies were sexed and sorted without anaesthesia by gentle aspiration within 2-6 h after emergence and kept in unisex groups of 7 females or 15 males per vial before experiments. Experimental flies were virgin and three or four days old, except for the experiments which involved Gal80^{ts}. For these experiments, some flies were raised at 18°C, sexed and sorted like the others but used at an age of 7 to 8 days.

Experiments were conducted under the same conditions as breeding (12 hrs daylight, 25°C ± 1°C, 59% ± 4%). Observer females were of different genotypes, while demonstrators and test males were always from the Canton-S strain. We created two artificial male phenotypes by randomly dusting males with green or pink powders, which created two contrasting phenotypes independent of any genetic variation. All males and females were used only once. Importantly, as females reject males they just saw copulating to avoid risks of sperm depletion,[61] males used in the mate-choice test always differed from those used in the demonstration. All fly manipulations were performed by gentle aspiration without anaesthesia.

METHOD DETAILS

Experimental protocol – Short-term memory context

Except when explicitly mentioned, we used the classical speed learning protocol developed by Dagaeff et al.[45] Experiments took place in double plastic tubes (1.1 cm x 3 cm each) separated by a microscopy cover slide (16 mm x 16 mm, for details please see Figure S1A). Each mate-copying experiment had two phases: a demonstration followed by a mate-choice test. Demonstrations consisted in a single virgin female (the demonstrator) placed with two virgin males, one of each colour, for 30 min on one side of the tubes, and a naïve, virgin observer female on the other side of the tubes separated by the glass partition. The copulation of the demonstrator female with one of the coloured males provided positive information for that male colour and negative information for the other male phenotype. As copulation lasts approx. 20 min in *D. melanogaster*, the observer female received enough information about the mate choice of the demonstrator female. When copulation ended, we removed the three demonstrator flies and immediately started the mate-choice test by inserting a new pair of males, one of each phenotype, in the demonstration side of the tube, then removing the partition so that the observer female could make her own choice within the next 30 min. To control for male competition that can never be excluded in free ranging individuals, we recorded whether both males courted the female, as this was the only situation when females were in a real situation of choice. All replicates were run as blocks of 6 trials with cardboard separations between experimental set-ups to prevent information exchange between the flies and prevent disturbance by the surrounding.

Replicates, where the observer female copulated with the male of the phenotype preferred during the demonstration (copied), were attributed a mate-copying score of 1, versus 0 in the opposite case. The mate-copying index (MCI) is the mean mate-copying score for each treatment, which corresponds to the proportion of females copulating with the male of the same phenotype as the one that was apparently selected by the demonstrator female. Mate-copying index around 0.5 indicated random choice by observer females, while values above 0.5 revealed mate copying.

For the analysis we took only replicates that fulfilled our minimum criteria of quality, which is that copulation occurred during the mate-choice test and that both males had courted the observer female before the onset of the copulation. Other situations were discarded from the analysis. We tested in total 3,963 observer females and discarded 2,738 replicates where only one male courted the female or no copulation was observed within the 30 min of the mate-choice test. We chose *a priori* to have a sample size of ~64 per treatment in order to have sufficient statistical power.

Proof of concept using photos during the demonstration

In addition to demonstrations with real flies, we also used photos of copulating flies as demonstration. The photos showed a copulating pair plus a rejected by-standing male of the opposite colour. For more details about the photos please see Nöbel et al.[49]

To show the photos, we added a third clip to our wooden device to hold the picture (for details please see Figure S1B). The end of the tube facing the photo was closed with a glued-on cover slide (using Uhu Patafix). The distance between the photo and the cover slide was 0.9 - 1.1 mm. The observer female was placed in the tube next to the photo and could watch the photo for 20 min. Afterwards the photo was covered with cardboard, and we immediately started the mate-choice test by inserting a pair of males, one of each phenotype, in the other side of the tube. Then, we removed the partition so that the observer female could make her own choice and proceeded as described above. We tested in total 575 observer females and discarded 382 replicates where only one male courted the female or no copulation was observed within the 30 min mate-choice test.

Treatments

As the aim of the study was to investigate which brain parts are involved in mate copying, we first looked globally at the CC and the MB. We chose the Gal4 driver line *C232*,[62] which targets the whole CC neurons, and the Gal4 driver *VT30559* for the entire MB neurons[63,64] and crossed it with the rutabaga mutant *rut*^{2080} [15] and *rut*;UAS-*rut*.[14] Female offspring from these crosses were used as observer females. We expected to find mate copying only in observer females that were *rut*^{2080} hemizygous, UAS-*rut* heterozygous and Gal4 heterozygous if the respective brain part is involved in social learning. As a control, we used an identical cross lacking the Gal4 driver or UAS-*rut*. Those females were expected to mate randomly. Wild type females acted as an additional control.

To investigate more the role of the MBs, we used Gal4 driver lines that are specific for one of the MB lobes. Gal4 *C739* is specific for the α/β neurons, Gal4 *G0050* targets the α′/β′ neurons and Gal4 *NP1131* is for the γ neurons.[36,42,43,62,65–69] We crossed these lines with *rut*;UAS-*rut* or wild type flies. If one of these lobes is involved in mate copying, we expected to rescue the behaviour only when observer females were rut^{2080} hemizygous, UAS-*rut* heterozygous and Gal4 heterozygous, while females carrying only the Gal4 should choose randomly.

When we found that one of these MB lobes was involved in mate copying with live demonstrations, we used the same line and tested it with photos during the demonstration to confirm our results. In addition, we expressed rutabaga with the help of the thermosensitive driver tub-$Gal80^{ts}$ only in adult flies to check if rutabaga is required during development or not.[16] For this, we bred rut^{2080} and the crosses with the respective Gal4 driver line and tub$Gal80^{ts}$ at 18°C and transferred 1-day old virgin females to 32°C for 6 or 7 days before we used them in mate-copying experiments. Control lines were kept for the same amount of time in 18°C.

Animal welfare note

Our study involved populations of D. melanogaster that have been maintained exclusively under laboratory conditions for hundreds of generations. The current study includes behavioural observations of *D. melanogaster* which required no ethical approval and complied with French laws regarding animal welfare. We handled flies by gentle aspiration without anesthesia to minimize damage and discomfort. After the experiments, individuals were euthanized in a freezer at −20°C.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

All statistical analyses were performed with the R software (version 4.0.2[70]). We included only cases in which both males courted the female, and we observed copulation within the 30 min of the test. The departure from the random choice was tested with a two-tailed binomial test. To test for a treatment effect, mate-copying scores were analysed in a generalized linear mixed model (GLMM) with binary logistic regression (package *lme4*[71]). All models included normalised air pressure as fixed effect as it was shown to influence mate copying in *D. melanogaster*,[45] however, accounting or not for air pressure did not change any conclusion. In experiments including pictures, we also included the photo ID as fixed effect. We also included a random block effect to account for non-independence of flies from the six trials that were run simultaneously. Significance of fixed effects was tested using Wald chi-square tests implemented in the ANOVA function of the *car* package.[72] All starting models included interactions between fixed effects. We applied a backward selection method using P-values, by dropping out non-significant effects one by one, starting with the highest order interaction. To compare post hoc specific groups, we used Fisher tests.