COGNITIVE NEUROSCIENCE

Memory enhancement by ferulic acid ester across species

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Cognitive impairments can be devastating for quality of life, and thus, preventing or counteracting them is of great value. To this end, the present study exploits the potential of the plant *Rhodiola rosea* and identifies the constituent ferulic acid eicosyl ester [icosyl-(2*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate (FAE-20)] as a memory enhancer. We show that food supplementation with dried root material from *R. rosea* dose-dependently improves odor-taste reward associative memory scores in larval *Drosophila* and prevents the age-related decline of this appetitive memory in adult flies. Task-relevant sensorimotor faculties remain unaltered. From a parallel approach, a list of candidate compounds has been derived, including *R. rosea*–derived FAE-20. Here, we show that both *R. rosea*–derived FAE-20 and synthetic FAE-20 are effective as memory enhancers in larval *Drosophila*. Synthetic FAE-20 also partially compensates for age-related memory decline in adult flies, as well as genetically induced early-onset loss of memory function in young flies. Furthermore, it increases excitability in mouse hippocampal CA1 neurons, leads to more stable context-shock aversive associative memory in young adult (3-month-old) mice, and increases memory scores in old (>2-year-old) mice. Given these effects, and given the utility of *R. rosea*—the plant from which we discovered FAE-20—as a memory enhancer, these results may hold potential for clinical applications.

INTRODUCTION

Whenever cognitive function is pathologically impaired, there is an obvious desire to remedy it. In this context, plants have the potential to provide us with therapeutic substances. Indeed, plant compounds that are widely used and that exert powerful effects on nervous system function, including morphine and codeine (*Papaver somniferum*), pseudoephedrine/ephedrine [genus *Ephedra* (e.g., *E. sinica*)], caffeine [genus *Coffea* (e.g., *C. arabica* and *C. canephora*)], and nicotine [genus *Nicotiana* (e.g., *N. tabacum* and *N. rustica*)], have been identified.

Preparations from the root of *Rhodiola rosea* L., a perennial plant of the family Crassulaceae that grows naturally in higher-altitude regions of the Northern Hemisphere, are traditionally used by humans for various alleged effects, including ones that arguably are related to nervous system function (*1*–*3*) [but see (*4*–*6*)]. Linnaeus (*2*), for example, mentions the use of *R. rosea* roots in cases including "cephalgia" and "hysteria." Contemporary studies in humans (*3*) and in rats (*7*) report that *R. rosea* extract enhances memory and/or attention [reviewed in (*8*)] and counteracts pharmacologically induced deficits in pre-pulse inhibition in rats (*9*), as well as fatigue (*3*) and milder forms of depression in humans (*3*). As yet lacking however, are comprehensive analyses of whether any one or more of the compounds contained in *R. rosea* root material or its extracts can individually bring about any one or more of these effects or produce the increases in life span that have been observed in various species fed with *R. rosea* powder or extract (*10*, *11*). This precludes proper quality control and thus imposes hard limits on the medical and scientific utility of *R. rosea* and of preparations derived from it. Further, the specific memory processes affected by *R. rosea* or its preparations have not been identified. In particular, it remains unknown whether memory acquisition, memory consolidation, or memory retrieval is enhanced.

To allow accelerated analyses of these issues, the present study uses three insect study cases [larval and adult *Drosophila melanogaster*, as well as the honeybee *Apis mellifera* (*12*–*15*)] as effective and placebo-free model systems to investigate the memory-enhancing effects of *R. rosea* root material and crude extracts. After demonstrating their dose-dependent effects in memory enhancement, we take advantage of a parallel study that has provided a list of neurobehaviorally active candidate compounds, including ferulic acid eicosyl ester [icosyl-(2*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate (FAE-20)], purified from *R. rosea* root material. Here, we study the effects of *R. rosea* root material, *R. rosea*–derived FAE-20, and, importantly, synthetic FAE-20 as an individual compound. We show that FAE-20 can indeed enhance memory in larval *Drosophila* and can partially compensate for memory impairments both in aged flies and in a case of genetically induced early-onset memory impairment in young flies. In addition, it can increase the excitability of hippocampal CA1 cells and enhance hippocampus-dependent contextual fear memory in mice. Given the commonalities

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in the mechanisms of learning and memory across species (*13*, *16*), we discuss the potential of these findings for clinical use.

RESULTS

Supplementing food with *Rhodiola*^{1} improves memory scores of larval *Drosophila*

We raised *Drosophila* larvae on either standard food medium (control) or food medium supplemented with different concentrations of *Rhodiola*^{1} root powder to test for the effects of this food supplementation on memory formed in an odor-sugar associative classical conditioning paradigm (Fig. 1A). For the highest concentration of *Rhodiola*^{1}, memory scores were almost tripled relative to control. This effect was confirmed twice by using a simplified, one-odor version of the paradigm (*17*) and by introducing a broader range of concentrations (Fig. 1, B and C). Together, these three independent datasets reveal a concentration-dependent enhancement of memory by *Rhodiola*^{1} food supplementation (Fig. 1, A to D, and fig. S1). In contrast, the behavior of experimentally naïve larvae toward the sugar reward [fructose (FRU)] and toward the odors [*n*-amyl acetate (AM) and 1-octanol (1OCT)] was unaffected (Fig. 2, A to C). Further, supplementing the food with *Rhodiola*^{1} did not affect the behavior of larvae toward the odors after training-like reward-only exposure or after training-like odor-only exposure (Fig. 2, D to G, and fig. S2). We conclude that food supplementation with *Rhodiola*^{1} improves associative memory scores of larval *Drosophila* in a dose-dependent manner and leaves task-relevant sensorimotor processing and the nonassociative effects of stimulus exposure unaffected.

A commercially available *Rhodiola* preparation does not increase larval memory scores

A tablet preparation containing the SHR-5 extract from *R. rosea* is commercially available ("Arctic root" tablets, Swedish Herbal Institute). This extract has been reported to counteract fatigue in humans (*3*), to be effective in the treatment of mild-to-moderate depression (*3*, *18*), and to increase life span and physical stress resistance in *Caenorhabditis elegans* and flies (*10*, *11*) [also see (*3*) for a review]. We therefore tested whether food supplementation with ground Arctic root tablets affects memory scores. This was found not to be the case (fig. S3; for our choice of concentrations, see Materials and Methods).

A subsequent experiment revealed a difference in memory performance between control larvae and larvae raised on food containing the abovementioned tablet or *Rhodiola* root accessions from different sources, named *Rhodiola*^{1} (accession 1), *Rhodiola*^{2} (accession 2, a separate batch of *Rhodiola*^{1}), and *Rhodiola*^{3} (accession 3, a root sample of different geographical origin) (fig. S4). Specifically, food supplementation with *Rhodiola*^{1}, *Rhodiola*^{2}, and *Rhodiola*^{3} increased memory scores, as did root material from a further additional source (accession 4: *Rhodiola*^{4}; fig. S1), but the tablet did not. We performed a separate, parallel chemical analysis and bioactivity correlation analysis aimed at identifying behaviorally active candidate compounds in *R. rosea* roots; this was based on the accession from which we had obtained the largest sample (*Rhodiola*^{4}). Within the present study, however, we first asked whether these effects can also be observed in young and/or aged adult flies.

Rhodiola^{4} improves memory scores in aged but not in young adult *Drosophila*

Food supplementation with *Rhodiola*^{4} did not improve the memory scores of young adult *Drosophila* in an odor-sugar associative learning paradigm (Fig. 3, A and B). Strikingly, however, the low memory scores in aged adult *Drosophila* can be remedied by food supplementation with *Rhodiola*^{4} (Fig. 3C and fig. S5).

We conclude that food supplementation with *Rhodiola*^{4} can compensate for age-related memory decline in adult *Drosophila*. To see whether feeding on *Rhodiola* can also have acute effects on memory function, and to disentangle the enhancing effect of *Rhodiola* on the acquisition of memory, memory consolidation, and memory retrieval, we decided to use the proboscis extension reflex conditioning paradigm of the honeybee, in which these processes can be conveniently investigated separately (*14*, *15*).

Feeding on *Rhodiola*^{4E} before training improves memory acquisition in the honeybee

We first asked whether *Rhodiola* has an acute impact on memory acquisition. To establish this, we fed the bees the day before training. One group of bees was fed with sugar solution plus *Rhodiola*^{4E}, whereas the second group received the same amount of plain sugar solution. On the next day, both groups were trained in five trials, pairing odor with plain sugar as the reward (Fig. 4A). The group previously fed with *Rhodiola*^{4E} showed statistically higher response rates to the conditioned odor during acquisition than the control group did. Since we could exclude effects of *Rhodiola*^{4E} on task-relevant sensorimotor processing such as sugar responsiveness and odor responsiveness (Fig. 4, B and C), the increase in conditioned responses reflects an enhancement of memory acquisition.

Feeding on *Rhodiola*^{4E} after training improves memory consolidation in the honeybee

Next, we tested whether acute feeding on *Rhodiola*^{4E} affects memory consolidation (*14*, *15*). In the current paradigm, consolidation to an early form of long-term memory (eLTM) takes place until 9 hours after training (*19*). Animals were fed with control or *Rhodiola*^{4E} solution 5 hours after training (i.e., during the time window of memory consolidation) and were tested for their memory the next day, by which time memory consolidation into eLTM was complete. Memory scores in *Rhodiola*^{4E}-treated bees were higher than those in the control group (Fig. 4D and fig. S6), in a dose-dependent manner (Fig. 4E).

Feeding on *Rhodiola*^{4E} does not affect memory retrieval in the honeybee

To see whether acute feeding on *Rhodiola*^{4E} also affects memory retrieval, we shifted *Rhodiola*^{4E} feeding to 29 hours after training and tested memory on the next day. This timing of experimental events ensures that memory consolidation processes had been completed when the animals were treated 29 hours after training (*19*), whereas the time interval between *Rhodiola*^{4E} feeding and the test remained constant relative to the previous experiment (i.e., 19 hours). If *Rhodiola*^{4E} feeding timed in this manner were to facilitate memory retrieval, increased scores should be observed. This, however, was not the case (Fig. 4F).

We conclude that acute feeding on *Rhodiola*^{4E} accelerates memory acquisition and its more effective consolidation into a stable form, whereas memory retrieval and task-relevant sensorimotor faculties remain unaltered. Together, the findings in *Drosophila* and the honeybee so far encouraged us to ask whether a specific memory-enhancing compound could be identified from *Rhodiola*.

Identifying ferulic acid eicosyl ester (FAE-20) as a candidate memory enhancer

Upon obtaining a large sample of dried *Rhodiola*^{4} root material, we first tested a crude extract for its memory-enhancing effect to ascertain that

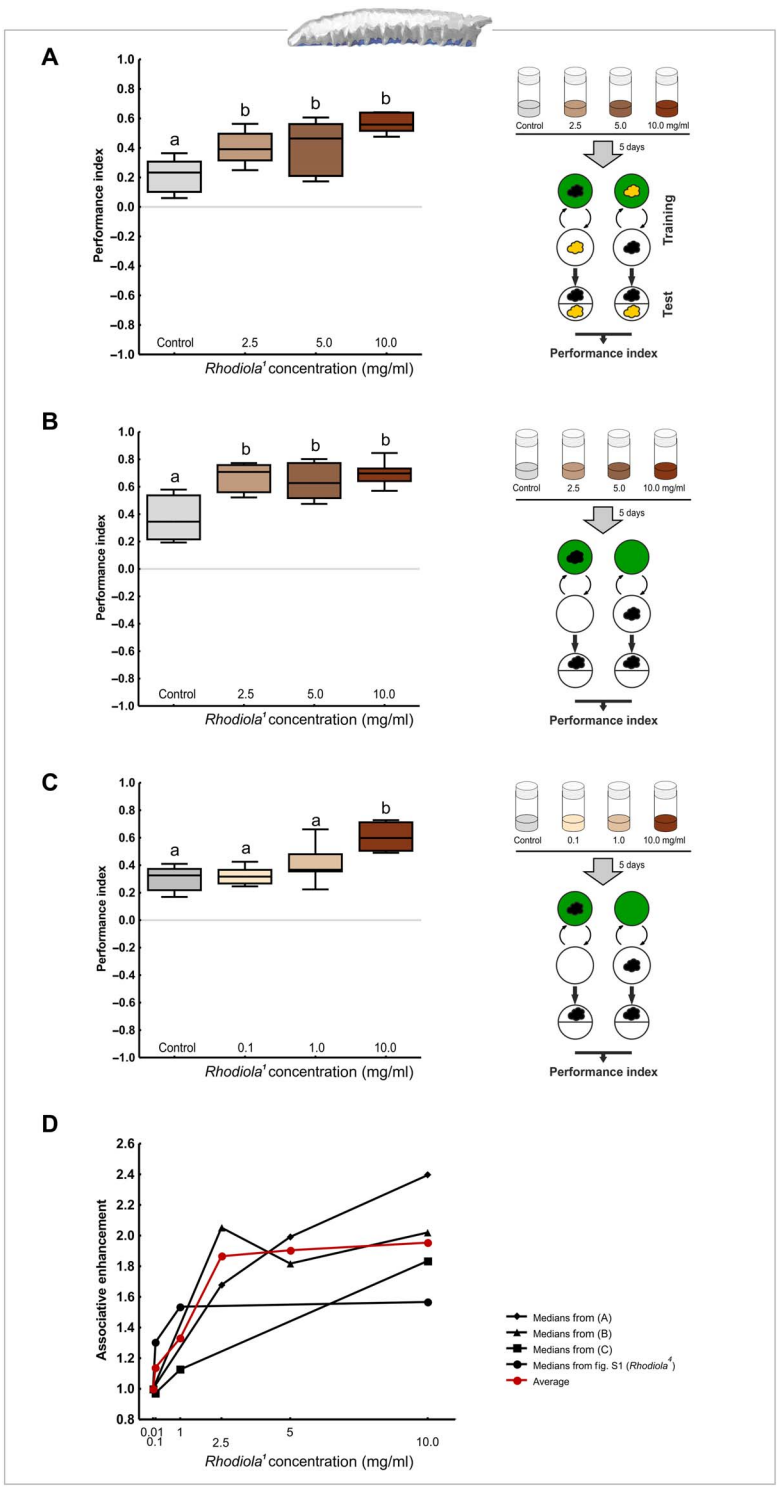

Fig. 1. *Rhodiola*[1] enhances memory in larval *Drosophila*. (**A** to **C**) Larvae reared in food vials with either control food (gray, control) or food supplemented with the indicated concentrations of *Rhodiola*[1] root (brown, *Rhodiola*[1]). (A) Larvae underwent differential training such that one of two odors (AM, black cloud; 1OCT, yellow cloud) was presented on a petri dish together with a sugar reward [green, fructose (FRU); in half of the cases, the training sequence was as indicated; in the other half, it was the reverse]. During testing, the choice between the odors was measured. The performance index (PI) quantifies associative memory as the difference in test behavior between reciprocally trained groups of larvae, revealing that *Rhodiola*[1] dose-dependently increases memory scores. n = 15, 12, 12, and 11. (B) As in (A), showing that *Rhodiola*[1] dose-dependently increases memory scores also in a nondifferential version of the paradigm. n = 11, 11, 11, and 11. (C) As in (B), testing lower levels of *Rhodiola*[1] concentration. n = 15, 15, 13, and 11. (**D**) Summary based on the median PIs using *Rhodiola*[1] (A to C) and *Rhodiola*[4] (fig. S1), normalized to the median of the respective control. Box plots represent the median as the middle line, with the 25th and 75th quantiles as box boundaries and the 10th and 90th quantiles as whiskers. "b" indicates a statistically significant difference from control ("a") in Bonferroni-corrected U tests ($P < 0.05/3$) preceded by a Kruskal-Wallis test ($P < 0.05$). Data are documented in data file S1.

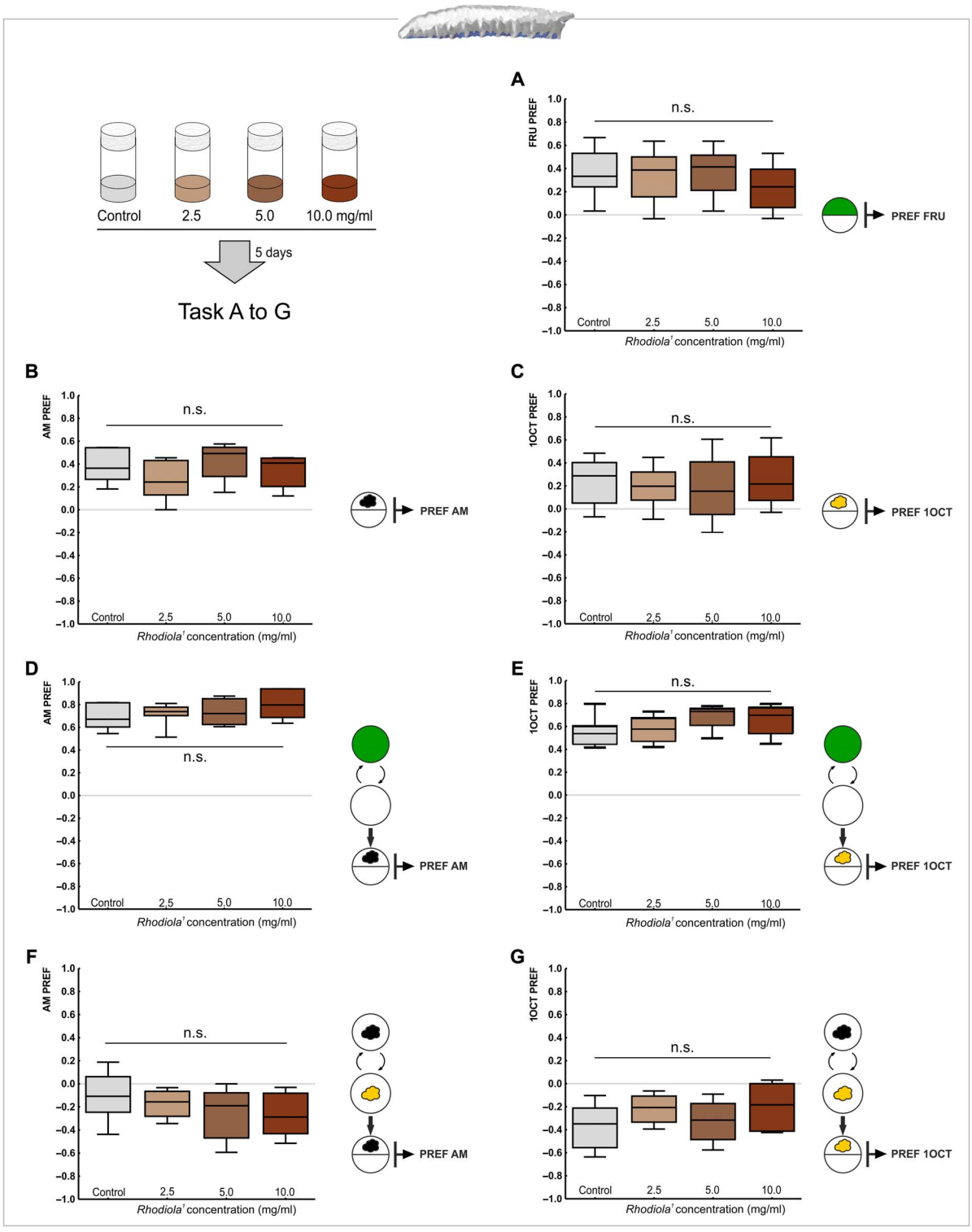

Fig. 2. *Rhodiola*[1] does not affect task-relevant sensorimotor faculties. Larvae reared in food vials with either control food or food supplemented with the indicated concentrations of *Rhodiola*[1] root performed the tasks indicated. These tasks test for sensorimotor faculties relevant for the odor-reward learning experiments shown in Fig. 1. In no case did *Rhodiola*[1] have an effect on performance. (**A** to **C**) Preference of experimentally naïve larvae toward the sugar reward [A: FRU, green (n = 17, 17, 17, and 17)] and toward the odors [B; AM, black cloud (n = 12, 12, 12, and 12); C: 1OCT, yellow cloud (n = 12, 12, 28, and 12)]. (**D** to **G**) Odor preference after training-like stimulus exposure to the reward but omitting the odors [D and E, testing for preferences for AM (n = 12, 12, 12, and 12) and 1OCT (n = 11, 12, 12, and 12), respectively] or to the odors but omitting the reward [F and G, testing for preferences for AM (n = 12, 11, 12, and 12) and 1OCT (n = 12, 12, 12, and 12), respectively]. In half of the cases, the sequence of exposure trials was as indicated; in the other half, it was the reverse. In fig. S2, odor preferences after exposure to only one of the odors are shown. n.s. indicates $P > 0.05$ (Kruskal-Wallis test). Further details as in Fig. 1. Data are documented in data file S1.

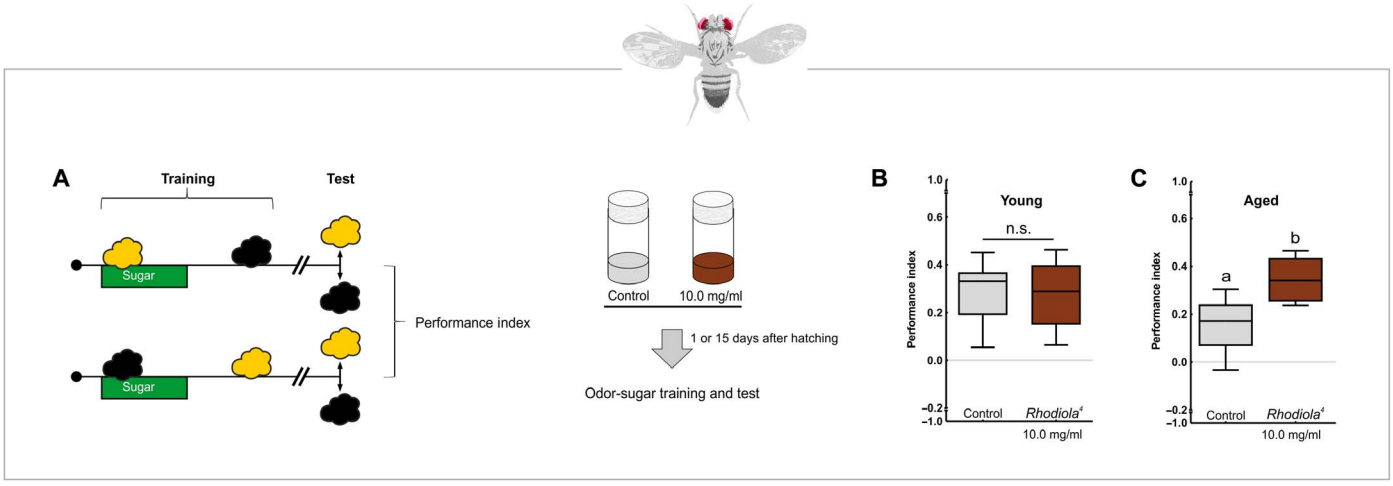

Fig. 3. *Rhodiola*^{4} compensates for age-dependent memory decline. (**A** to **C**) Flies reared until 1 or 15 days after hatching in food vials with either control food or food supplemented with *Rhodiola*^{4} root underwent differential training such that one of two odors [black and yellow clouds; in half of the cases, benzaldehyde (BA); in the other half, 3-octanol (3OCT)] was presented with a sugar reward [green; sucrose (SUC)], and the other odor was presented without the reward (in half of the cases, the training sequence was as indicated; in the other half, it was the reverse). During the test, choice between the odors was measured. The PI quantifies associative memory as the difference in test behavior between reciprocally trained groups of flies. *Rhodiola*^{4} leaves memory scores in young flies unaffected (B; $n = 24$ and 46) yet compensates for the decline in memory scores of aged flies (C; $n = 23$ and 22). n.s. indicates $P > 0.05$ (*U* test). b indicates statistically significant difference from control (a) in a *U* test ($P < 0.05$). Further details as in Fig. 1. Data are documented in data file S1.

functional components of *Rhodiola* are extractable. Supplementing food with *R. rosea* crude extract (*Rhodiola extract*^{crude}) indeed improved memory scores in larval *Drosophila* (Fig. 5A), showing that a soluble component conferred such an effect. This encouraged us to adopt a bioassay-guided isolation and reverse metabolomics approach using activity correlation algorithms (*20*). However, the present food supplementation paradigm requires, impractically, large amounts of extract for such an approach. Although, in principle, the bee paradigm would have been a suitable and resource-friendly alternative, we reasoned that, in the longer run, it would be an advantage to work in a genetically more accessible model system. In a parallel study, therefore, we developed an alternative memory-related screen for larval *Drosophila* that takes advantage of the rewarding effect of *Rhodiola*^{4} and requires much less material. This screen suggested a list of candidate compounds, including β-sitosterol-β-D-glucoside (BSSG) and ferulic acid eicosyl ester (FAE-20). Consequently, both compounds were synthesized to obtain sufficient and pure material for causal verification in the present test for memory enhancement. Given that food supplementation with synthetic BSSG and its derivatives had no impact on larval memory scores (fig. S7), and given that we had ascertained the presence of FAE-20 in *Rhodiola*^{4} (figs. S8 and S9), we focused on synthetic FAE-20 in the following experiments.

FAE-20 as a memory enhancer in *Drosophila*

Drosophila larvae were raised on either control food medium or food medium supplemented with different concentrations of synthetic FAE-20. The latter improved memory scores in a dose-dependent manner. Memory scores were doubled at a 0.71 μM concentration of FAE-20, whereas 10-fold lower or 10-fold higher concentrations of FAE-20 were of no statistically significant effect (Fig. 5B). A similar dose dependency had been observed earlier when using *Rhodiola extract*^{crude} and *Rhodiola*^{4E} (Figs. 4E and 5A). The effect of *R. rosea* food supplementation on the *Drosophila* life span follows an optimum function as well (*11*).

The next question was whether not only FAE-20 but also structurally related and naturally occurring derivatives would improve memory scores in larval *Drosophila* (Fig. 5C). Larvae were raised either on control food or on food supplemented with FAE-20, hexadecyl-(2*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate (FAE-16), dodecyl-(2*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate (FAE-12), octyl-(2*E*)-3-(4-hydroxy-3-methoxyphenyl)-prop-2-enoate (FAE-8), icosyl-(2*E*)-3-(4-hydroxyphenyl)-prop-2-enoate (4-OH-CAE-20), or 7,8-dihydro-ferulic acid eicosyl ester (DH-FAE-20). The results confirm that feeding on FAE-20 leads to higher memory scores than in controls and suggest that substances with a shorter alkyl chain length (FAE-16, FAE-12, and FAE-8) have no statistically significant effect. In addition, food supplementation with substances similar to FAE-20 but lacking the methoxy group of FAEs (4-OH-CAE-20) or substances with a hydrogenated double bond (DH-FAE-20) exerted no measurable effect on memory scores. Food supplementation with FAE-20 leaves the memory scores of young adult flies unchanged (Fig. 6, A and B) but partially compensates for age-related memory decline (Fig. 6C). In contrast, the behavior of experimentally naïve aged flies toward the odors BA and 3OCT (Fig. 6D) and toward the sugar reward (Fig. 6E) was unaffected by FAE-20 food supplementation.

A preliminary proteomics approach suggested that food supplementation with *Rhodiola*^{4} decreases the levels of the active zone protein Bruchpilot in aged adult *Drosophila* (BRP, coded by the *brp* gene). We found this intriguing because, in *Drosophila*, BRP expression has been reported to increase with age, and up-regulation of BRP protein in young flies has been found to elicit a premature impairment of memory function in an aversive learning paradigm (*21*). This raised the question whether, relative to the baseline condition with two genomic copies of the *brp* gene (2×BRP, baseline), the establishment of an early-onset condition of high BRP expression in young flies with four genomic copies of the *brp* gene (4×BRP, "control") (*21*) would lead to an early-onset decrease in memory scores in our appetitive paradigm as well. This was indeed found to be the case at the behavioral level (Fig. 6F; 2×BRP, baseline versus 4×BRP, control). Food supplementation with FAE-20 in 4×BRP flies (4×BRP, FAE-20) was able to partially compensate for this genetically induced "pathological" condition.

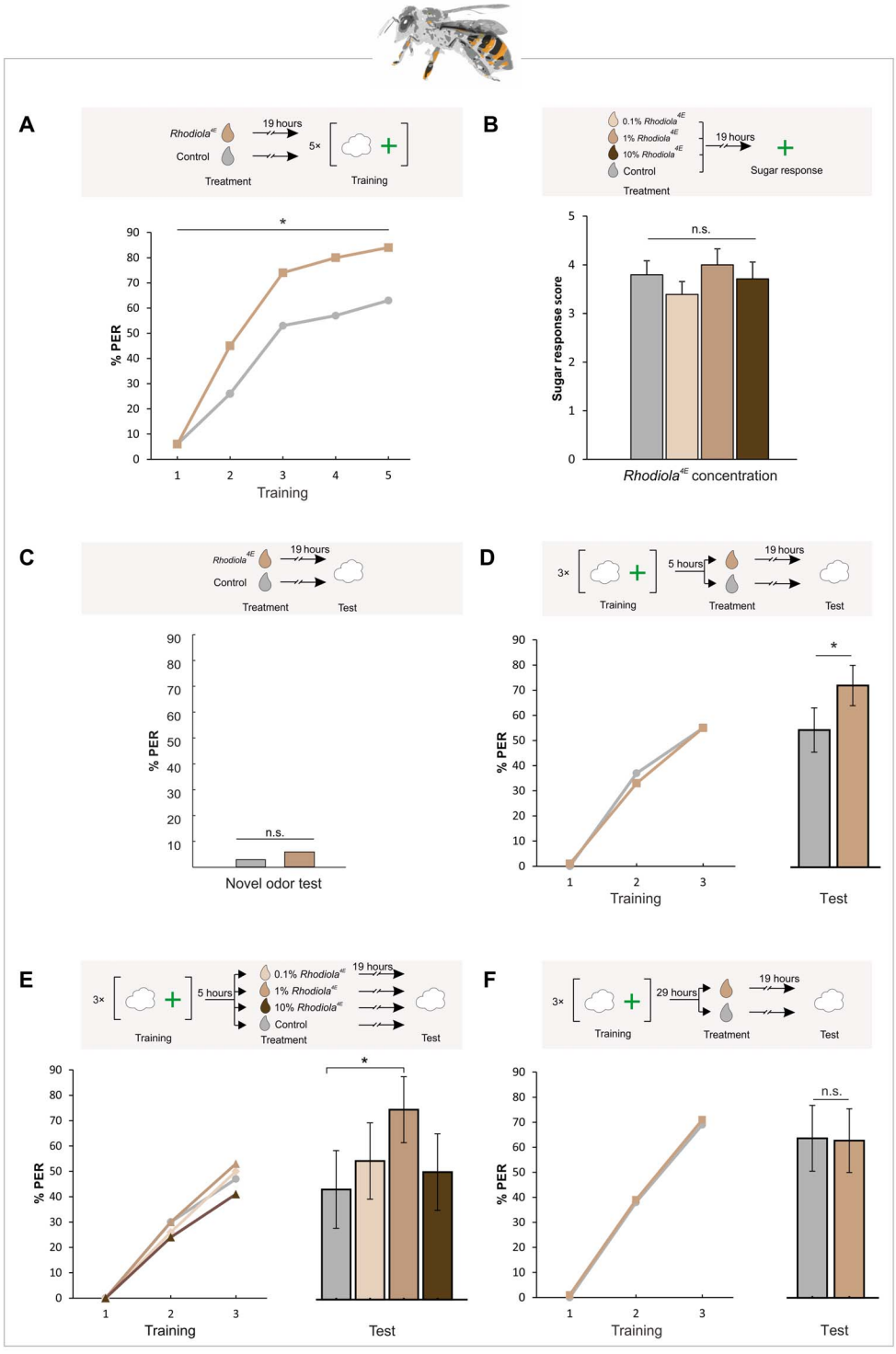

Fig. 4. *Rhodiola*^{4E} improves acquisition and consolidation, but not retrieval, in bees. (**A**) Bees were fed with sugar solution plus *Rhodiola*^{4E} extract (brown), or sugar solution (gray, control). On the next day, the bees underwent classical conditioning of the proboscis extension response (PER) through paired odor-reward presentations. Acquisition rates for PERs toward the odor are higher after feeding on *Rhodiola*^{4E} [logistic regression: $\chi^2(1) = 13.33$, $P = 0.00026$; $n = 73$ and 117]. (**B** and **C**) Feeding on *Rhodiola*^{4E} affects neither sugar responsiveness [B; one-way analysis of variance (ANOVA), $P > 0.05$; $n = 49$, 51, 47, and 45] nor odor responsiveness [C; logistic regression: $\chi^2(1) = 0.01$, $P = 0.98$; $n = 157$ and 114). (**D**) When bees were fed with *Rhodiola*^{4E} during memory consolidation, levels of PER during testing were higher than that in controls [logistic regression: $\chi^2(1) = 8.50$, $P = 0.0036$; $n = 129$ and 130]. Data shown include data from experiments in (E) and fig. S6. (**E**) As in (D), showing that memory is improved in accordance with the *Rhodiola*^{4E} concentration. The memory of bees fed with 1% *Rhodiola*^{4E} differs from the control [logistic regression: $\chi^2(1) = 16.08$, $P = 0.00006$; $n = 44$, 46, 47, and 46]. (**F**) *Rhodiola*^{4E} has no effect on memory retrieval when fed at the same time before the test as in (D) and (E) but does at a time after training when memory consolidation is already over. Rates of PER were not different for bees fed later on with *Rhodiola*^{4E} versus control [logistic regression: $\chi^2(1) = 0.01$, $P = 0.92$; $n = 59$ and 55]. *$P < 0.05$. Bars indicate confidence intervals. Data are documented in data file S1.

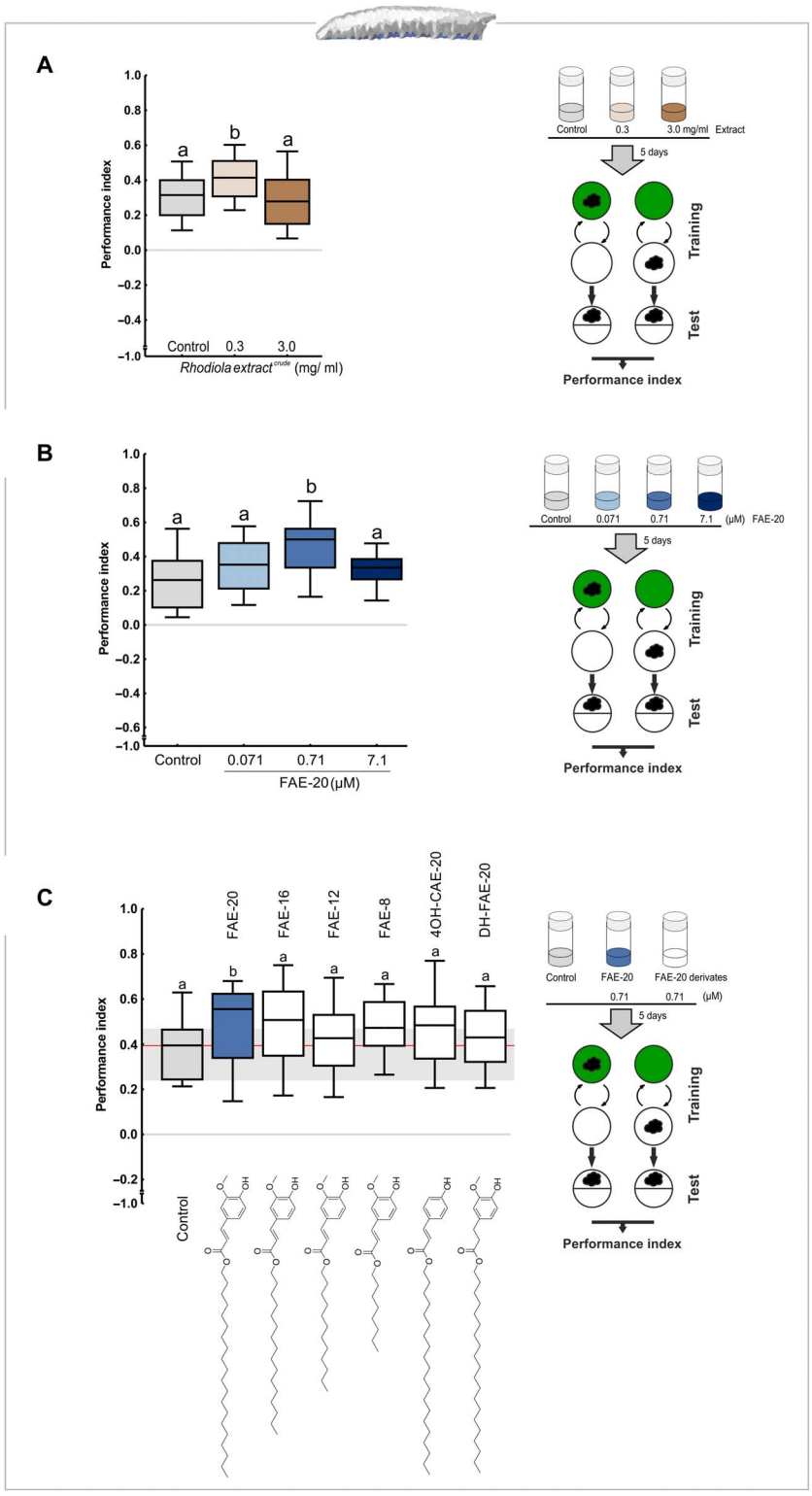

Fig. 5. *Rhodiola extract*^{crude} and FAE-20 improve memory in larval *Drosophila*. (**A** and **B**) As in Fig. 1B, food supplementation with *Rhodiola extract*^{crude} (A; brown) or with FA eicosyl ester (B; FAE-20, blue) improves the memory of larval *Drosophila* in a dose-dependent manner. Similar to what was found for memory consolidation in bees (Fig. 4E), both for *Rhodiola extract*^{crude} and for FAE-20, we observed memory enhancement at intermediate concentrations. b indicates statistically significant difference from control (a) in Bonferroni-corrected *U* tests [A: $P < 0.05/2$ ($n = 33$, 34, and 27); B: $P < 0.05/3$ ($n = 17$, 16, 19, and 18)] preceded by Kruskal-Wallis tests ($P < 0.05$). (**C**) As above, for food supplementation with FAE-20 or the FAE-20 derivatives indicated. Of the tested compounds, only FAE-20 leads to enhanced memory scores relative to control ($P = 0.007$), replicating the results from (B). "b" indicates statistically significant difference from control ("a") in Bonferroni-corrected *U* tests [$P < 0.05/6$ ($n = 35$, 35, 35, 35, 35, and 35)] preceded by a Kruskal-Wallis test ($P = 0.055$). Gray shading indicates the values between the 25th and 75th quantiles of the control PI scores. Further details as in Fig. 1. Data are documented in data file S1.

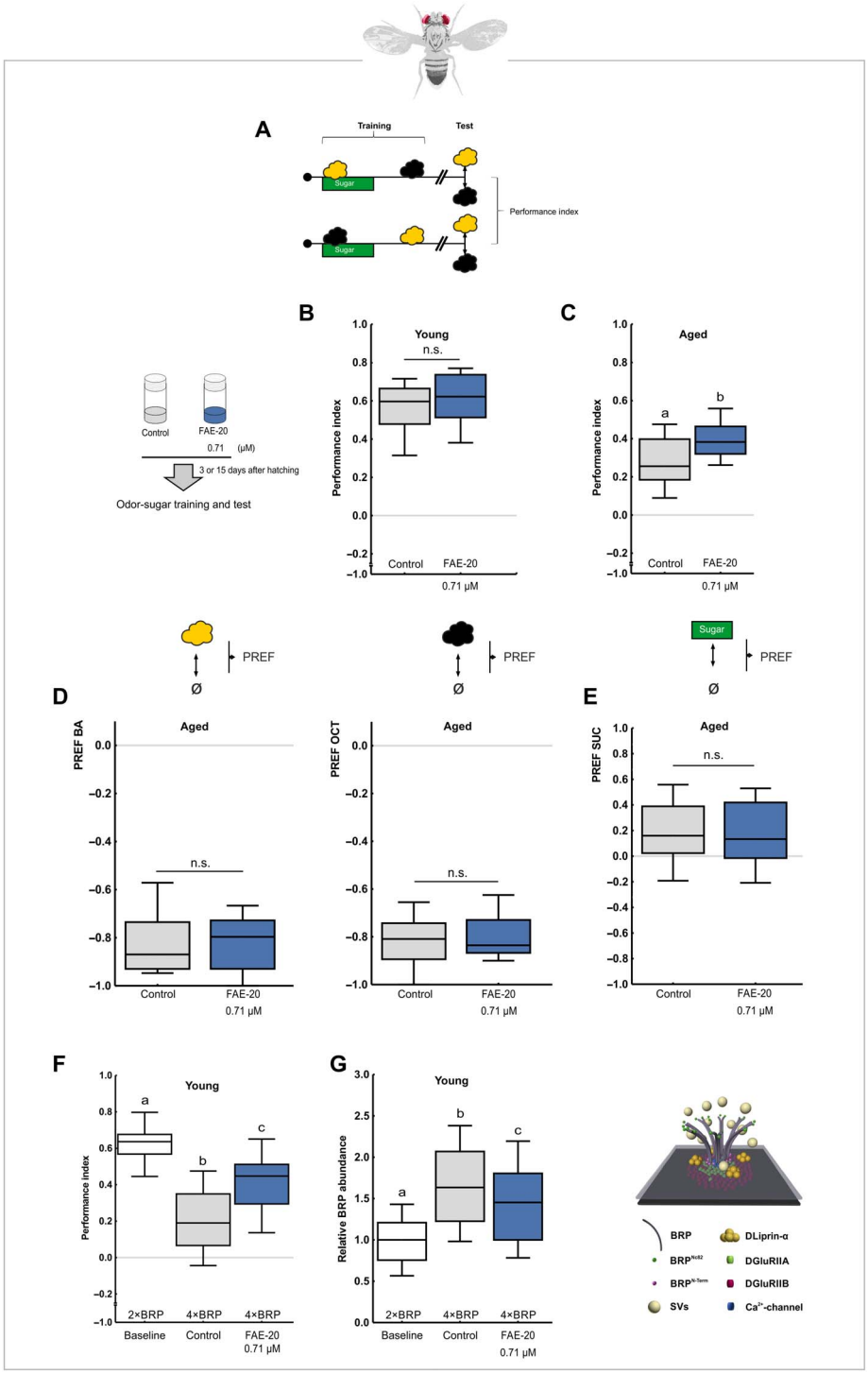

Fig. 6. FAE-20 improves memory in aged flies and modulates levels of the BRP protein. (**A** to **C**) As in Fig. 3, food supplementation with FAE-20 (blue) leaves memory scores in young flies unaffected (B; $n = 24$ and 28) yet partially compensates for the memory impairment of aged flies (C; $n = 29$ and 28). n.s. and b, respectively, indicate $P > 0.05$ and $P < 0.05$ in U tests versus control (a). (**D** and **E**) Feeding on FAE-20 does not affect odor preference [D: BA (yellow cloud) and 3OCT (black cloud)] or SUC preference (E, green) in aged flies. n.s. indicates $P > 0.05$ in U tests ($n = 28$ and 28; 28 and 28; and 40 and 40). (**F**) As in (B) and (C), showing that relative to baseline conditions of BRP (2×BRP, baseline), memory scores in young flies are impaired upon BRP overexpression when raised on control food (4×BRP, control), which is partially compensated for by raising the animals on FAE-20 food (4×BRP, FAE-20). b indicates a difference from baseline (a), "c" indicates a difference from control in Bonferroni-corrected U tests [$P < 0.05/2$ ($n = 20$, 28, and 27)] preceded by a Kruskal-Wallis test ($P < 0.05$). (**G**) Quantitative mass spectrometry (MS) shows that BRP overexpression can be partially compensated for by FAE-20 feeding. "b" indicates a difference from baseline ("a"), "c" indicates a difference from control in Bonferroni-corrected U tests [$P < 0.05/2$ ($n = 95$, 96, and 102)] preceded by a Kruskal-Wallis test ($P < 0.05$). The inset illustrates the topology of BRP at the presynapse [© from (*47*); originally published in the *Journal of Cell Biology*. https://doi.org/10.1083/JCB.200812150]. Further details as in Fig. 1. Data are documented in data file S1.

To see whether feeding FAE-20 to 4×BRP animals indeed reduces levels of BRP protein, we used quantitative MS of adult fly heads (Fig. 6G). This confirmed the increase in BRP levels in young 4×BRP control flies when compared with young 2×BRP baseline flies (*21*). Critically, FAE-20 food supplementation reduced this increase in BRP in 4×BRP flies.

We conclude that supplementing food with FAE-20 can improve memory scores in larval *Drosophila*. Furthermore, FAE-20 can partially compensate for both memory impairments in aged flies and early-onset memory impairments caused by a genetically induced, premature increase in BRP levels in young flies. Our next question was whether FAE-20 has neurocognitive effects in mice as well.

FAE-20 increases excitability of CA1 neurons and improves contextual fear memory

Given the role of hippocampal CA1 neurons in learning and memory in rodents and humans (*22*, *23*), we tested whether the physiological properties of these neurons are altered by acute application of *Rhodiola extract*^{crude}. It turned out that cell excitability, measured as the number of action potentials upon current injection, is increased (Fig. 7, A and B). Likewise, application of 4 μM FAE-20 increases the excitability of CA1 neurons (Fig. 7, C and D). This prompted us to test whether acute application of FAE-20 modulates hippocampus-dependent memory in the mouse. Because we were concerned that the acidic conditions in parts of the mammalian digestive system might incapacitate FAE-20 by acidic hydrolysis, mice were intraperitoneally injected with FAE-20 solution (6 or 12 mg/kg) or solvent and trained in a contextual fear conditioning paradigm 30 min later. On the next day, they were tested for freezing behavior first in a novel context and then in the training context (Fig. 7, E to G). After injection of FAE-20 in young adult mice (3 months), the FAE-20–treated groups and the control groups showed a similarly low level of freezing in the novel context, and initially, equally elevated freezing in the training context. Contextual fear memory was more stable across the testing period in FAE-20–treated animals (Fig. 7, E and F). Application of FAE-20 in aged mice (>2 years) resulted in higher levels of contextual fear memory throughout the test, as compared with controls (Fig. 7G). We conclude that acute FAE-20 treatment can improve hippocampus-dependent contextual fear memory in the mouse, possibly related to increased excitability of the neurons involved.

DISCUSSION

Translational potential

The starting point of the present study was that preparations from *R. rosea* roots reportedly increase the life span in various animal species (*10*, *11*), are used in traditional human medicine (*1*–*3*), and have been reported to have positive effects on memory function in rodents (*7*) and humans (*3*) [but see (*4*–*6*)]. As regards the effects on memory function, however, the specific memory processes affected by *Rhodiola* (acquisition, consolidation, and retrieval) and the bioactive compound(s) in the root causing these effects remained unknown. We have systematically investigated the effects of *R. rosea*, its extracts, and single compounds present in these extracts on associative learning and memory. We conclude that *R. rosea* improves memory scores in a dose-dependent manner in *Drosophila* larvae and that it compensates for age-related memory decline in aged flies. In bees, feeding on *R. rosea* specifically improves memory acquisition and memory consolidation but not its retrieval. Task-related sensorimotor faculties remain unchanged in all cases. We specifically attribute a memory enhancement effect in *Drosophila* to the bioactive compound FAE-20, which we show to be present in the *R. rosea* extract. We confirm these effects with synthetic pure FAE-20, which also ameliorates age-related as well as genetically caused early-onset memory impairments in adult flies.

Critically, from a translational medicine perspective, FAE-20 is also effective in a rodent model: Mouse hippocampal CA1 neurons showed increased excitability, and mice treated with FAE-20 showed improved hippocampus-dependent memory. In the light of the abovementioned reports of neurocognitive effects of *R. rosea* in traditional medicine, and given that FAE-20 was identified from *R. rosea* as a memory-enhancing compound, trials into the effects of FAE-20 in humans now seem warranted. Once toxicity tests are passed and the pharmacokinetics of FAE-20 are known, such tests might be particularly promising because of the reported associations between neuronal excitability, synaptic plasticity, and learning (*24*, *25*). A possible mechanism by which an increase in neuronal excitability might lead to improved synaptic plasticity is that the higher number of action potentials generated during the induction of synaptic plasticity by more excitable cells can cause an increased Ca^{2+} influx via *N*-methyl-D-aspartate types of glutamate receptors. Pyramidal cells from area CA1 of the dorsal hippocampus, for example, are less excitable in old than in young mice (*26*); thus, old mice should benefit more from increases in excitability than young mice, which is consistent with the apparently more pronounced behavioral effects of FAE-20 we observe in old mice. Moreover, several compounds that increase the intrinsic excitability of these neurons, such as the cholinesterase inhibitor galantamine and the L-type calcium channel blocker nimodipine, improve the performance of old mice in hippocampus-dependent behavioral tasks (*26*).

FAE-20 as a memory enhancer

FAE-20 has so far not been reported as a constituent of *R. rosea* [regarding other plants, see (*27*) and (*28*)], but in general, ferulic acid (FA) and related phenolic compounds in free form or as conjugates are ubiquitous antioxidants in plants. While FA is discussed as a therapeutic agent against oxidative stress–related diseases, including neurodegenerative disorders (*29*), no neurocognitive effects have previously been reported for alkyl esters of FA such as FAE-20. Synthetic alkyl esters of FA (and caffeic acid) have been shown to inhibit in vitro tumor cell proliferation, the activity of cyclooxygenase enzymes (COX-1 and COX-2), and lipid peroxidation (*30*)—effects that are consistent with the increase in life span and the reduction in body weight observed upon *R. rosea* food supplementation in a number of species (*10*, *11*, *31*, *32*). These effects were enhanced by ester formation, probably because bioavailability and membrane permeation are increased by this process. In particular, long-chain esterification might increase the bioavailability or even membrane accumulation of FA (because it increases lipophilicity and thus facilitates localization to and crossing of membranes and of the blood-brain barrier). Accordingly, we find memory enhancement for FAE-20 rather than for shorter-chain alkyl esters of FA (see also the "Could FAE-20 reach the brain?" section).

The identification of FAE-20 as a memory enhancer does not invalidate previous claims regarding the activity of other compounds from *R. rosea* (*4*–*6*). In most previous studies, the so-called SHR-5 extract of *R. rosea* root material was used, which is typically marketed in tablet form (*3*–*6*, *10*, *11*). This extract is standardized to the content of salidroside [2-(4-hydroxyphenyl)ethyl β-D-glucopyranoside] (*4*–*6*). It is also reported to contain other phenyl ethanoids and propanoids (e.g., tyrosol, rosavin, and tiandrin), and bioactivity has been attributed to

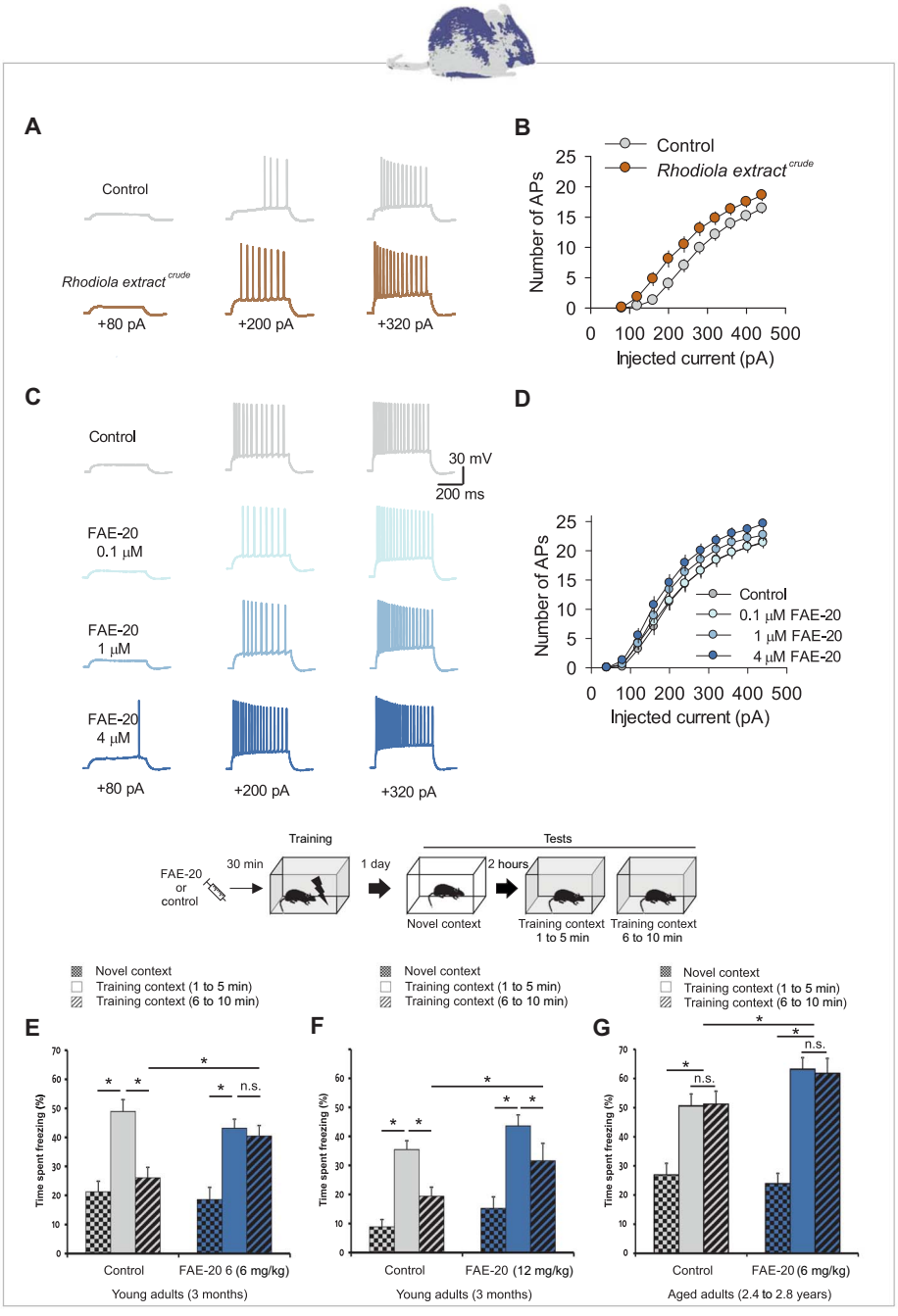

Fig. 7. FAE-20 increases hippocampal excitability and leads to more stable contextual fear memory in mice. (**A** to **D**) *Rhodiola extract*^{crude} (A and B) or FAE-20 (C and D) was applied to acute murine hippocampal slices at the concentrations indicated, and the excitability of CA1 neurons was determined as the number of action potentials (APs) upon current injection. Excitability was increased by *Rhodiola extract*^{crude} [$P < 0.05$, two-way repeated-measures ANOVA (rmANOVA); $n = 10$] and by FAE-20 [two-way rmANOVA with $P > 0.05$ for the two lower concentrations ($n = 11$ to 18) and $P < 0.05$ for the highest FAE-20 concentration ($n = 13$)], relative to the application of solvent as a control ($n = 10$ to 14). (**E**) Mice (3 months old) were intraperitoneally injected with either FAE-20 (6 mg/kg; $n = 14$) or solvent as a control ($n = 13$) 30 min before contextual fear conditioning training. On the next day, freezing behavior was tested in a novel context (to assess unspecific fear) and in the training context (to assess conditioned contextual fear). Animals from both groups discriminated equally well between the neutral and the training context (rmANOVA, all P values < 0.05). In control animals, contextual fear decreased over time [$P < 0.05$, Fisher's least significant difference (LSD) post hoc test], yet memory remained stable upon FAE-20 treatment ($P > 0.05$) and was higher than that in control animals ($P = 0.0007$). (**F**) If animals were treated with a higher concentration of FAE-20, they again discriminated between the two contexts as well as control animals did (rmANOVA, all P values < 0.05). They showed a slight but statistically significant ($P = 0.04$) decline in freezing over time in the training context but, importantly, exhibited more freezing in the second half of the training context exposure than the control group ($P < 0.0001$), again indicating a more stable fear memory in FAE-20–treated mice (Fisher's LSD post hoc comparisons; control, $n = 13$; FAE-20, $n = 9$). (**G**) As in (E), for 2.4- to 2.8-year-old mice. Animals from both groups discriminated equally well between the neutral and the training context (rmANOVA, all P values < 0.05). Animals treated with FAE-20 exhibited higher levels of fear memory in the training context (i.e., showed more freezing) than the control animals throughout the testing phase ($P < 0.05$, Fisher's LSD post hoc test; control, $n = 15$; FAE-20, $n = 16$). Data are documented in data file S1.

these compound classes (*3*, *33*) [but see (*4*–*6*)]. In the case of larval *Drosophila* at least, and to the extent tested, our data provide no evidence for a memory enhancement effect of SHR-5–containing tablet material. Our attempts to determine whether this is because of insufficient amounts of FAE-20 in the SHR-5–containing tablets were unsuccessful, however, because signal overlap and matrix effects precluded proper quantitative analysis.

Can FAE-20 account for the effects of *Rhodiola*?

FAE-20 enhances memory in larval *Drosophila* at a concentration of 0.71 μM (Fig. 5B), indicating effectiveness in the low micromolar range. This concentration corresponds to 0.34 μg of FAE-20 per milliliter of food. According to our analytical investigations, the FAE-20 content in the *Rhodiola extract*^{crude} is estimated to be less than 1%. Range-wise, this is consistent with what we found for the memory-enhancing effect of *Rhodiola extract*^{crude} at 0.3 mg of extract per milliliter of food (Fig. 5A), as this would correspond to less than 3 μg of FAE-20 per milliliter of food. Given that we obtained about 25 to 30% of the weight of the dried *R. rosea* root material as extract, reaching roughly the same FAE-20 dose by adding *Rhodiola*^{1} root material would require about three to four times as much *Rhodiola*^{1} root material as compared with the *Rhodiola extract*^{crude}, which amounts to a few milligrams of *Rhodiola*^{1} per milliliter of food. This is about what we have found to be effective (Fig. 1, B to D). Due caveats include the error margins of the above estimations, the combined effects of structurally related compounds that individually have subthreshold effects, the possibility of inverted U-shaped dose-effect relationships, and errors arising from quantitatively relating behavioral experiments that were not performed in parallel. Bearing these caveats in mind, we think it is likely that FAE-20 mediates a substantial part of the memory-enhancing effect of *R. rosea* and its extract, and we emphatically refrain from making more quantitative claims.

Molecular effects of *Rhodiola*/FAE-20

A process previously proposed to mediate the enhancement of memory by *R. rosea* is the inhibition of acetylcholinesterase (AChE) activity (*34*, *35*). However, in bees, AChE inhibitors either slightly impair memory acquisition (*36*) or leave it unaltered (*37*). Furthermore, memory retrieval in the bee was improved by inhibiting AChE (*37*). Without further assumptions, these effects of AChE inhibition do not readily match our findings in bees because *Rhodiola*^{4E}, by contrast, enhances memory acquisition and leaves retrieval unaffected.

Polar *R. rosea* extracts and compounds isolated therefrom, such as rosiridin, have been reported to influence the levels and/or to moderate the effects of biogenic amines (*38*) [see also (*39*, *40*)]. As biogenic amines are themselves evolutionarily conserved and powerful modulators of memory processes [*Drosophila*, (*13*, *41*, *42*); bee, (*14*, *15*); mammals, (*43*)], the biogenic amine systems indeed appear to be promising candidates to mediate the memory-related effects of polar *R. rosea* extracts across species. We should point out, however, that FAE-20 is rather nonpolar, so a different or an additional mechanism may be expected to bring about memory enhancement by FAE-20.

Our present results show that, upon an increase in the levels of the synaptic protein BRP in aged adult flies and in young flies overexpressing the BRP protein, FAE-20 can reduce BRP levels back toward normal. In both cases, this effect on BRP levels is accompanied by a partial compensation for memory decline. This is consistent with previous findings showing that maintaining adaptive levels of BRP expression is necessary for proper memory function in flies (*21*, *44*) and arguably in bees as well (*45*). Why might BRP levels matter for memory function? BRP localizes to the presynaptic active zone (*46*). It is required for concentrating synaptic vesicles close to Ca^{2+} channels and thus for the fine-tuning of synaptic transmission (*47*, *48*). As the N terminus of *Drosophila* BRP has sequence homology to the vertebrate ELKS/CAST/ERC protein (*46*), BRP appears to be a promising candidate to mediate the memory-related effects of FAE-20 across species.

It did not escape our attention that the effects of FAE-20 on memory as well as on BRP levels in adult flies resemble the effects of feeding flies with the autophagy-promoting polyamine spermidine (*49*). However, preliminary proteomic analyses remained inconclusive as to whether there is a change in the abundance of proteins that are involved in spermidine synthesis or autophagy after feeding the flies with FAE-20. Further, the two substances differ drastically in their physicochemical properties and are therefore unlikely to have the same molecular target. Still, it remains possible that FAE-20 and spermidine affect overlapping processes, such as autophagy. This is an attractive idea given the common role of autophagy in plasticity and learning across species (*21*, *49*–*51*).

Together, the available evidence shows that FAE-20 is a potent memory enhancer in different species and paradigms and prompts at least four nonexclusive working hypotheses for a mechanism of its action, namely, modulation of neuronal excitability, of the biogenic amine systems, of BRP/ELKS/CAST/ERC function, and of homeostatic autophagy. To identify the mode(s) of action of FAE-20, these working hypotheses now need to be investigated in detail.

Could FAE-20 reach the brain?

Assuming the brain to be the site ultimately affected by FAE-20 treatment, one wonders whether the FAE-20 would actually reach the brain. With pharmacokinetic analyses pending, we note that, in *Drosophila*, digestion and absorption are predominantly accomplished in the mid-gut, which is much less acidic than in mammals (*52*), making the acidic hydrolysis of esters unlikely. If FAE-20 is indeed taken up into the hemolymph ("blood"), then lipoproteins such as lipophorin (*53*) could transport it to close to anywhere in the body. Because of its highly nonpolar lipophilic properties, the interaction of transported FAE-20 with cell membranes could then allow it to pass through the blood-brain barrier (*54*). In the case of mice, the latter interaction might also allow FAE-20 to reach the brain upon FAE-20 intraperitoneal injection.

Are larvae young or old?

The effects of memory enhancers are often easier to detect when, for whatever reason, memory function is compromised (*49*, *55*, *56*). Accordingly, in our study, the effects of FAE-20 were revealed by compensating for genetically induced mnemonic impairments in adult *Drosophila* and by enhancing memory function that is compromised by age. But why, then, is memory enhancement through FAE-20 so readily revealed in larval *Drosophila*? A possible reason for this is that the late, stage 3 larvae used in the present experiments are, in a sense, "aged," representing a life stage at the end of a 5-day continuous feeding frenzy and shortly before the "larval death" followed by rebirth as a young adult (*57*).

Caveats

We note that, under healthy conditions, the use of memory enhancers entails risks. (i) As argued above, memory-enhancing effects are easier to obtain whenever memory function is compromised. This might prompt healthy subjects to use higher concentrations in an attempt nevertheless to achieve an increase in memory function, increasing the

likelihood of unintended side effects. The rewarding effects of *Rhodiola* and FAE-20 that we observed in larval *Drosophila* in a parallel study may indicate an addictive potential and caution against their nonmedical use. (ii) Under healthy conditions, memory enhancement may induce excessively rapid acquisition and consolidation for only spuriously associated events, leading to superstitious behavior not adaptively grounded in experience. Last but not least, (iii) it may distort the adaptive balance between memory acquisition and forgetting/extinction, or the adaptive balance between the discrimination and generalization of memories. In other words, faster learning or more robust or more specific memory does not necessarily equal better cognition and may not always be adaptive. Bearing these caveats in mind, memory enhancement may still be desirable, for example, to compensate for age-dependent memory loss or when mnemonic abilities are pathologically impaired.

MATERIALS AND METHODS

Drosophila experiments, *R. rosea* materials

D. melanogaster genotypes

Canton-S wild-type *D. melanogaster* larvae or adults were used for all experiments, unless mentioned otherwise. Flies carrying P(acman) *brp83* have been described previously (*58*), and because they carry two additional copies of the gene coding for BRP, these animals are referred to as "4×BRP" for simplicity. The "2×BRP, baseline" genotype, having only the genomic copies of BRP-coding genes, corresponds to the Canton-S strain mentioned above.

Food media and fly keeping

Procedures followed those of (*59*). To prepare standard food medium, 34 liters of water were mixed to 5.9 kg of cornmeal (Mühle Hofmann, Röthlein, Germany), boiled for 5 min, and automatically stirred gently for 4 hours. On the next day, 400 g of soya flour (Mühle Hofmann, Röthlein, Germany), 750 g of dried yeast powder (Heirler Cenovis, Radolfzell, Germany), and 250 g of agar-agar (Carl Roth, Karlsruhe, Germany) were added to 6 liters of water; after stirring, 1.8 liters of malt (Ulmer Spatz, Bingen am Rhein, Germany) and 1.8 liters of sugar beet molasses (Grafschafter Krautfabrik, Meckenheim, Germany) were added and boiled for 5 min with the cornmeal mixture while being gently stirred. Upon cooling to 70° to 80°C, 100 g of antifungal agent (methyl-4-hydroxybenzoate; Merck, Darmstadt, Germany) was added.

To prepare fly culture vials, this food medium was boiled in a microwave oven, and, for control vials, aliquots (20 ml) were poured into plastic vials and kept at 4°C for later use. For the experimental groups, each of the following substances was added 5 min after boiling to reach the specified concentrations of material per volume of food. These vials were stored for later use at 4°C

(1) *Rhodiola*^{1}: Dried *R. rosea* roots (collected by O.L. in 2009 in the Carpathian Mountains near Mount Pip Ivan; 48°2′31″N, 24°37′32″E) were ground for 60 s with a commercial coffee mill. The powder was added to the vials 5 min after boiling to reach the specified concentrations; then, vials were stored at 4°C.

(2) Tablet: Ground "Arctic root" tablets (Swedish Herbal Institute, Gothenburg, Sweden; lot no. 60363, purchased via s.a.m. Pharma, Vienna, Austria; expiration date: November 2011, implying harvest of plant material before that date) were added to the food to reach the indicated concentrations. According to the manufacturer's specifications, 28% of the tablets' weight consists of the patented SHR-5 extract of *R. rosea*. Assuming that this extract is enriched from the dried root ingredients by at least a factor of 10, a concentration of 2.8 mg/ml of tablet should thus correspond to ~10 mg/ml of *Rhodiola*^{1}; a pilot experiment had shown that higher concentrations of the hygroscopic ground tablet powder compromised the viability of *Drosophila*.

(3) *Rhodiola*^{2} refers to a second crop of dried *R. rosea* roots, also collected by O.L. in 2009 in the Carpathian Mountains near Mount Pip Ivan (48°2′31″N, 24°37′32″E).

(4) *Rhodiola*^{3} refers to dried *R. rosea* roots of unspecified Russian origin purchased by O.L. before 2011 and thus also harvested before that date.

(5) *Rhodiola*^{4} root was purchased by B.M. in 2011 from the Eveline24.de online shop (Maardu, Estonia; Ch./lot no. 18621; expiration date: 6 July 2014 and thus harvested before that date).

Voucher samples of all accessions or purchases are deposited at the Leibniz Institute of Plant Biochemistry and the Leibniz Institute for Neurobiology: Arctic root tablets (QGB005), *Rhodiola*^{1} (QGB001), *Rhodiola*^{2} (QGB003), *Rhodiola*^{3} (QGB004), and *Rhodiola*^{4} (QGB011).

In all cases, vials were retrieved from the 4°C store at around noon, and 2 hours afterward, approximately 100 Canton-S wild-type flies were added to the vial, which was then maintained at 25°C and 60 to 70% relative humidity under a 14-hour light/10-hour dark cycle. On the next day, these flies were removed; after an additional 4 days, the larvae were harvested from the food slurry for experiments.

Preparation of Rhodiola extract^{crude} for food medium

Finely chopped *Rhodiola*^{4} root (1.565 kg) was exhaustively extracted with 80% aqueous undenatured ethanol (3 × 6 liters) at room temperature. The extracts were combined and filtered, and the solvent was evaporated under reduced pressure. Food medium was then prepared as described above, at the concentrations mentioned in Results.

Synthesis of ferulic acid esters and derivatives for food medium

The ferulic acid esters FAE-8, FAE-12, FAE-16, and FAE-20 were synthesized by the Mitsunobu reaction from FA and long-chain alcohols as described by Maresca *et al.* (*60*). In the same fashion, 4-OH-CAE-20 was obtained starting from *trans-p*-coumaric acid, and icosanol. DH-FAE-20 was obtained by hydrogenation of the double bond of FA eicosyl ester, affording the product in quantitative yield. Synthesis details and compound data are summarized in Supplementary Materials and Methods. Food medium was then prepared as described above, at the concentrations mentioned in Results.

Synthesis of BSSG and derivatives for food medium

The synthesis of β-sitosterol-β-D-glucoside and β-sitosterol-β-D-galactoside was performed following a procedure described by Kunz and Harreus (*61*), starting from acetobromo-α-D-glucose or acetobromo-α-D-galactose and sitosterol. The remaining compounds were purchased from commercial sources: β-sitosterol (Honeywell-Fluka via Fisher Scientific, Schwerte, Germany), stigmasterol (Acros Organics, Geel, Belgium), stigmasterol-β-D-glucoside (ChemFaces, Wuhan, Hubei, China), and cholesterol-β-D-glucoside and stevioside (both from Sigma-Aldrich, Taufkirchen, Germany). Food medium was then prepared as described above, at the concentrations mentioned in Results.

Behavioral experiments in larval Drosophila

The procedures for behavioral experiments in larval *Drosophila* follow those of (*59*) and are further specified below.

Two-odor learning paradigm in larval Drosophila

The learning experiments follow the procedures of (*62*) (see sketch in Fig. 1A): Petri dishes (Sarstedt, Nümbrecht, Germany) with an inner diameter of 85 mm were filled with 1% agarose (electrophoresis grade; Carl Roth, Karlsruhe, Germany), which was allowed to solidify. The

dishes were covered with their lids and then left untreated at room temperature until the following day. As the sugar reward, 2 mol of FRU (purity: 99%, Carl Roth, Karlsruhe, Germany) was used, which was added to 1 liter of agarose 10 min after boiling.

Experiments were performed under natural light at 21° to 24°C. Before the experiments, the regular lids of the petri dishes were replaced by lids perforated in the center by 15 holes (diameter, 1 mm) to improve aeration.

Odor was applied by adding 10 µl of odor substance into custom-made Teflon containers (inner diameter, 5 mm; these could be closed by a perforated lid with seven holes, 0.5 mm in diameter each). As odors, we used AM [Chemical Abstracts Service (CAS) no. 628-63-7; purity, 98.5%, diluted 1:50 in paraffin oil] and 1OCT [CAS no. 111-87-5; purity, 99% (undiluted)], both from Merck (Darmstadt, Germany), unless stated otherwise.

A spoonful of food medium containing larvae was taken from the food vial and transferred to a droplet of tap water on a petri dish. Thirty animals were collected, briefly washed in tap water, and transferred as a group to the assay plates for the start of training; in half of the cases, we started with a FRU-containing petri dish, and in the other half of the cases, we started with an agarose-only (PURE)–containing petri dish.

Immediately before the first training trial, two containers both loaded with the same odor were placed onto the assay plate on opposite sides of the plate (7 mm from the edges). Within each reciprocal training condition, we started with AM in half of the cases and with 1OCT in the other half, unless stated otherwise. Then, the petri dish was closed, and the larvae were allowed to move freely for 5 min. The larvae were then transferred to a petri dish with the alternative odor and the respective other substrate for 5 min (e.g., AM was presented on a FRU-containing plate and 1OCT on a PURE petri dish: AM+/1OCT training). This cycle was repeated two more times. Fresh petri dishes were used for each trial.

After this training, the animals were tested for their choice between the odors. The larvae were placed in the middle of a PURE petri dish; unless mentioned otherwise, a container with AM was placed on one side and a container with 1OCT was placed on the other side to create a choice situation. After 3 min, the number of animals on the "AM" or "1OCT" side was counted. After this test was completed, the next group of animals was run and trained reciprocally (e.g., AM/1OCT+).

For both groups, the odor preference ranging from −1 to 1 was calculated. To this end, the number of animals observed on the AM side ($\#_{AM}$) minus the number of animals observed on the 1OCT side ($\#_{1OCT}$) was determined, divided by the total number ($\#_{TOTAL}$)

$$\mathrm{PREF} = (\#_{AM} - \#_{1OCT})/\#_{TOTAL} \quad (1)$$

To determine whether these preferences vary according to the training regimen (i.e., whether they reflect associative memory), the data from alternately run, reciprocally trained groups were taken, and the PI ranging from −1 to 1 was calculated as

$$\mathrm{PI} = (\mathrm{PREF}_{AM+/1OCT} - \mathrm{PREF}_{AM/1OCT+})/2 \quad (2)$$

Data for control and experimental groups were gathered alternately.

One-odor learning paradigm in larval Drosophila

In two experimental series, we used a one-odor training regimen (*17*) by omitting 1OCT from the experiment. That is, the animals in one group received presentations of AM with the reward, alternating with presentations of an empty odor container (EM) on a PURE petri dish (AM+/ EM); the animals trained reciprocally received unpaired presentations of odor and reward (AM/ EM+). During the test, the animals were allowed to choose between AM and EM; the data were then treated, with due adjustments, as described in the preceding section.

Behavior toward odors and sugar in experimentally naïve larval Drosophila

To test for the behavioral specificity of *Rhodiola*[1] treatment, we determined the behavior of experimentally naïve larvae toward the stimuli to be associated for each of the rearing conditions indicated. To test behavior toward FRU, split petri dishes of 85 mm inner diameter were prepared: One-half contained PURE, while in the other half, FRU was present in addition (see sketch in Fig. 2A).

Regarding the odors, the larvae had the choice either between AM and EM or between 1OCT and EM (see sketch in Fig. 2, B and C). In both cases, the larvae were placed in the middle, and after 3 min, the number of larvae on either side was counted; then, the preference index (PREF) values were calculated, with due adjustments, according to Eq. 1.

Olfactory behavior after training-like stimulus exposure in larval Drosophila

As we have argued before (*63*), the mere exposure to the training stimuli (i.e., odor exposure per se and reward exposure per se) can have nonassociative effects on test behavior. Therefore, the behavior of animals from the control and *Rhodiola*[1] groups toward AM (diluted 1:50 in paraffin oil) and 1OCT, respectively, was assayed after either of two exposure treatments. Either the larvae were exposed to the reward but not to the odors in an otherwise training-like way (see sketches in Fig. 2, D and E) or they were exposed to the odors but not to the reward (Fig. 2, F and G). Then, the PREF scores for AM and 1OCT, respectively, versus EMs were determined, with due adjustments, according to Eq. 1.

Learning experiments in adult Drosophila

Flies were raised and kept on standard fly food (control) or on fly food supplemented with either ground *Rhodiola*[4] root (10 mg/ ml) or FAE-20 (final concentration, 0.71 µM) at 25°C and 60 to 70% relative humidity under a 12-hour light/12-hour dark cycle. For the learning experiments, we used either freshly hatched flies (1 to 3 days after hatching) or 15-day-old flies (after hatching). Every 3 to 5 days after hatching, the "15-day-old" group was transferred to fresh food vials. One day before the behavioral experiments, the flies were starved overnight for 18 to 20 hours at 25°C and 60 to 70% relative humidity in vials equipped with a moist tissue paper to prevent desiccation.

The experimental setup and protocol followed those of (*64*). As odors, 90 µl of BA (CAS no. 100-52-7, Merck, Darmstadt, Germany) and 340 µl of 3OCT (CAS no. 589-98-0, Merck, Darmstadt, Germany) were applied in 1-cm-deep Teflon containers of 5- and 14-mm diameters, respectively. Two training trials were applied. Each trial started by loading a group of 50 to 100 flies into the setup (0:00 min). One minute later, the flies were transferred to a tube lined with a filter paper that had been soaked the previous day with 2 ml of 2 M SUC solution; then, BA, for example, was shunted into the permanent air flow running through this tube (in half of the cases, 3OCT was used). After 45 s, odor stimulation was terminated, and after an additional 15 s, the flies were taken out of the tube. At the end of a 1-min waiting period, the flies were transferred into another tube lined with a filter paper that had been soaked with pure water the previous day, and the respective other odor

was presented. After 45 s, stimulation with this odor was terminated, and 15 s later, the flies were taken out of this second tube. The second trial started immediately. In half of the cases, both training trials started with an odor-sugar presentation; in the other half, both trials started with an odor-alone presentation. After this BA+/ 3OCT training and an additional waiting period of 3 min, the flies were transferred to a T maze, where they could choose between the previously rewarded and the previously unrewarded odor. After 2 min, the choice point of the maze was closed, the flies on each side were counted, and the PREF was calculated

$$\mathrm{PREF} = (\#_{\mathrm{BA}} - \#_{\mathrm{3OCT}})/\#_{\mathrm{TOTAL}} \quad (3)$$

A second group was trained reciprocally (BA/ 3OCT+), and the PI was calculated as a measure of associative memory based on their PREF values

$$\mathrm{PI} = (\mathrm{PREF}_{\mathrm{BA+/3OCT}} - \mathrm{PREF}_{\mathrm{BA/3OCT+}})/2 \quad (4)$$

The experiments were performed at 22° to 25°C and 75 to 85% relative humidity. Training took place under light; the test was performed in complete darkness, preventing the flies from seeing.

Behavior toward odors and SUC in experimentally naïve adult Drosophila

To test for the behavioral specificity of FAE-20 effects, the preference of control or FAE-20–reared aged flies toward the stimuli to be associated was determined. Flies were tested for responsiveness to the odors BA and 3OCT and to SUC in the same T maze setup used for the learning experiments. To test the preference for olfactory cues, the flies were given 2 min to choose between the two arms of the T maze: one scented with the respective odor used for conditioning and the other one unscented. For each experiment, the number of flies was counted in both arms, and a PREF for each odor was calculated, with due adjustments, according to Eq. 3. To test the preference for SUC, the flies were given 2 min to choose between one arm of the T maze lined with a SUC solution–soaked filter paper and the other arm lined with a water-soaked filter paper. The PREF was calculated, with due adjustments, in the same way.

Statistical analyses of Drosophila behavioral experiments

All statistical analyses were performed with Statistica (version 11; StatSoft Inc., Tulsa, OK, USA). In a conservative approach, nonparametric tests at a statistical significance level of 5% were used throughout. For multiple-group comparisons, Kruskal-Wallis (H) tests were used, followed by pairwise comparisons with Mann-Whitney *U* tests. For these follow-up pairwise comparisons, the statistical significance level of 5% was maintained with a Bonferroni correction ($P < 0.05$ divided by the respective number of pairwise tests). Data are displayed as box plots representing the median as the middle line, with the 25th and 75th quantiles as box boundaries and the 10th and 90th quantiles as whiskers.

Quantitative MS of adult Drosophila heads

Single heads of adult *Drosophila* were resolubilized in 20 µl of water containing 8 M of freshly deionized urea. Tissue and cell destruction was achieved by means of a microglass potter and pulsed sonification on ice for 1 hour. After centrifugation at 21,000*g* for 15 min at 15°C, 15 µl of the resulting supernatant was transferred to a fresh tube and supplemented with 60 µl of 50 mM NH_4HCO_3 buffer (pH 8.0) and 2 mM dithiothreitol. After incubation for 1 hour at 20°C, 10 mM methyl methane thiosulfonic acid was added for an additional 1 hour for thiomethylation of previously reduced cysteines. Limited proteolysis was started by adding 250 ng of trypsin (Trypsin Gold, Promega, Mannheim, Germany), followed by incubation at room temperature for 12 hours. Resulting peptides were purified with reversed-phase C18 ZipTip nano-columns (Millipore/Merck, Darmstadt, Germany), eluted with 0.1% trifluoroacetic acid (TFA)/70% acetonitrile (ACN), and dried in a vacuum evaporator centrifuge (Savant, Thermo Fisher Scientific, Waltham, MA, USA).

Proteome analysis was performed on a hybrid dual-pressure linear ion trap/orbitrap mass spectrometer (LTQ Orbitrap Velos Pro, Thermo Fisher Scientific) equipped with an EASY-nLC ultrahigh-performance liquid chromatography (Thermo Fisher Scientific). Samples were resolubilized in 12 µl of 0.1% TFA and 2% ACN and subjected to a 75-µm-inner-diameter, 25-cm PepMap C18 column, packed with 2-µm resin (Thermo Fisher Scientific). Separation was achieved by applying a gradient from 2 to 35% ACN in 0.1% formic acid over a 120-min gradient at a flow rate of 300 nl/min. The LTQ Orbitrap Velos Pro MS used exclusively collision-induced dissociation fragmentation. The spectra acquisition consisted of an orbitrap full MS [Fourier transform MS (FTMS)] scan, followed by up to 15 LTQ tandem MS/MS experiments (TOP15) on the most abundant ions detected in the full MS scan. Essential MS settings were as follows: FTMS (resolution, 60,000; mass/charge ratio range, 400 to 2000) and MS/MS (linear trap; minimum signal threshold, 500; dynamic exclusion time setting, 30 s; singly charged ions were excluded from selection). Normalized collision energy and activation time were set to 35% and 10 ms, respectively.

Raw data processing and protein identification were performed by PEAKS Studio 8.0 (Bioinformatics Solutions; Waterloo, Canada). False discovery rate was set to <1%.

BRP quantification was performed on the basis of the six most abundant tryptic BRP peptides within the MS datasets obtained. Relative protein quantification was achieved using the Skyline analysis platform (*65*) for MS peak integration on extracted ion chromatograms of the following selected peptide masses:

(1) TQGTLQTVQER: 630.8308^{2+} (precursor), 631.3322^{2+} (precursor [M + 1]), 631.8335^{2+} (precursor [M + 2]), and 632.3347^{2+} (precursor [M + 3]).

(2) SLQTQGGGAAAAGELNK: 786.9025^{2+} (precursor), 787.4039^{2+} (precursor [M + 1]), 787.9052^{2+} (precursor [M + 2]), and 788.4064^{2+} (precursor [M + 3]).

(3) VTYELER: 455.2374^{2+} (precursor) and 455.7389^{2+} (precursor [M + 1]).

(4) LQQSSVSPGDPVR: 685.3571^{2+} (precursor), 685.8586^{2+} (precursor [M + 1]), 686.3599^{2+} (precursor [M + 2]), 686.8611^{2+} (precursor [M + 3]), and 687.3624^{2+} (precursor [M + 4]).

(5) LLQLVQMSQEEQNAK: 879.9564^{2+} (precursor), 880.4578^{2+} (precursor [M + 1]), and 880.9587^{2+} (precursor [M + 2]).

(6) IEMEVQNMESK: 669.3074^{2+} (precursor) and 669.8088^{2+} (precursor [M + 1]).

The monoisotopic precursor mass and one or more ^{13}C-isotopic variants ([M + 1], [M + 2] …) were chosen for more accurate and confident quantification. The peak qualities of the quantified peptides were controlled by the "isotope dot product" (idotp), set to >0.95. Idotp provides a measure for precursor isotope distribution and the correlation between the expected and the observed pattern, with optimal matching resulting in an idotp value of "1" (*66*).

To analyze the relative abundance of the BRP protein in 2×BRP baseline, 4×BRP control, and 4×BRP flies fed with FAE-20, the six BRP peptides showing the highest intensities of all BRP peptides in the MS raw data were taken into consideration. Having separated by

gender each of these six peptide intensities, data from $n = 7$ to 9 replicates were normalized to the median of the respective peptide intensity of the female or male control. For each group of flies, normalized data of the six peptides were pooled and analyzed with nonparametric statistics.

In all cases, experimenters were blinded to the treatment conditions (food supplementation, genotypes of the animals). These were decoded only after the experiments.

Honeybee experiments

Animals

Forager honeybees (*A. mellifera carnica*) were collected at 2 p.m. at the hive entrance or in an indoor flight room. The bees were immobilized by cooling and mounted in plastic tubes. At 4 p.m., the bees were fed to saturation, and at 4 p.m. on the following day, with 16 µl of SUC solution. All the SUC solutions mentioned in the context of the bee experiments refer to a concentration of 30% (w/v), unless stated otherwise. The animals were kept in a dark humidified chamber overnight at 20° to 24°C.

Learning experiments and Rhodiola feeding in bees

Odor-sugar associative learning experiments were conducted on harnessed bees, as previously described (*67*). All experiments were performed in the morning (10 to 12 a.m.). The animals were placed next to the training site 30 min before the experiment. Experiments consisted of a five-trial (Fig. 4A) or a three-trial (Fig. 4, D to F, and fig. S6) classical conditioning phase, followed by a single-trial (Fig. 4, D to F) or a three-trial (fig. S6) odor-only presentation test phase. A training trial consisted of the bee being placed in front of an exhaust, followed after 10 s by the presentation of the odor for 5 s and then 3 s later by the presentation of SUC solution to the antennae and the proboscis lasting for 4 s (i.e., an overlap of 2 s between odor and SUC). An extension of the proboscis to the odor was considered to be a response (PER). The total duration of a trial was 30 s; the time between two trials was 10 min. The odor was presented manually with a 20-ml syringe containing a filter paper (1 cm in diameter) with 4 µl of either 1-hexanol (CAS no. 111-27-3) or 1-nonanol (CAS no. 143-08-8) (both 98%; Sigma-Aldrich, Munich, Germany) for each bee.

Bees were fed 16 µl of SUC solution, of which 8 µl contained 0.1, 1, or 10% (w/v) *Rhodiola*^{4E} root extract. The feeding took place 19 hours before training (1% extract: Fig. 4A), 5 hours after training (i.e., 19 hours before the test; Fig. 4, D and E, and fig. S6), or 29 hours after training (i.e., 19 hours before the test; Fig. 4F). In the control group, the bees were fed 16 µl of SUC solution at these respective time points.

Sugar response experiments in experimentally naïve bees

Bees were fed with 16 µl of SUC solution, of which 8 µl contained 0.1, 1, or 10% (w/v) *Rhodiola*^{4E} root extract; in the control group, the bees were fed 16 µl of SUC solution. On the following day, the PERs of treated and control bees were noted to concentrations of SUC solution presented to the antenna in an ascending order [0, 0.1, 0.3, 1, 3, 10, and 30% (w/v), according to (*68*)]. The SUC response score was calculated for each bee by summing the number of PERs it had shown.

Odor response experiments in experimentally naïve bees

Bees were fed 16 µl of SUC solution, of which 8 µl contained 1% (w/v) *Rhodiola*^{4E} root extract; in the control group, the bees were fed 16 µl of SUC solution. On the following day, the bees were tested for their PER in response to odor (4 µl of either 1-hexanol or 1-nonanol for each bee).

Preparation of Rhodiola^{4E}

To produce *Rhodiola*^{4E}, dried *Rhodiola*^{4} roots were ground in a commercial coffee mill for 3 min and subsequently in a porcelain mortar. Ground roots (100 mg) were stirred for 18 hours at room temperature in 10 ml of ethanol (99%, undenatured) in a light-protected glass bottle. After solvent evaporation, the remaining material was added to SUC solution, vortexed, and heated to 90°C for 60 s.

Stock solutions were kept in darkness at 4°C for up to 7 days. The SUC solution for the control groups was prepared and treated in parallel.

Statistical analyses of behavioral experiments on bees

Included in the analysis were bees that fulfilled two criteria: (i) showing a PER to the SUC solution throughout training and (ii) showing a PER upon SUC stimulation at the very end of the respective experiments. rmANOVA was used with Fisher's LSD as a post hoc analysis (Statistica version 8.0, StatSoft Inc., Tulsa, OK, USA). We used R version 3.4.1 (*69*) and lme4 (*70*) to perform a logistic mixed-effects analysis of the relationship between *Rhodiola* treatment and the number of PERs in honeybees. To model several trials, we added as fixed effects the time points of testing and the different treatment groups (without an interaction term) to the model. As random effects, we included an intercept for subjects. To model only the test trials, we entered the different treatment groups as fixed effects into the model. As random effects, we had an intercept for subjects. To model the test trials in the concentration dependency experiment, we entered the different treatment groups as fixed effects into the model and added the last trial of the acquisition as a fixed effect. As random effects, we had an intercept for subjects. As optimizers for the model, we used BOBYQA, *nloptwrap*, and R's standard. No obvious deviations from homoscedasticity or normality were found by visual inspection of residual plots. We obtained all P values by likelihood ratio tests of the full model with treatment groups against the model without groups as a fixed effect. In Fig. 4 (B to F), bars and error bars represent means and confidence intervals with confidence levels of 95%.

Mouse experiments

Mice and slice preparation for electrophysiology

Experiments were performed on 4- to 6-week-old male C57BL/6J mice as described in (*71*). Mice were treated in accordance with the ethical guidelines for the use of animals in experiments; experiments were approved by the local animal care committee (Landesverwaltungsamt Sachsen-Anhalt). The animals were decapitated after cervical dislocation. The brain was rapidly removed and placed in ice-cold solution containing 230 mM SUC, 2.5 mM KCl, 7 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 26.6 mM $NaHCO_3$, 0.5 mM $CaCl_2$, and 10 mM D-glucose (all chemicals here and below were from Sigma-Aldrich, Steinheim, Germany). The frontal lobe was removed, and the brain was glued to a vibratome stage. Horizontal or transverse hippocampal slices were cut at 350 µm with a vibrating microtome (VT1200S, Leica). The slices were then incubated at room temperature (23° to 25°C) for at least 1 hour in a submerged chamber to recover in artificial cerebrospinal fluid (ACSF) containing 113 mM NaCl, 2.38 mM KCl, 1.24 mM $MgSO_4$, 0.95 mM NaH_2PO_4, 24.9 mM $NaHCO_3$, 1 mM $CaCl_2$, 1.6 mM $MgCl_2$, and 27.8 mM D-glucose. Subsequently, the slices were transferred to the recording chamber and were continuously perfused (2 to 3 ml/min) with carbogen-bubbled ACSF containing 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11 mM D-glucose. All solutions were saturated by 95% O_2/5% CO_2 and adjusted to a pH of 7.4 and an osmolarity of 295 ± 5 mosM. Before whole-cell patch-clamp recording, *Rhodiola extract*^{crude} (0.28 µg/ml) or FAE-20 (0.1, 1.0, and 4 µM) was bath applied. The vehicle (80% ethanol) was applied in the same way.

Whole-cell patch-clamp recordings and data analysis

Recordings were obtained using an EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany). The data were digitized at 20 kHz and filtered at 2 to 3 kHz. All recordings were made at room temperature in whole-cell configuration from CA1 pyramidal cells visually identified with an infrared differential interference contrast microscope (SliceScope, Scientifica, Kings Grove, UK). Whole-cell patch-clamp recordings were performed with glass pipettes (4 to 5 megohms; Hilgenberg, Malsfeld, Germany) filled with internal solution containing 140 mM K-gluconate, 8 mM NaCl, 0.2 mM $CaCl_2$, 10 mM Hepes, 2 mM EGTA, 0.5 mM NaGTP, and 2 mM MgATP (pH 7.2 with KOH; 290 mosM). To evoke action potentials, current pulses were applied using a patch amplifier in current-clamp mode. A series of 14 current pulses (500-ms duration, from −80 to 440 pA in 40-pA increments) were applied. Membrane potential was held at −70 mV during interpulse intervals by injecting direct current using the Patchmaster software (HEKA Elektronik). The numbers of action potentials at each current step were counted.

Excel (Microsoft, USA) and SigmaPlot 12.3 (Systat Software Inc., Erkrath, Germany) were used for statistical analyses and graphical presentation of electrophysiological data, presented as the means ± SEM. Statistical significance was determined using two-way rmANOVA.

Contextual fear conditioning in mice

For the contextual fear conditioning experiments, young adult (3 months old) or aged (2.4 to 2.8 years old) C57BL/6J male mice (Charles River, Sulzfeld, Germany) were used as mentioned in Results. They were housed in groups of three to four animals and had free access to food and water. All experiments took place during the light phase of the 12-hour light/12-hour dark cycle. The experiments were carried out in accordance with the European Committee Council Directive (86/609/EEC) and were approved by the local animal care committee (Landesverwaltungsamt Sachsen-Anhalt 42502-2-1191).

We used an automated system (TSE Systems, Bad Homburg, Germany) for fear conditioning, located in a sound-attenuating chamber. We used cubic test boxes (23 cm by 23 cm), which were surrounded by an array of infrared light beams to detect the movements of the animals. The floor consisted of a grid, by which the unconditioned stimulus (1-s, 0.5-mA scrambled foot shock) could be delivered. To provide distinct contexts, the color of the test boxes (black or transparent), the floor (grid or plastic floor), the odor [70% alcohol or Deskosept (Dr. Schumacher GmbH, Melsungen)], and the background noise (provided by a fan) could be changed. To avoid any bias, these different contextual stimuli were randomly changed.

On the first day, the animals were placed into the conditioning chamber for a total of 10 min. After a 2-min habituation period, they received three scrambled foot shocks (1 s, 0.5 mA) at random intervals (1.5 to 4 min). After the last foot shock, the animals remained in the chamber for the last 2 min. Twenty-four hours after the conditioning, the animals were first tested for unspecific fear by exposing them for 5 min to a neutral novel context and scoring their freezing behavior. One hour later, we exposed the animals for 10 min to the context in which the training had been carried out (training context) and scored their freezing behavior.

To apply FAE-20 to the animals, 3 ml of a 4.06 mM FAE-20 solution [10% (v/v) ethanol in phosphate-buffered saline (PBS)] per kilogram of body weight (i.e., 6 mg of FAE-20 per kilogram of body weight) or, for the control, 3 ml of an ethanol solution [10% (v/v) ethanol in PBS] per kilogram of body weight was injected intraperitoneally 30 min before the fear conditioning started. To test an FAE-20 concentration that was twice as high, 6 ml of the abovementioned 4.06 mM FAE-20 solution per kilogram of body weight (i.e., 12 mg of FAE-20 per kilogram of body weight) or of an ethanol solution [10% (v/v) ethanol in PBS] per kilogram of body weight for the control was injected.

The behavioral data of the mice were analyzed by rmANOVA using the intraperitoneally injected "drug" as the between-subject factor and the "context" of testing as the within-subject factor. For the detailed group comparisons, the ANOVA was followed by Fisher's LSD post hoc comparisons. $P < 0.05$ was considered a statistically significant difference.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/10/eaat6994/DC1

Supplementary Materials and Methods

Fig. S1. *Rhodiola*^{4} produces memory enhancement in *Drosophila* larvae.

Fig. S2. *Rhodiola*^{1} does not affect olfactory behavior after one-odor exposure.

Fig. S3. A *Rhodiola* tablet preparation has no memory-enhancing effect in larval *Drosophila*.

Fig. S4. Different levels of memory enhancement by various *Rhodiola* materials.

Fig. S5. Confirming that *Rhodiola*^{4} improves memory in aged flies.

Fig. S6. *Rhodiola*^{4E} leads to enhanced memory performance across extinction trials in bees.

Fig. S7. No memory-enhancing effects of synthetic BSSG or its derivatives in larvae.

Fig. S8. Analytical data on the ferulic acid ester fraction isolated from *Rhodiola* roots.

Fig. S9. NMR comparison of isolated and synthetic FAE-20.

Data file S1. Raw data of all experiments (Excel).

References (*72*–*74*)

REFERENCES AND NOTES

1. R. P. Brown, P. L. Gerbarg, Z. Ramazanov, *Rhodiola rosea*. A phytomedicinal overview. *HerbalGram* **56**, 40–52 (2002).
2. C. Linnaeus, Materia medica. Liber I, De plantis (Lars Salvius, 1749), p. 168.
3. A. Panossian, G. Wikman, J. Sarris, Rosenroot (*Rhodiola rosea*): Traditional use, chemical composition, pharmacology and clinical efficacy. *Phytomedicine* **17**, 481–493 (2010).
4. European Medicines Agency, *Assessment report on Rhodiola rosea L., rhizoma et radix* (London, United Kingdom, 2012); www.ema.europa.eu/docs/en_GB/document_library/Herbal_-_HMPC_assessment_report/2012/05/WC500127861.pdf.
5. National Institutes of Health, *Rhodiola* (National Center for Complementary and Integrative Health, 2016); https://nccih.nih.gov/health/rhodiola.
6. S. Ishaque, L. Shamseer, C. Bukutu, S. Vohra, *Rhodiola rosea* for physical and mental fatigue: A systematic review. *BMC Complement. Altern. Med.* **12**, 70 (2012).
7. V. D. Petkov, D. Yonkov, A. Mosharoff, T. Kambourova, L. Alova, V. V. Petkov, I. Todorov, Effects of alcohol aqueous extract from *Rhodiola rosea* L. roots on learning and memory. *Acta Physiol. Pharmacol. Bulg.* **12**, 3–16 (1986).
8. S. K. Hung, R. Perry, E. Ernst, The effectiveness and efficacy of *Rhodiola rosea* L.: A systematic review of randomized clinical trials. *Phytomedicine* **18**, 235–244 (2011).
9. A. Coors, E. Kahl, R. Khalil, B. Michels, B. Gerber, M. Fendt, *Rhodiola rosea* root extract has antipsychotic-like effects in rodent models of sensorimotor gating, poster at the 11th FENS Forum of Neuroscience (July 2018).
10. F. A. C. Wiegant, S. Surinova, E. Ytsma, M. Langelaar-Makkinje, G. Wikman, J. A. Post, Plant adaptogens increase lifespan and stress resistance in *C. elegans*. *Biogerontology* **10**, 27–42 (2009).
11. S. E. Schriner, K. Lee, S. Truong, K. T. Salvadora, S. Maler, A. Nam, T. Lee, M. Jafari, Extension of *Drosophila* lifespan by *Rhodiola rosea* through a mechanism independent from dietary restriction. *PLOS ONE* **8**, e63886 (2013).
12. S. Diegelmann, B. Klagges, B. Michels, M. Schleyer, B. Gerber, Maggot learning and Synapsin function. *J. Exp. Biol.* **216**, 939–951 (2013).
13. R. L. Davis, Olfactory memory formation in *Drosophila*: From molecular to systems neuroscience. *Annu. Rev. Neurosci.* **28**, 275–302 (2005).
14. R. Menzel, U. Müller, Learning and memory in honeybees: From behavior to neural substrates. *Annu. Rev. Neurosci.* **19**, 379–404 (1996).
15. M. Schwärzel, U. Müller, Dynamic memory networks: Dissecting molecular mechanisms underlying associative memory in the temporal domain. *Cell. Mol. Life Sci.* **63**, 989–998 (2006).
16. E. R. Kandel, Y. Dudai, M. R. Mayford, The molecular and systems biology of memory. *Cell* **157**, 163–186 (2014).
17. T. Saumweber, J. Husse, B. Gerber, Innate attractiveness and associative learnability of odors can be dissociated in larval *Drosophila*. *Chem. Senses* **36**, 223–235 (2011).
18. S. M. Ross, *Rhodiola rosea* (SHR-5), Part 2: A standardized extract of *Rhodiola rosea* is shown to be effective in the treatment of mild to moderate depression. *Holist. Nurs. Pract.* **28**, 217–221 (2014).

19. D. Wüstenberg, B. Gerber, R. Menzel, Long- but not medium-term retention of olfactory memories in honeybees is impaired by actinomycin D and anisomycin. *Eur. J. Neurosci.* **10**, 2742–2745 (1998).
20. S. Hielscher-Michael, C. Griehl, M. Buchholz, H.-U. Demuth, N. Arnold, L. A. Wessjohann, Natural products from microalgae with potential against Alzheimer's disease: Sulfolipids are potent glutaminyl cyclase inhibitors. *Mar. Drugs* **14**, E203 (2016).
21. V. K. Gupta, U. Pech, A. Bhukel, A. Fulterer, A. Ender, S. F. Mauermann, T. F. M. Andlauer, E. Antwi-Adjei, C. Beuschel, K. Thriene, M. Maglione, C. Quentin, R. Bushow, M. Schwärzel, T. Mielke, F. Madeo, J. Dengjel, A. Fiala, S. J. Sigrist, Spermidine suppresses age-associated memory impairment by preventing adverse increase of presynaptic active zone size and release. *PLOS Biol.* **14**, e1002563 (2016).
22. O. J. Ahmed, M. R. Mehta, The hippocampal rate code: Anatomy, physiology and theory. *Trends Neurosci.* **32**, 329–338 (2009).
23. K. M. Igarashi, H. T. Ito, E. I. Moser, M.-B. Moser, Functional diversity along the transverse axis of hippocampal area CA1. *FEBS Lett.* **588**, 2470–2476 (2014).
24. H. K. Titley, N. Brunel, C. Hansel, Toward a neurocentric view of learning. *Neuron* **95**, 19–32 (2017).
25. W. Zhang, D. J. Linden, The other side of the engram: Experience-driven changes in neuronal intrinsic excitability. *Nat. Rev. Neurosci.* **4**, 885–900 (2003).
26. X.-W. Yu, M. M. Oh, J. F. Disterhoft, CREB, cellular excitability, and cognition: Implications for aging. *Behav. Brain Res.* **322**, 206–211 (2017).
27. A. M. Baldé, M. Claeys, L. A. Pieters, V. Wray, A. J. Vlietninck, Ferulic acid esters form stem bark of *Pavetta owariensis*. *Phytochemistry* **30**, 1024–1026 (1991).
28. L. Hennig, G. M. Garcia, A. Giannis, R. W. Bussmann, New constituents of *Baccharis genistelloides* (Lam.) Pers. *ARKIVOC* **2011**, 74–81 (2011).
29. A. Sgarbossa, D. Giacomazza, M. di Carlo, Ferulic acid: A hope for Alzheimer's disease therapy from plants. *Nutrients* **7**, 5764–5782 (2015).
30. B. Jayaprakasam, M. Vanisree, Y. Zhang, D. L. Dewitt, M. G. Nair, Impact of alkyl esters of caffeic and ferulic acids on tumor cell proliferation, cyclooxygenase enzyme, and lipid peroxidation. *J. Agric. Food Chem.* **54**, 5375–5381 (2006).
31. C. Chen, J. Song, M. Chen, Z. Li, X. Tong, H. Hu, Z. Xiang, C. Lu, F. Dai, *Rhodiola rosea* extends lifespan and improves stress tolerance in silkworm, *Bombyx mori*. *Biogerontology* **17**, 373–381 (2016).
32. M. Wang, L. Luo, L. Yao, C. Wang, K. Jiang, X. Liu, M. Xu, N. Shen, S. Guo, C. Sun, Y. Yang, Salidroside improves glucose homeostasis in obese mice by repressing inflammation in white adipose tissues and improving leptin sensitivity in hypothalamus. *Sci. Rep.* **6**, 25399 (2016).
33. A. Panossian, R. Hamm, G. Wikman, T. Efferth, Mechanism of action of *Rhodiola*, salidroside, tyrosol and triandrin in isolated neuroglial cells: An interactive pathway analysis of the downstream effects using RNA microarray data. *Phytomedicine* **21**, 1325–1348 (2014).
34. Y. Cao, L. Liang, X. Jian, J. Wu, Y. Yan, P. Lin, Q. Chen, F. Zheng, Q. Wang, Q. Ren, Z. Gou, Y. Fan, Y. Du, Memory-enhancing effect of *Rhodiola rosea* L extract on aged mice. *Trop. J. Pharm. Res.* **15**, 1453–1457 (2016).
35. B. J. Hillhouse, D. S. Ming, C. J. French, G. H. N. Towers, Acetylcholine esterase inhibitors in *Rhodiola rosea*. *Pharm. Biol.* **42**, 68–72 (2004).
36. S. M. Williamson, C. Moffat, M. A. E. Gomersall, N. Saranzewa, C. N. Connolly, G. A. Wright, Exposure to acetylcholinesterase inhibitors alters the physiology and motor function of honeybees. *Front. Physiol.* **4**, 13 (2013).
37. D. Guez, H. Zhu, S. W. Zhang, M. V. Srinivasan, Enhanced cholinergic transmission promotes recall in honeybees. *J. Insect Physiol.* **56**, 1341–1348 (2010).
38. D. van Diermen, A. Marston, J. Bravo, M. Reist, P.-A. Carrupt, K. Hostettmann, Monoamine oxidase inhibition by *Rhodiola rosea* L. roots. *J. Ethnopharmacol.* **122**, 397–401 (2009).
39. Q. G. Chen, Y. S. Zeng, Z. Q. Qu, J. Y. Tang, Y. J. Qin, P. Chung, R. Wong, U. Hägg, The effects of *Rhodiola rosea* extract on 5-HT level, cell proliferation and quantity of neurons at cerebral hippocampus of depressive rats. *Phytomedicine* **16**, 830–838 (2009).
40. C. Mannucci, M. Navarra, E. Calzavara, A. P. Caputi, G. Calapai, Serotonin involvement in *Rhodiola rosea* attenuation of nicotine withdrawal signs in rats. *Phytomedicine* **19**, 1117–1124 (2012).
41. C. J. Burke, W. Huetteroth, D. Owald, E. Perisse, M. J. Krashes, G. Das, D. Gohl, M. Silies, S. Certel, S. Waddell, Layered reward signalling through octopamine and dopamine in *Drosophila*. *Nature* **492**, 433–437 (2012).
42. C. Schroll, T. Riemensperger, D. Bucher, J. Ehmer, T. Völler, K. Erbguth, B. Gerber, T. Hendel, G. Nagel, E. Buchner, A. Fiala, Light-induced activation of distinct modulatory neurons triggers appetitive or aversive learning in *Drosophila* larvae. *Curr. Biol.* **16**, 1741–1747 (2006).
43. W. Schultz, Neuronal reward and decision signals: From theories to data. *Physiol. Rev.* **95**, 853–951 (2015).
44. S. Knapek, S. Sigrist, H. Tanimoto, Bruchpilot, a synaptic active zone protein for anesthesia-resistant memory. *J. Neurosci.* **31**, 3453–3458 (2011).
45. K. B. Gehring, K. Heufelder, H. Depner, I. Kersting, S. J. Sigrist, D. Eisenhardt, Age-associated increase of the active zone protein Bruchpilot within the honeybee mushroom body. *PLOS ONE* **12**, e0175894 (2017).
46. D. A. Wagh, T. M. Rasse, E. Asan, A. Hofbauer, I. Schwenkert, H. Dürrbeck, S. Buchner, M.-C. Dabauvalle, M. Schmidt, G. Qin, C. Wichmann, R. Kittel, S. J. Sigrist, E. Buchner, Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. *Neuron* **49**, 833–844 (2006).
47. W. Fouquet, D. Owald, C. Wichmann, S. Mertel, H. Depner, M. Dyba, S. Hallermann, R. J. Kittel, S. Eimer, S. J. Sigrist, Maturation of active zone assembly by *Drosophila* Bruchpilot. *J. Cell Biol.* **186**, 129–145 (2009).
48. R. J. Kittel, C. Wichmann, T. M. Rasse, W. Fouquet, M. Schmidt, A. Schmid, D. A. Wagh, C. Pawlu, R. R. Kellner, K. I. Willig, S. W. Hell, E. Buchner, M. Heckmann, S. J. Sigrist, Bruchpilot promotes active zone assembly, Ca^{2+} channel clustering, and vesicle release. *Science* **312**, 1051–1054 (2006).
49. V. K. Gupta, L. Scheunemann, T. Eisenberg, S. Mertel, A. Bhukel, T. S. Koemans, J. M. Kramer, K. S. Y. Liu, S. Schroeder, H. G. Stunnenberg, F. Sinner, C. Magnes, T. R. Pieber, S. Dipt, A. Fiala, A. Schenck, M. Schwaerzel, F. Madeo, S. J. Sigrist, Restoring polyamines protects from age-induced memory impairment in an autophagy-dependent manner. *Nat. Neurosci.* **16**, 1453–1460 (2013).
50. L. Álvarez-Arellano, M. Pedraza-Escalona, T. Blanco-Ayala, N. Camacho-Concha, J. Cortés-Mendoza, L. Pérez-Martinez, G. Pedraza-Alva, Autophagy impairment by caspase-1-dependent inflammation mediates memory loss in response to β-amyloid peptide accumulation. *J. Neurosci. Res.* **96**, 234–246 (2017).
51. Y. G. Zhao, L. Sun, G. Miao, C. Ji, H. Zhao, H. Sun, L. Miao, S. R. Yoshii, N. Mizushima, X. Wang, H. Zhang, The autophagy gene *Wdr45/Wipi4* regulates learning and memory function and axonal homeostasis. *Autophagy* **11**, 881–890 (2015).
52. M. J. Lehane, P. F. Billingsley, *Biology of the Insect Midgut* (Springer Netherlands, 1996).
53. W. Palm, J. L. Sampaio, M. Brankatschk, M. Carvalho, A. Mahmoud, A. Shevchenko, S. Eaton, Lipoproteins in *Drosophila melanogaster*—Assembly, function, and influence on tissue lipid composition. *PLOS Genet.* **8**, e1002828 (2012).
54. T. Stork, D. Engelen, A. Krudewig, M. Silies, R. J. Bainton, C. Klämbt, Organization and function of the blood–brain barrier in *Drosophila*. *J. Neurosci.* **28**, 587–597 (2008).
55. L. Eleore, J. C. López-Ramos, P. J. Yi, J. M. Delgado-García, The cognitive enhancer T-588 partially compensates the motor associative learning impairments induced by scopolamine injection in mice. *Behav. Neurosci.* **121**, 1203–1214 (2007).
56. Y. Wang, L. Wang, J. Wu, J. Cai, The in vivo synaptic plasticity mechanism of EGb 761-induced enhancement of spatial learning and memory in aged rats. *Br. J. Pharmacol.* **148**, 147–153 (2006).
57. M. Tissot, R. F. Stocker, Metamorphosis in *Drosophila* and other insects: The fate of neurons throughout the stages. *Prog. Neurobiol.* **62**, 89–111 (2000).
58. T. Matkovic, M. Siebert, E. Knoche, H. Depner, S. Mertel, D. Owald, M. Schmidt, U. Thomas, A. Sickmann, D. Kamin, S. W. Hell, J. Bürger, C. Hollmann, T. Mielke, C. Wichmann, S. J. Sigrist, The Bruchpilot cytomatrix determines the size of the readily releasable pool of synaptic vesicles. *J. Cell Biol.* **202**, 667–683 (2013).
59. D. Mishra, "The content of olfactory memory in larval *Drosophila*," thesis, University of Würzburg (2011).
60. A. Maresca, G. Akyuz, S. M. Osman, Z. AlOthman, C. T. Supuran, Inhibition of mammalian carbonic anhydrase isoforms I–XIV with a series of phenolic acid esters. *Bioorg. Med. Chem.* **23**, 7181–7188 (2015).
61. H. Kunz, A. Harreus, Glycosidsynthese mit 2,3,4,6-tetra-*O*-pivaloyl-α-D-glucopyranosylbromid. *Justus Liebigs Ann. Chem.* **1982**, 41–48 (1982).
62. B. Michels, T. Saumweber, R. Biernacki, J. Thum, R. D. V. Glasgow, M. Schleyer, Y.-c. Chen, C. Eschbach, R. F. Stocker, N. Toshima, T. Tanimura, M. Louis, G. Arias-Gil, M. Marescotti, F. Benfenati, B. Gerber, Pavlovian conditioning of larval *Drosophila*: An illustrated, multilingual, hands-on manual for odor-taste associative learning in maggots. *Front. Behav. Neurosci.* **11**, 45 (2017).
63. B. Michels, S. Diegelmann, H. Tanimoto, I. Schwenkert, E. Buchner, B. Gerber, A role for Synapsin in associative learning: The *Drosophila* larva as a study case. *Learn. Mem.* **12**, 224–231 (2005).
64. A. Yarali, B. Gerber, A neurogenetic dissociation between punishment-, reward-, and relief-learning in *Drosophila*. *Front. Behav. Neurosci.* **4**, 189 (2010).
65. B. MacLean, D. M. Tomazela, N. Shulman, M. Chambers, G. L. Finney, B. Frewen, R. Kern, D. L. Tabb, D. C. Liebler, M. J. MacCoss, Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics* **26**, 966–968 (2010).
66. B. Schilling, M. J. Rardin, B. X. MacLean, A. M. Zawadzka, B. E. Frewen, M. P. Cusack, D. J. Sorensen, M. S. Bereman, E. Jing, C. C. Wu, E. Verdin, C. R. Kahn, M. J. Maccoss, B. W. Gibson, Platform-independent and label-free quantitation of proteomic data using MS1 extracted ion chromatograms in skyline: Application to protein acetylation and phosphorylation. *Mol. Cell. Proteomics* **11**, 202–214 (2012).
67. N. Stollhoff, R. Menzel, D. Eisenhardt, Spontaneous recovery from extinction depends on the reconsolidation of the acquisition memory in an appetitive learning paradigm in the honeybee (*Apis mellifera*). *J. Neurosci.* **25**, 4485–4492 (2005).

68. R. Scheiner, Responsiveness to sucrose and habituation of the proboscis extension response in honey bees. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* **190**, 727–733 (2004).
69. R Core Team, R: A language and environment for statistical computing (R Foundation for Statistical Computing, 2017); www.R-project.org/.
70. D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 48 (2015).
71. I. Pavlov, L. P. Savtchenko, I. Song, J. Koo, A. Pimashkin, D. A. Rusakov, A. Semyanov, Tonic $GABA_A$ conductance bidirectionally controls interneuron firing pattern and synchronization in the CA3 hippocampal network. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 504–509 (2014).
72. N. Mamidi, D. Manna, $Zn(OTf)_2$-promoted chemoselective esterification of hydroxyl group bearing carboxylic acids. *J. Org. Chem.* **78**, 2386–2396 (2013).
73. C. Anselmi, F. Bernardi, M. Centini, E. Gaggelli, N. Gaggelli, D. Valensin, G. Valensin, Interaction of ferulic acid derivatives with human erythrocytes monitored by pulse field gradient NMR diffusion and NMR relaxation studies. *Chem. Phys. Lipids* **134**, 109–117 (2005).
74. K. Nishimura, Y. Takenaka, M. Kishi, T. Tanahashi, H. Yoshida, C. Okuda, Y. Mizushina, Synthesis and DNA polymerase α and β inhibitory activity of alkyl *p*-coumarates and related compounds. *Chem. Pharm. Bull.* **57**, 476–480 (2009).

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