HHS Public Access

Author manuscript

Neurobiol Learn Mem. Author manuscript; available in PMC 2021 July 01.

Published in final edited form as:

Neurobiol Learn Mem. 2020 July ; 172: 107233. doi:10.1016/j.nlm.2020.107233.

Repeating or Spacing learning sessions are strategies for memory improvement with shared molecular and neuronal components

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Abstract

Intellectual disability is a common feature in genetic disorders with enhanced RAS-ERK1/2 signaling, including neurofibromatosis type 1 (NF1) and Noonan syndrome (NS). Additional training trials and additional spacing between trials, respectively, restores memory deficits in animal models of NF1 and NS. However, the relationship between the underlying mechanisms in these strategies remain obscure. Here, we developed an approach to examine the effect of adding training trials or spacing to a weak training protocol and used genetic and behavioral manipulations in *Drosophila* to explore such question. We found that repetition and spacing effects are highly related, being equally effective to improve memory in control flies and sharing mechanistic bases, including the requirement of RAS activity in mushroom body neurons and protein synthesis dependence. After spacing or repeating learning trials, memory improvement depends on the formation of long-term memory (LTM). Moreover, a disease-related gain-of-function *RasV152G* allele impaired LTM. Using minimal training protocols, we established that

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CRediT roles:
VC, BDG, MRP: Conceptualization;
VC, MRP: Data curation;
VC, MRP: Formal analysis;
BDG, MRP: Funding acquisition;
VC, MRP: Investigation;
VC, ASM, SEL, BDG, MRP: Methodology;
MRP: Project administration;
SEL, BDG, MRP: Resources;
ASM, SEL, MRP: Software;
BDG, MRP: Supervision;
VC, ASM, MRP: Validation;
VC, ASM, MRP: Visualization;
MRP: Roles/Writing - original draft;
VC, ASM, SEL, BDG, MRP: Writing - review & editing.

both learning strategies were also equally effective for memory rescue in the *Ras V152G* mutant and showed non-additive interaction of the spacing and repetition effects. Memory improvement was never detected after Ras inhibition. We conclude that memory improvement by spacing or repeating training trials are two ways of using the same molecular resources, including RAS-ERK1/2-dependent signaling. This evidence supports the concept that learning problems in RAS-related disorders depend on the impaired ability to exploit the repetition and the spacing effect required for long-term memory induction.

Keywords

Memory improvement strategies; memory rescue; RASopathies; *Drosophila*

1. Introduction

Intellectual disability is common in RASopathies, a group of genetic disorders with enhanced activity of the small G-protein RAS/extracellular signal-regulated kinase (ERK1/2) signaling, including neurofibromatosis type 1 (NF1) and Noonan syndrome (NS) [1,2]. Cognitive impairments, including learning and memory, have been studied in patients and animal models of RASopathies. However, the mechanistic basis is poorly understood [3,4], reviewed in [5]. Our studies in *Drosophila* have suggested that memory deficit in NS is mediated by an alteration in the molecular timing required for the spacing effect [6]. The *spacing effect* refers to a higher memory obtained after learning spaced over time, compared with the same amount of training presented without spacing (massed learning) [7]. Additional spacing between training trials rescued memory deficits in a *Drosophila* model of NS [6]. In contrast, additional training trials rescued memory deficits in NF1 mice model and patients [8]. The *repetition effect* (improvement of memory by repeating training trials) is a fundamental property of learning and memory detected in vertebrates and invertebrates [7,9,10]. The relationship between the underlying mechanisms of the repetition and spacing effects for memory improvement remains unclear.

The spacing effect, controlled by the tyrosine phosphatase SHP2 and ERK1/2, was consistently associated with a long-lasting and protein synthesis-dependent memory known as long-term memory (LTM) [6,11]. RAS is a key component involved in RASopathies, memory and regulates structural synaptic plasticity after spaced stimulation [5,12,13]. Neurofibromin, the protein product of *Nf1*, is the main regulator of RAS and is essential in learning and memory in vertebrates and invertebrates [8,14–16]. However, whether SHP2 and ERK1/2 control the spacing effect through RAS-ERK1/2 signaling it is unknown (For review see [12]). For instance, SHP2, a regulator of many tyrosine kinase receptors, may control the spacing effect through a combined action of signaling pathways other than RAS-ERK1/2 [17–19]. Indeed, plastic changes involved in memory depend on a diversity of growth factors [20]. Moreover, during spaced training in *Aplysia*, two distinct tyrosine kinase receptors mediate ERK activation, acting independently at the soma and nerve terminals [21].

Studies on regulators of small G-proteins, including guanine nucleotide exchange factors and GTPase-activating proteins, suggested that RAS is involved in LTM and NMDAR-dependent synaptic plasticity (reviewed in [22]). These studies provided indirect and inconclusive evidence since the effects reported can be attributed to others small G-proteins. Direct manipulation of RAS provided ambiguous evidence on its role in memory. Heterozygote $Kras^{+/-}$ mice showed normal memory and synaptic plasticity, however, LTM and synaptic plasticity were more sensitive to a MEK inhibitor [23]. Mice expressing the constitutively active allele $Hras^{G12V}$ showed enhancement in both NMDAR-independent synaptic potentiation and spatial memory in the Morris water maze [24]. Moreover, $Hras^{G12V}$ mice showed enhanced short-term memory and LTM in contextual fear conditioning [24]. Therefore, although it is clear that RAS is involved in memory, its role is uncertain.

To understand the relationship between the memory improvement mediated by additional training or additional spacing, we developed an approach to examine the effect of adding training trials or spacing to a weak training protocol. Here, we show that both strategies share the same mechanisms, and produce RAS-dependent memory improvement in control genotypes or memory rescue in disease-related RAS mutants.

2. Materials and Methods

2.1 Fly stocks

Flies were raised at room temperature (25 °C) on standard cornmeal medium with 12:12 light-dark cycles and 65% humidity. *Drosophila UAS-Ras-V152G* stocks were generated as previously reported [17]. *UAS-Ras85D.K* (RRID:BDSC_5788), *UAS-Ras85D-N17* (RRID:BDSC_4845), *UAS-Ras85D-RNAi* (RRID:BDSC_34619), c747-GAL4 (RRID:BDSC_6494), 247-GAL4 (RRID:BDSC_50742) and tub-GAL80ts (RRID:BDSC_7019) were obtained from the Bloomington Drosophila Stock Center and are described at FlyBase (http://www.flybase.org). All of the transgenes were in the genetic background of the control genotype w^{1118}. Note that the transgenes in the genotypes are written without representation of the chromosome involved.

2.2 Behavioral assay

Behavioral experiments were performed using a standard olfactory conditioning procedure [25]. Briefly, groups of ~100 flies two- to four-day-old were trained and tested under red light illumination (LED, 625 nm) undetected by fruit flies, at 70% humidity and 25 °C. For experiments using the TARGET system, for which flies raised at 18 °C were incubated at 29 °C for 4 days to allow transgene expression, training and testing was made at 29 °C. A single training trial consisted in the following procedure: Flies were exposed to a first odor #1 (CS+) temporally paired with electric shock (US) (1 min), followed by a second odor #2 (CS−) without electric shock (1 min). In parallel a second group of ~100 flies were subjected to a similar procedure where the odor #2 (CS+) was paired with the electric shock (US), followed by the odor #1 (CS−) without electric shock. Both groups were used to estimate a single behavioral performance index (PI) to obtain a balanced design of the experiment. Animals were trained with one or more training trials, in a massed pattern or in

a spaced pattern, including 15, 30, 45 or 60 min of inter-trial intervals. Odors were delivered by an air stream of 750 ml/min passing through mineral oil containing octanol-3 [1.5×10^{-3}] or methylcyclohexanol (MCH) [1.0×10^{-3}] (Sigma Aldrich). Electric shocks (60 V) were delivered through a copper grid using an S88 Grass stimulator. Training protocols were delivered by an electro-mechanic system controlled by a computer.

2.3 Performance index

Memory tests were performed at the corresponding retention time, were each group of ~100 flies were force to choose between odor #1 and #2 in a T-maze. Conditioned odor avoidance responses were assessed for 2 min in a T-maze, where the CS+ and CS− were delivered simultaneously on each arm of the T-maze. Then, flies from each arm were counted to estimate the behavioral performance index as PI= [(CS−)-(CS+)/(CS+)+(CS−)]x100. The PI obtained from the two groups trained and tested in parallel (in the balanced experiment) were averaged to obtain a single PI (n=1). In all cases, the sample size was eight independent experiments (n=8), with a statistical power larger than 0.90 in all tests [26].

2.4 Drug feeding and cold shock treatment

Drug feeding and cold shock anesthesia were performed as previously reported [6]. Groups of ~100 flies were fed cycloheximide (CXM) 35 mM (Sigma Aldrich) or vehicle (sucrose 5%) for 12 h before training. Cold shock (2 min) was applied 2 h after training by submerging flies kept in a glass tube in ice water at 0 °C.

2.5 ERK activity analysis

ERK activity was examined by western blot experiments. For each data point 30 head were homogenized, run on a 12 % SDS PAGE and transferred to PVDF (Thermo Scientific). Mouse anti-activated pERK1/2 1:1000 (Sigma #M8159) and rabbit anti-total ERK1/2 1:15000 (sigma #M5670) were used. HRP-conjugated secondary antibody goat anti-mouse 1:1000 (Sigma #A5278) and goat anti-rabbit 1:5000 (Sigma #A0545) and ECL kit Super Signal West Pico (Thermo Scientific) were used for signal detection in an ImageQuant RT ECL device (GE Healthcare Life Sciences). Activation was quantified by ImageJ as pERK normalized to total-ERK.

2.6 Statistical Analysis

Statistical analyses were performed with Prism GraphPad 6.01 and G*Power3 software [26]. Comparisons for more than two groups were performed with one-way ANOVA or two-way ANOVA according to the experimental design, followed by planned multiple comparisons (Boferroni) and by t-tests when comparing two groups. Differences were considered significant when $p < 0.05$.

3. Results

3.1 Rationale of the strategy to examine the effect of repeating or spacing training trials

To determine the effect of training repetition and the effect of spacing training trials, we developed an approach to detect memory improvement by repeating or spacing training

trials. To do so, wetook a weak protocol of two trials spaced by 15 min (2S-15min) and added 15 min of additional spacing (2S-30min) or five additional training trials (7S-15min) (Fig 1A).

We used as starting point the two-trial protocol (2S-15min) since does not produce a better memory at 24 h compared with a single training trial [27].

The inter-trial interval of 30 min was based on the properties of activation of *rolled*, the ortholog of ERK1/2, which is required for LTM induction [6]. In *Drosophila,* LTM is detectable at 24 h after five to ten training trials spaced by 15 min [27,28]. We previously showed that a complete activation of ERK requires 20 min after a training trial of olfactory conditioning and that a second training trial removes the activated ERK induced by the first trial [6]. Therefore, we used an inter-trial interval of 30 min in the two-trial protocol, aimed at obtaining maximal ERK activation after both training trials.

In addition, we used an atypical seven-trial protocol to add training repetition since the classic protocols of five or ten trials spaced by 15 min were unsuitable, for the following reasons: First, to make this study comparable with our previous investigations on the spacing effect [6], we wanted to use the same intensity of stimuli (*i.e.* same odor concentrations and 60 V of electric shock). Second, using 60 V of electric shock we had to provide more than five trials of training to obtain a consistent level of 24 h memory. This might explain why the classic five-trial protocol usually uses 90V of electric shock [29]. Third, the classic protocol of ten-trials was incompatible to detect subtle memory enhancements because it produces some degree of overtraining, since it was designed to produce the best possible memory [27].

3.2 Two trials of spaced training induce spacing effect in 24-h memory

Whereas we expected that the 7S-15min protocol produces a better memory compared with the weak training protocol (2S-15min) [27], the effect of the 2S-30min protocol was not previously reported. To characterize this new training protocol, we began examining the basic learning conditions. First, we documented that the weak protocol of two trials (2S-15min) produced a significantly 24-h memory compared with the untrained group (Fig 1B). The addition of spacing between training trials (2S-30min) or the addition of training trials (7S-15min) produced a memory enhancement compared with the two-trial protocol (2S-15min) (Fig 1B). Then, we characterized 24-h memory after one trial (1T) or two trials of massed training (2M). These protocols produced a conditioned behavioral response indistinguishable from each other, but significantly higher compared with the untrained group (Fig 1C). The 2S-30min protocol produced a higher memory than that produced by two massed trials, showing a significant spacing effect (Fig 1C).

The spacing effect in 24-h memory after the 2S-30min protocol resulted from the spacing and not from the total time of training, since eleven trials of massed training (11M) (which spans the total time required for the 2S-30min protocol) did not produce a better memory than 2M trials (Fig 1C).

Then, we contrasted the behavioral performance after the weak training protocol (2S-15min), with that containing additional training (7S-15min). We examined 24-h memory after seven trials of massed training (7M) and the 7S-15min protocol. As expected, the 7S-15min protocol produced a higher memory than 2S-15min and spacing effect compared with the 7M training protocol (Fig 1D) [27]. Moreover, 24-h memory after 1T, 2M or 11M trials were indistinguishable from each other, indicating the existence of a ceiling effect in the absence of inter-trial intervals (Fig 1C). Hence, the memory induced at 24 h after massed training, previously identified as anesthesia-resistant memory (ARM) [28], was insensitive to additional training.

Of note, the 2S-30min training was as good as 7S-15min to induce the spacing effect (Fig 1E), suggesting that these strategies (additional spacing or additional trials of training) are equally effective to trigger memory improvement.

3.3 Two trials of training spaced by 30 min induced LTM

We asked whether the memory improvement produced by the 2S-30min protocol was LTM-dependent. LTM is characterized as a protein synthesis-dependent memory also sensitive to anesthesia [28]. Hence, we examined whether 24-h memory after the 2S-30min protocol was sensitive to the protein synthesis inhibitor cycloheximide (CXM). We fed fruit flies with CXM or vehicle (sucrose 5%) and both groups were subjected to 2M or 2S-30min training. CXM produced no effect on 24-h memory after 2M training (Fig 2A), consistent with the classical model postulating that after massed training only ARM is detectable [28]. In contrast, CXM reduced 24-h memory after the 2S-30min protocol compared with the control group fed with vehicle (Fig 2A). These results indicated that two spaced trials are enough to induce LTM if a long inter-trial interval is included. The remaining 24-h memory after 2S-30min training in the group fed with CXM was indistinguishable from that after 2M training (Fig 2A). This is also consistent with the coexistence of ARM and LTM at 24 h after spaced training [28].

To further analyze the properties of the 24-h memory after the 2S-30min training, we examined its sensitivity to anesthesia. Exposing fruit flies to a 2-minute cold-shock in ice water, 2 h after massed or spaced training, we found that only 24-h memory after spaced training was significantly reduced (Fig 2B). These results further supported the notion that two spaced trials produce LTM and ARM, while two massed trials only produce ARM [28].

Taken together, the 2S-30min training protocol produced a 24-h memory with similar properties to the memory produced with more conventional training protocols, including protein synthesis-dependence and sensitivity to anesthesia [28].

3.4 ARM does not improve with additional training trials or additional spacing between trials

Whereas memory was improved by additional spacing or additional training trials through LTM formation, ARM was not affected by increasing massed training trials or additional spacing (Figs 1C–D and 2A–B). Therefore, it remained to be determined whether in this conditions ARM can be affected by additional training trials in the long protocol; 7S-15min. We examined the effect of anesthesia (2 min cold-shock) on 24-h memory after this protocol

and found no enhancement in the levels of ARM (Fig 2C), being similar to the memory after massed training (Fig 2B). Thus, ARM is not susceptible to improvement by variations in spacing or training trials (Figs 1C–D and 2), confirming that memory improvement is mediated by LTM induction.

3.5 Memory improvement by two spaced trials requires Ras activity in mushroom body neurons

As mentioned above, mutations in RASopathies enhance activity of the RAS-ERK1/2 signaling pathway and *neurofibromin* is the main regulator of RAS (for review, see [2,5,16]). Nevertheless, the role of RAS in memory is not clear in general and much less with respect to the spacing and repetition effects. In order to understand the role of RAS in memory improvement, we examined the effect of expressing a dominant negative allele of Ras (Ras-N17) in mushroom body (MB) neurons using a previously characterized GAL4 driver, c747-GAL4 [30,31]. Suppression of RAS activity did not affect immediate memory or 24-h memory after 2M training (Fig 3 A and B). In contrast, 24-h memory after the 2S-30min training protocol was strongly reduced (Fig 3B). These observations suggested that RAS is not required for immediate memory or ARM, but it is essential for LTM. To further support this, we expressed *Ras-RNA* interference (RNAi) harpin construct in MB neurons using the same GAL4 driver (c747-GAL4). *Ras-RNAi* had no effect on immediate or 24-h memory after 2M training, whereas it significantly reduced 24-h memory after the 2S-30min protocol compared with the control group (Fig 3 A and C). Thus, reducing RAS in mushroom body neurons through two distinct approaches suggested its requirement for LTM, but not for other forms of memory.

3.6 Acute suppression of RAS in MB of adult fruit flies precludes memory improvement

The above experiments suggested the involvement of RAS in 24-h memory improvement after additional spacing, however, a RAS-dependent defect in development could also produce a similar result. To test whether RAS affected 24-h memory independently of structural alterations during development, we examined the effect of suppressing RAS in adult flies immediately before training. For that we used the TARGET system (GAL80ts;GAL4;UAS-transgene) [32]. We combined the temperature-sensitive GAL4 repressor, *Tubulin-GAL80ts*, with the mushroom body GAL4 driver (*247-GAL4*) and a dominant negative form of Ras (*UAS-Ras-N17*) [33]. When these flies and control genotypes were developed and tested at permissive temperature (18 °C), no 24-h memory phenotype was observed (Fig 4A). In contrast, when we expressed Ras-N17 in adult flies, during four days at the restrictive temperature (29 °C), 24-h memory was normal after 2M training, but strongly reduced after the 2S-30min protocol (Fig 4B). These results show that the memory improvement mediated by additional spacing requires acutely RAS activity in MB neurons.

Then, we examined whether the inducible suppression of Ras in adult flies affected 24-h memory after the long protocol 7S-15 min. Similar to the behavior observed after two training trials, we found no effect of Ras-N17 at the permissive temperature (18 °C) (Fig 4C). However, the expression of Ras-N17 during four days before training at a restrictive temperature (29 °C) reduced 24-h memory after the 7S-15min but not after 7M (Fig 4D).

Taken together, these studies showed that RAS is required in MB neurons for LTM induced by additional spacing (2S-30min) or additional training trials (7S-15min). Therefore, these results further documented that these strategies for memory improvement share similar properties.

3.7 Overexpression of RAS-WT in MB produces LTM after massed training

To further analyze the role of RAS in memory improvement, we examined the effect of overexpressing a wild-type *Ras* allele (RAS-WT) in MB neurons driven by c747-GAL4. We expected that the effect of overexpressing RAS-WT would be especially informative, because the overexpression of a WT allele of *Shp2* has no effect after spaced training and induced LTM even after massed training [6]. Thus, if SHP2 acts mainly through a downstream pathway involving RAS, overexpression of RAS-WT might produce a similar effect. Alternatively, if RAS is not acting as SHP2 does, the overexpression of RAS-WT should provide evidence of a differential role of RAS and SHP2. First, overexpressing RAS-WT did not affect immediate memory or 24-h memory after the 2S-30min training protocol (Fig 5A–B). However, RAS-WT enhanced 24-h memory after the 2M protocol (Fig 5B), suggesting that RAS induces LTM after massed training, as is the case after overexpression of SHP2. To test whether the memory enhancement after massed training had properties of LTM, we examined its sensitivity to CXM. We fed flies overexpressing RAS-WT or control genotype (c747;+) with CXM or vehicle (sucrose 5%) before training and all groups were trained with the massed protocol 2M. In control fruit flies (c747;+) CXM had no effect compared with vehicle (Fig 5C). In contrast, in flies overexpressing RAS-WT, CXM prevented 24-h memory enhancement, indicating that the memory enhancement was LTM (Fig 5C). Together, these observations indicated that RAS modulates the spacing effect replicating the effects of SHP2 manipulation. Most importantly, our observations presented here and previous reports [6,11]suggest a mechanism controlling the spacing effect through a signaling pathway involving SHP2, RAS and ERK1/2 and predict that RASopathies may have an alteration in the spacing and the repetition effects as well.

3.8 Acute overexpression of RAS-WT enhanced 24-h memory after massed training

Next, we examined the acute effect of overexpressing RAS-WT in MB of adult flies immediately before training. In these experiments, we combined *Tubulin*-GAL80ts with the MB GAL4 driver (247-GAL4) and the WT allele of Ras (UAS-Ras-WT). When these flies and control genotypes were developed, trained and tested at a permissive temperature (18 °C), no 24-h memory phenotype was observed after massed or spaced training with the two-trial (2M and 2S-30min) or seven-trial (7M and 7S-15min) protocols (Fig 6A and B). When we induced the expression of RAS-WT in adult fruit flies, during four days at the restrictive temperature (29 °C), 24-h memory was normal after two training trials (2M and 2S-30min) (Fig 6C). This is in contrast to the memory enhancement after massed training detected in flies with constitutive expression of Ras-WT (Fig 5). However, the inducible expression of RAS-WT enhanced 24-h memory after seven massed trials (7M) (Fig 6D). Together, these results that the degree of induction after 4 days at 29 °C might be insufficient for memory enhancement. To test that, we examined 24-h memory after the 2M protocol after inducing the expression of Ras-WT for 6 days at 29 °C. In such conditions, the transient expression of Ras-WT enhanced 24-h memory after massed training (Fig 6E). However, we cannot rule

out that additional molecular or cellular changes occurred during the 6 days of induction. In summary, RAS-WT acutely promotes memory enhancement after massed training.

3.9 GOF Ras V152G impairs 24-h memory after spaced training

The data presented above showed that additional training trials and additional spacing were equally effective for memory improvement (Figs 1 and 2) and in both cases RAS is required in MB neurons (Figs 3 and 4). Next, we examined whether these training strategies (additional spacing or additional training) were equally effective to rescue memory deficit in a disease related gain-of-function (GOF) Ras allele V152G [34]. We targeted the expression of Ras-V152G to MB neurons with the c747-GAL4 driver. First, we documented that Ras-V152G had no effect on immediate memory compared with control flies (Fig 7A). Then, we used the 2-trial protocol to examine 24-h memory, since this is a weak training producing LTM and therefore we expected to be especially sensitive to memory deficits or enhancement. The GOF Ras allele V152G had no effect on 24-h memory after massed training (2M) (Fig 7B). In contrast, it impaired 24-h memory after 2S-30min training compared with the control groups (Fig 7B), suggesting that this GOF Ras mutation impairs LTM, leaving ARM intact. To test this possibility, we subjected GOF Ras mutant flies to the 2S-30min protocol followed by anesthesia. Whereas in the control genotype, cold shock reduced 24-h memory to the level of ARM, the memory in the GOF Ras mutants was unaffected (Fig 7C). These results led us to conclude that the GOF Ras mutant impaired LTM after spaced training, without affecting immediate memory or ARM. Importantly, the effect of GOF Ras resembles that produced by GOF SHP2 [6].

3.10 Comparable and non-additive memory rescue by additional training or spacing in GOF Ras V152G

Next, we examined whether increasing the number of training trials or the spacing between trials restored 24-h memory in the GOF Ras mutants. We used the 2S-30min protocol as reference, as it allowed us to detect 24-h memory impairment in the GOF Ras mutants (Fig 7B). Increasing the number of training trials from two up to four had no effect in the control group, but partially rescued the memory deficit in the GOF Ras mutants (Fig 7D). Similarly, prolonging the inter-trial interval from 30 to 45 or 60 min had no effect in the control group, but partially rescued the memory deficit in the GOF Ras mutants (Fig 7E). These results showed that memory improves by increasing the number of training trials or the spacing between trials, and therefore GOF Ras mutants have a deficit in the spacing and the repetition effect for memory formation.

We next examined whether the combination of those strategies elicited a complete restoration of memory. Unexpectedly, 24-h memory after three trials spaced by 45 min (3S-45min) did not elicit a complete memory rescue, showing no additive effects (Fig 7F). These data indicated that the effect of additional training in the 3S-30min protocol and the additional spacing in the 2S-45min are additive when they were combined in the 3S-45min protocol. However, a similar result would have been obtained if memory cannot be rescued beyond the level observed in this GOF Ras mutant. We tested this possibility by using a stronger protocol of seven training trials spaced by 45 min. After this stronger protocol, we found a normal 24 h behavioral performance in the GOF Ras mutant compared with the

control (Fig 7F). Together, these experiments using the minimal training protocols support the idea that both strategies are equally effective for improving memory, presumably through the same molecular resources (*i.e.* RAS-dependent signaling pathway).

To test whether RAS activity was required for the memory improvement, we examined whether the long protocol (7S-45min) could improve 24-h memory after suppression of RAS activity. Whereas 7S-45min improved 24-h memory in the GOF Ras mutant (Fig 7F), it had no effect in fruit flies expressing *Ras-N17* or *Ras-RNAi* in MB neurons (Fig 7G), indicating that RAS activity was required for memory rescue. Altogether, these experiments support the idea that both strategies (additional trials or spacing) are equally effective for improving memory through a RAS-dependent signaling pathway in a non-additive manner in a context of minimal training protocols. In other words, both strategies appear to contribute to improve memory with the same underlying molecular mechanisms.

3.11 Underlying mechanisms in the spacing and the repetition effect

The prolonged spacing in the 2S-30min protocol substituted the multiple training repetitions of the 7S-15min protocol in control genotypes (Fig 1A and B). How do these protocols contribute equally to 24-h LTM formation? The most parsimonious explanation appears to be that the short protocol of two trials (*i.e.* 2S-30min) is just enough to induce 24-h LTM and the long protocol of seven trials has some degree of over-training. However, the over-training must be quite small since five trials of training spaced by 15 min produced an inconsistent behavioral performance (see section 3.1).

We propose that both training protocols provide enough amounts of the same requirements for LTM formation (*e.g.,* activation of ERK), where the length of the inter-trial interval determines the magnitude of the signaling pathway activation. Such condition of ERK activation for LTM induction is never reached by a single training trial or two trials spaced by the standard inter-trial interval of 15 min owing to the negative effect of the second trial of training on the levels of ERK activation [6].

One prediction of this interpretation is that the concentration of activated ERK produced by the 2S-30min protocol should be enough for LTM induction and in some extend similar to that produced by the long protocol (*i.e.* 7S-15min). To explore this possibility, we analyzed the time course of ERK activation produced by training in western blot experiments [39]. After a single trial of training with an inter-trial interval of 15 min, the area under the curve (AUC) of activated-ERK was 324 ± 13.08 (Fig. 8A). Using this value, the activated ERK produced by the training protocols of five or seven trails spaced by 15 min can be estimated as 324×5 = 1620 ± 65.4 or 324×7 = 2268 ± 91.56 (Fig. 8A). Similarly, after a single trial of training with an inter-trial interval of 30 min the AUC was 802 ± 23.91, significantly higher than that after 15 min of inter-trial interval, p=0.004, Mann Whitney test (Fig. 8B). Again, using this value, the total activated ERK in the protocols of 2S-30min can be estimated as 802×2= 1604 ± 47.82(Fig. 8B).

Interestingly the 2S-30min protocol activated as much ERK as five trials spaced by 15 min (AUC 1604 vs. 1620), the second most common protocol [35]. Thus, this observations are consistent with the idea that the length of the inter-trial interval determines the magnitude of

signaling and memory levels. Therefore, both learning strategies might contribute similarly to memory improvement because they produce the required magnitude of signaling, including ERK activation, in two distinct patterns. One pattern with seven small pulses of ERK activation and the other with two large pulses of ERK activation.

4. Discussion

This study investigates the relationship between memory improvement by additional training and by additional spacing between training trials. Both strategies successfully used to rescue memory deficits in animal models and humans were previously viewed as unrelated phenomena [6,8,36–39]. However, this study shows that these two learning strategies are highly related. Both training strategies involved the same mechanisms (RAS- and protein synthesis-dependent mechanism, compatible specifically with LTM) (Figs 1–4) and had similar effectiveness for memory improvement in control genotypes (Fig 1 and 2). In addition, both strategies had the same effectiveness for rescuing memory deficits in a RASopathy-related GOF Ras mutant (Fig 7). Notably, these training strategies did not show an additive effect when combined (Fig 7D–F), indicating that the underlying mechanisms interact, and therefore are highly related or are the same. Thus, it seems that these training strategies exploit the same mechanisms through different learning patterns. Moreover, a variety of genetic manipulations consistently showed that RAS is only involved in LTM in a spaced-dependent manner, without affecting other memory components (Fig 3–7). In addition, genetic manipulations of Ras or increasing the number of training trials or the spacing between them did not affect ARM (Figs 1–6). The evidence presented here, together with previous investigations [6,11], support a model where the spacing effect is regulated by a molecular mechanism involving SHP2, RAS and ERK, a key signaling pathway involved in RASopathies and LTM [5]. Thus, we conclude that both strategies for memory improvement use the mechanisms of the spacing effect in different magnitudes depending on the length of the spacing.

However, the 2S-30min protocol produced a consistent level of memory, whereas the five trials did not (see section 3.1). Thus, even when ERK is required for LTM and it is larger with longer inter-trial intervals, the concentration of ERK alone cannot explain such difference. Possibly, the time course of activation of other signaling pathways after each trial might explain such difference.

Of note, two trials with large spacing produced LTM sensitization in *Aplysia*[40]]as well as olfactory LTM in *Drosophila*, showing that LTM formation after two training trials is a property of memory across phyla. Altogether, this study suggests that RASopathies might have learning problems because of a deficit to exploit the spacing and the repetition effect, which affect LTM induction.

4.1 Ras in learning and memory and relevance for Ras-related disorders

Genetic manipulations of Ras, including GOF Ras, Ras-RNAi and Ras-N17 impaired specifically LTM after spaced training without affecting immediate or other consolidated memories as ARM (Fig 3–7). Why did overexpression of RAS-WT not affect LTM, whereas RAS-V152G did? One explanation is that the WT proteins have nearly normal properties of

activation and inactivation and did not affect signaling timing [6]. This fact contradicts a few cases of copy number variation for *KRAS* and other related genes [41]. Another possibility previously considered was that protocols of ten trials overcome some defects in the signaling mechanisms by over-training. However, we did not detect memory impairment after a weak protocol of two training trials in animals overexpressing RAS-WT (Fig 5B). Therefore, our observations might support the idea that phenotypes in RASopathies are mediated by altered activation, and not by a dosage effect, as previously suggested [6,42].

The fact that overexpressing RAS-WT enhanced memory after massed training, suggested that RAS is a limiting factor. However, overexpressing SHP2 also enhance memory after massed training [6]. If RAS and SHP2 would be limiting factors, the overexpression of only one of them cannot produce a memory enhancement. An alternative explanation is that a larger availability of SHP2-WT or RAS-WT produced a stronger and faster activation of the molecular components downstream of SHP2 and RAS, including ERK1/2 as previously detected [6]. Additionally, since RAS has the ability to spread to neighbor sub-cellular areas by diffusion [43], with a higher concentration of RAS-WT a larger diffusion potential take place and a larger signal amplification as well, promoting a more efficient nuclear ERK activation [44].

Why did the GOF Ras-V152G show LTM deficits? One possibility arises from the effect of the Ras-V152G allele on synaptic plasticity. We previously showed that GOF Ras-V152G flies appear to be able to trigger a significant amount of structural synaptic plasticity after spaced stimulation [13]. Nevertheless, in contrast to the control genotype, the GOF Ras-V152G allele produce a significant number of new synaptic boutons even after a more massed protocol, which normally does not produce synaptic plasticity. Of note, the Ras-V152G triggers a similar plasticity after the spaced and the more massed protocol with an abnormal enhanced variability. All together, these studies suggested that the LTM deficits in the Ras-V152G flies would be mediated not by absence of MAPK signaling or synaptic plasticity, but by a defect leading to inappropriate plasticity, for instance with poor specificity and precision [13].

In summary, we conclude that the repetition and the spacing effect are related in the sense that just the mechanisms of the spacing (mediated by a SHP2/RAS/ERK signaling during the inter-trial intervals) are used with different temporal patterns.

Acknowledgments

We are grateful to Y. Zhong and F. Ceriani for essential materials required in the experiments, G. Ortega for technical assistance, L. Rela and E. Merlo for comments on the manuscript and members of the GNS lab for discussion of the experiments.

This work was supported by the National Institutes of Health (Grant R03-TW009010 to B.G.) and the National Agency of Scientific and Technological Promotion (Grant PICT-2011-2267 to M.R.P.).

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Highlights

- Memory improvement by learning repetition or spaced learning shares mechanisms.
- Memory is rescue in disease-related GOF RAS mutant.
- Memory improvement and memory rescue is mediated by LTM formation.
- Non-additive interaction of the spacing and repetition effects.

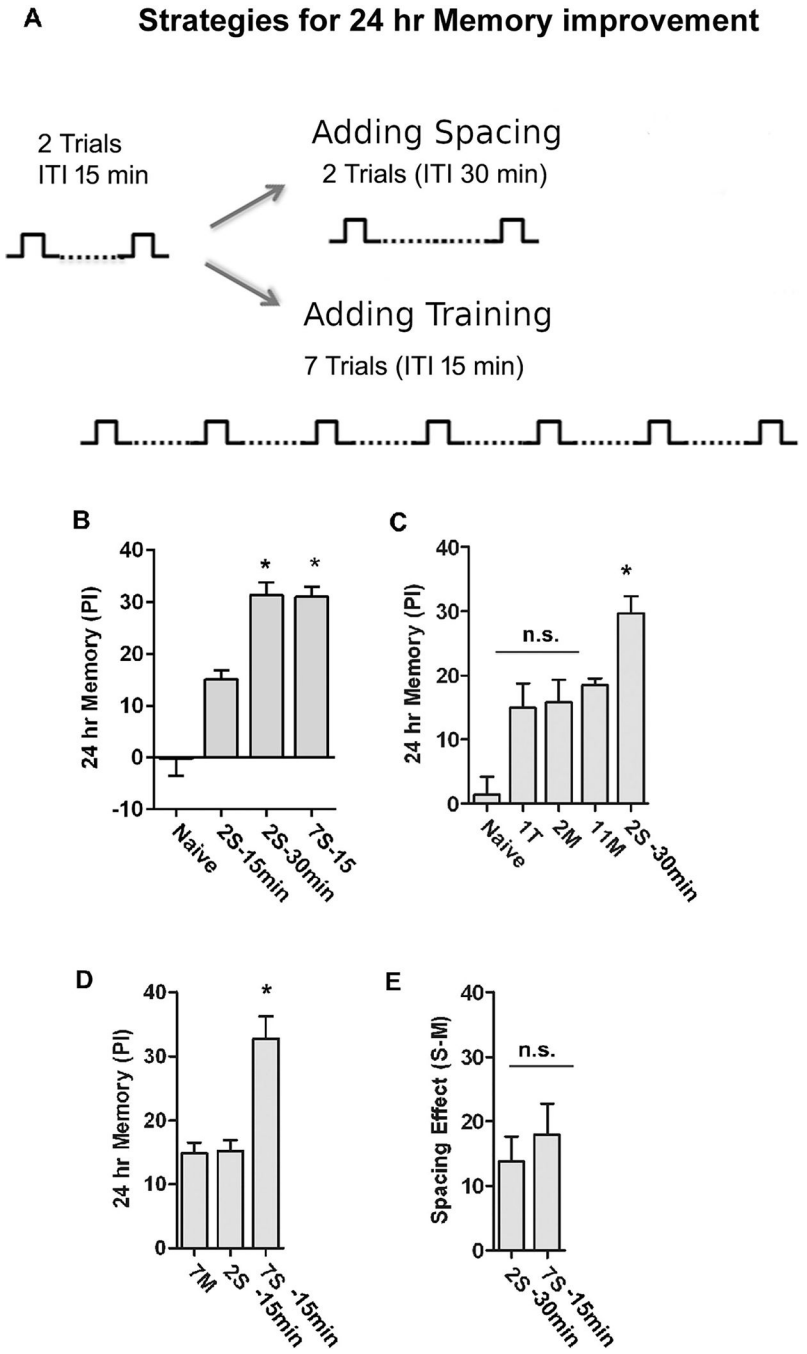

Fig. 1.

Two trials of spaced training induced spacing effect in 24-h memory. (A) Schematic representation of the strategy for examining memory improvement. The starting point is the protocol of two training trials spaced by 15 min (2S- 15 min) (left), which does not improve memory compared with a single learning trial. Then, it was included additional spacing (2S-30 min) (top)or additional training trials (7S-15 min) (bottom). (B–E) Memory performance in control fruit flies w1118. (B) Response of untrained animals to memory testing (Naive) and 24-h memory after the training protocols depicted in (A), 2S- 15 min,

2S-30 min and 7S-15 min. Asterisk indicates significant memory im-provement compared with 2S-15 min protocol, $p < 0.0001$, One-way ANOVA followed by planned multiple comparison Bonferroni test. (C) Response of untrained animals to memory testing (Naive) and 24-h memory after 2 and 11 trials of massed training (2 M and 11 M, respectively) and 2 trials of training spaced by 30 min (2S-30 min); ($F(4,35) = 11.86$, $p < 0.0001$, one-way ANOVA) followed by planned multiple comparison Bonferroni test. Asterisk indicates significant difference ($p = 0.0060$ for 2M vs. 2S-30 min). (D) 24-h memory after 7 trials of massed training (7M) and 2 or 7 trials spaced by 15 min (2S-15 min and 7S-15 min). Asterisk indicates significant difference compared with 7M and 2S-15 min, ($F(2,21) = 17.85$, $p < 0.0001$, one-way ANOVA) followed by planned multiple comparison Bonferroni test. (E) Spacing effect at 24 h after 2 or 7 trials of spaced training, (2S-30 min) and (7S-15 min) re-spectively. The spacing effect, estimated as the difference between 24-h memory after spaced and massed training, showed no difference (n.s.) ($t(14) = 0.6603$, $p = 0.5198$ two-tailed t-test). Bars, mean ± standard error of the mean (SEM), ($n = 8$).

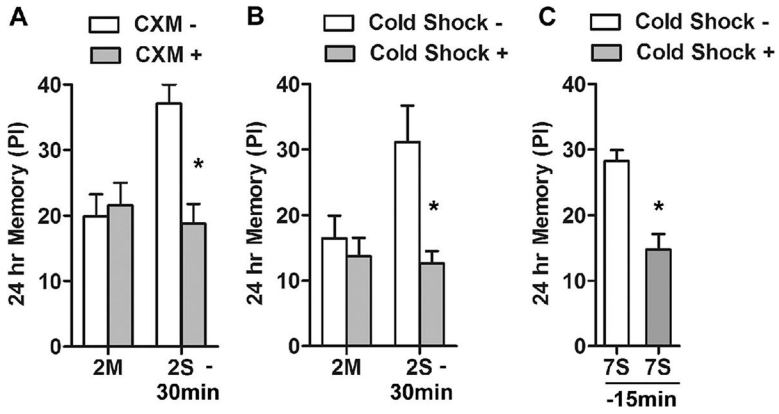

Fig. 2.

Two trials of spaced training induce a protein synthesis-dependent 24-h memory. (A) Effect of the protein synthesis inhibitor cycloheximide (CXM+), or vehicle (CXM−) on 24-h memory after two trials of massed training (2M) or two trials of training spaced by 30 min (2S-30 min). The effect of drug treatments (CXM+ and CXM−) and training protocols (2M and 2S-30 min) was analyzed with two-way ANOVA with drug ($F(1,28) = 6.78$, $p = 0.0146$) and training ($F(1,28) = 5.095$, $p < 0.0320$) as main effects and the interaction ($F(1,28) = 9.803$, $p = 0.0041$), followed by planned multiple comparison Bonferroni test. Asterisk indicates significant memory reduction compared with control treatment ($p = 0.0007$ for 2S-30 min CXM− vs. CXM+). (B) Effect of the cold shock (Cold shock+) or control group (Cold shock−)($F(1,28) = 8.315$, $p = 0.0075$) on 24-h memory after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min) ($F(1,28) = 3.435$, $p = 0.0744$) and the interaction ($F(1,28) = 4.578$, $p = 0.0412$). Asterisk in-dicates significant memory reduction compared with control treatment ($p = 0.0028$ for 2S-30 min Cold shock - vs. Cold shock +), two-way ANOVA followed by planned multiple comparison Bonferroni test. (C) Effect of the cold shock (Cold shock +) or control group (Cold shock-) on 24-h memory after 7 trials of massed training (7M) or seven trials spaced by 15 min (7S-15 min). Asterisk indicates significant memory reduction compared with control treat-ment ($t(14) = 4.636$, $p = 0.0004$, two-tailed t-test). Bars, mean ± SEM ($n = 8$).

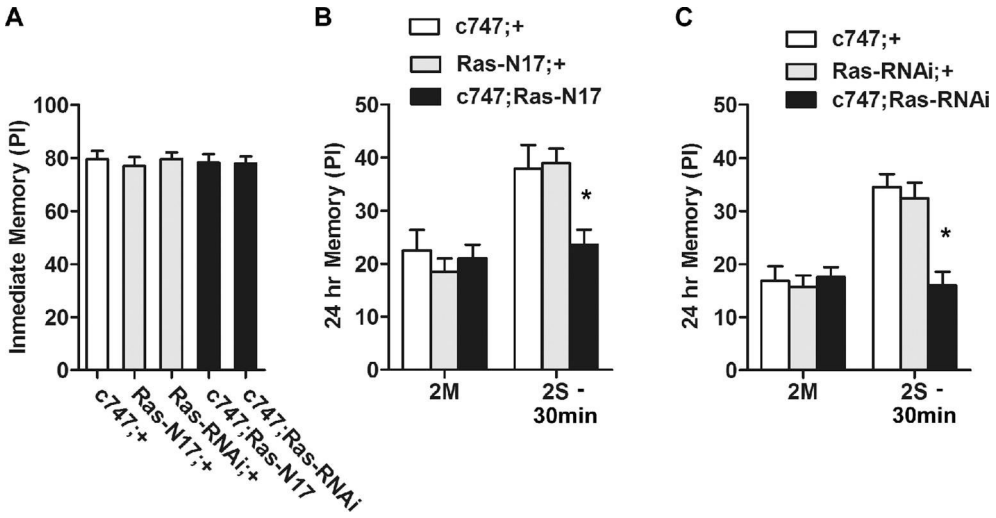

Fig. 3.

RAS is required in the spacing effect for 24-h memory. (A) Immediate memory after 1 trial of training (1T) in flies over-expressing the Ras dominant negative allele Ras-N17 or Ras-RNAi in mushroom bodies using the c747-GAL4 driver (c747;Ras-N17 and c747;Ras-RNAi) and parental lines (c747;+, Ras-N17;+ and Ras-RNAi;+) ($F(4, 35) = 0.1324$, $p = 0.9694$, one-way ANOVA). (B) Effect of genotype (c747;Ras-N17) and parental lines (c747;+ and Ras-N17;+) ($F(2,42) = 3.341$, $p = 0.0450$) on 24-h memory after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min) of training ($F(1, 42) = 23.22$, $p < 0.0001$) and the interaction ($F(2,42) = 4.043$, $p = 0.0248$). Asterisk in-dicates significant memory reduction com-pared with control genotypes ($p = 0.0052$ for 2S-30 min c747;Ras-N17 vs. Ras-N17;+). (C) Effect of genotype (c747;Ras-RNAi) and parental lines (c747;+ and Ras-RNAi;+) ($F(2,42) = 7.327$, $p = 0.0019$) on 24-h memory after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min) ($F(1,42) = 29.13$, $p < 0.0001$) and the interaction ($F(2,42) = 9.607$, $p = 0.0004$). Asterisk indicates significant memory reduction compared with control genotypes ($p < 0.0001$ for 2S-30 min c747;Ras-RNAi vs. Ras-RNAi;+). (B-C) Two-way ANOVA followed by planned multiple comparison Bonferroni test. Bars, mean ± SEM ($n = 8$).

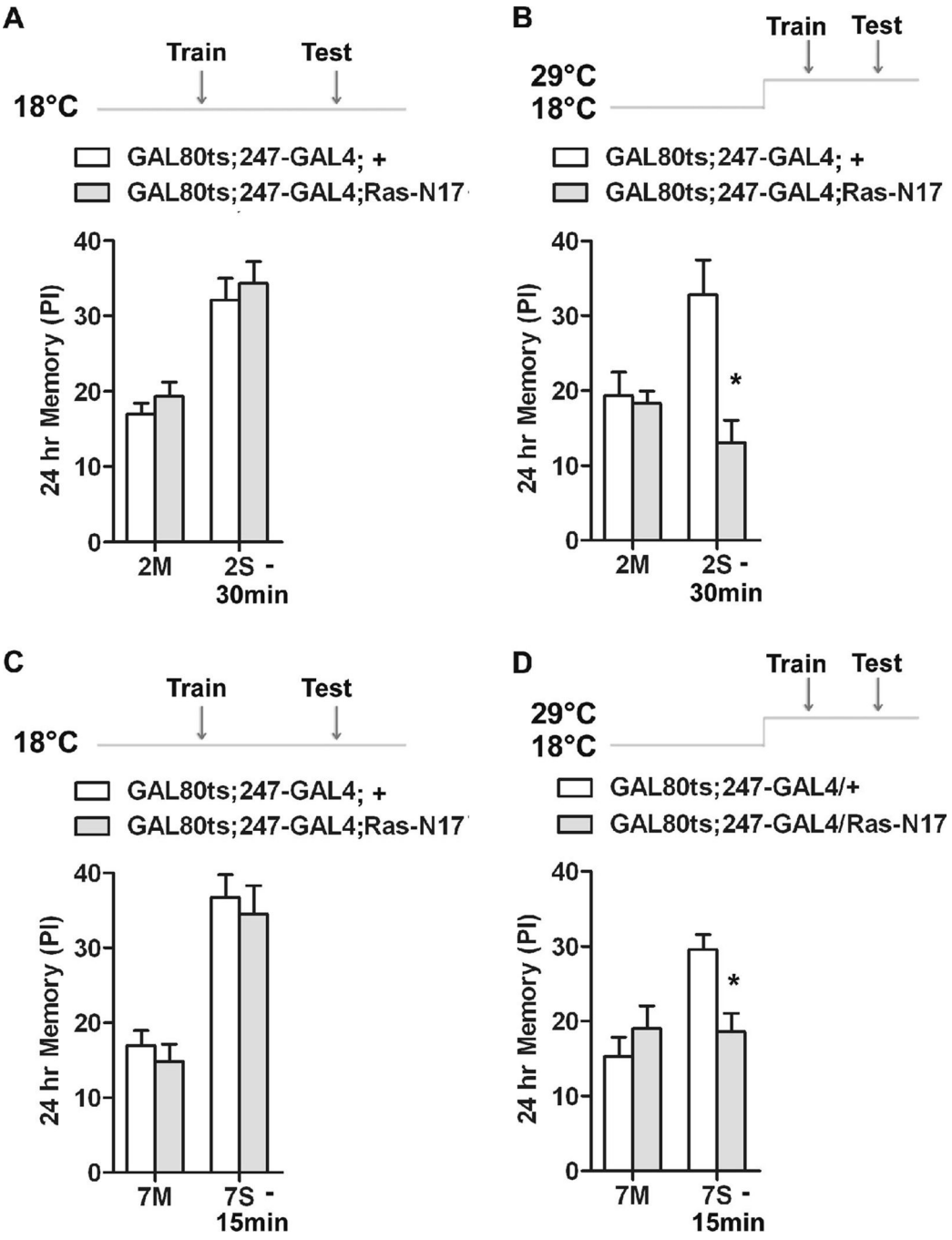

Fig. 4.

RAS disrupt the spacing effect and LTM independently of developmental defects. (A–D) Temperature protocol to control the expression of the transgenes in adult flies (top) and effect on memory (bottom)in flies carrying the tem-perature-sensitive GAL4 repressor, Tubulin-GAL80ts, combined with the mushroom body GAL4 driver (247-GAL4) and the dominant negative of Ras (UAS-Ras-N17) (GAL80ts;247-GAL4;Ras-N17) or the control group (GAL80ts;247-GAL4;+). (A-B) 24-h memory after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min). (A) At permissive temperature (18 °C) main

effect of training ($F(1,28) = 40.03$, $p < 0.0001$), genotype ($F(1,28) = 0.9299$, $p = 0.3432$) and interaction ($F(1,28) = 0.0005$, $p = 0.9817$). (B) At restrictive temperature (29 °C) main effect of genotype ($F(1,28) = 10.17$, $p = 0.0035$), training ($F(1,28) = 1.591$, $p = 0.2176$) and interaction ($F(1,28) = 8.230$, $p = 0.0078$). Asterisk indicates significant memory reduction ($p = 0.0004$ for 2S-30 min between genotypes). (C-D) 24-h memory after 7 trials of massed training (7M) or seven trials spaced by 15 min (7S-15 min). (C) At permissive temperature (18 °C) main effect of training ($F(1,28) = 48.26$, $p < 0.0001$), genotype ($F(1,28) = 0.5924$, $p = 0.4479$) and interaction ($F(1,28) = 0.0007$, $p = 0.9782$). (D) At restrictive temperature (29 °C) a main effect of genotype ($F(1,28) = 7.453$, $p = 0.0108$), training ($F(1,28) = 2.067$, $p = 0.1616$) and interaction ($F(1,28) = 8.460$, $p = 0.0070$). Asterisk indicates significant memory reduction ($p = 0.0094$ for 7S-15 min between genotypes). (A-D) Two-way ANOVA followed by planned multiple comparison Bonferroni test. Bars, mean ± SEM ($n = 8$).

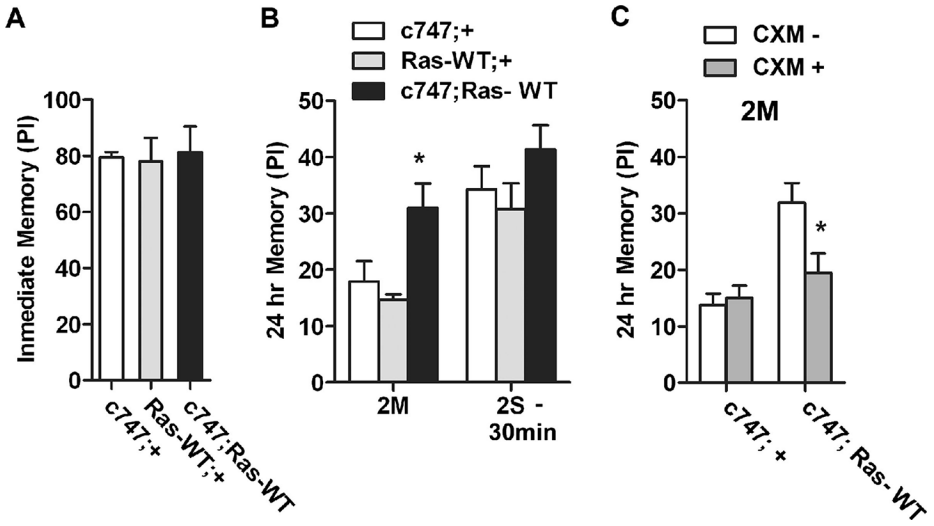

Fig. 5.

Wild-type RAS overexpression enhanced 24-h memory after massed training. (A) Immediate memory after 1 trail of training (1T) in flies over-expressing the Ras-WT in mushroom bodies using the c747-GAL4 driver (c747; Ras-WT) and parental lines (c747;+ and Ras-WT;+) ($F(2,21) = 0.3469$, $p = 0.7109$, one-way ANOVA). (B) Effect of geno-type (c747;Ras-WT) and parental lines (c747;+ and Ras-WT;+) ($F(2,42) = 6.650$, $p = 0.0031$) on 24-h memory after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min) of training ($F(1,42) = 20.68$, $p < 0.0001$) and the interaction ($F(2,42) = 0.3800$, $p = 0.6862$). Asterisk indicates significant memory enhancement compared with control genotypes ($p = 0.0274$ for 2M c747;Ras-WT vs. Ras-WT;+). (C) The effect of drug treatments (CXM+ and CXM−)($F(1,28) = 15.45$, $p = 0.0005$) and genotype (c747;Ras-WT and c747;+) ($F(1,28) = 3.748$, $p = 0.0630$) and the interaction ($F(1,28) = 5.683$, $p = 0.0242$) on 24-h memory after two trials of massed training (2M). Asterisk indicates significant memory reduction compared with control treatment ($p = 0.0098$ for c747; Ras-WT CXM+ vs. CXM−). (B-C) Two-way ANOVA followed by planned multiple comparison Bonferroni test. Bars, mean ± SEM ($n = 8$).

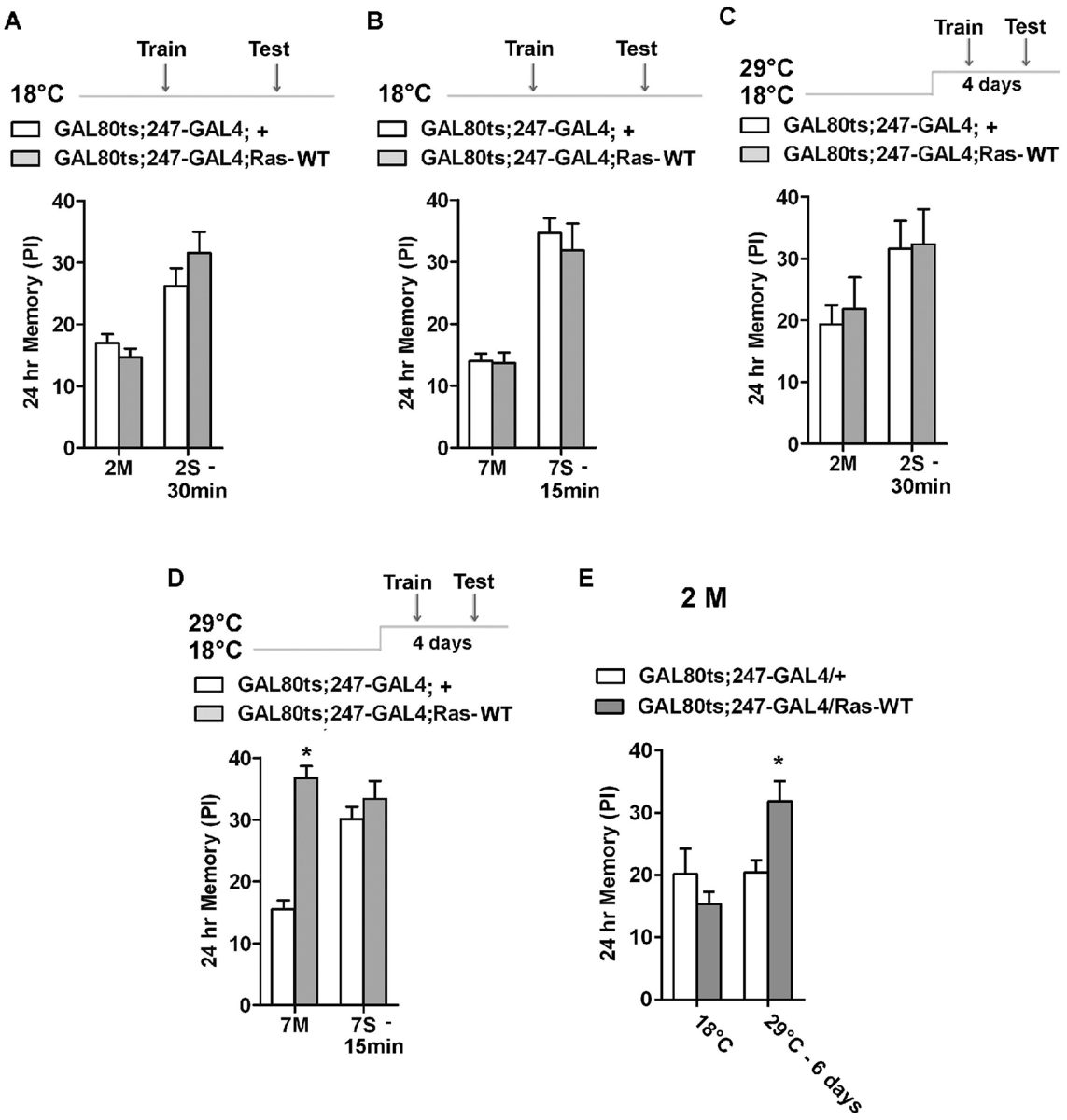

Fig. 6.

Acute overexpression of RAS-WT enhanced 24-h memory after massed training. (A–D) Temperature protocol to control the expression of the transgenes in adult flies (top) and effect on 24-h memory (bottom)in flies carrying the temperature-sensitive GAL4 repressor, Tubulin-GAL80ts, combined with the mushroom body GAL4 driver (247-GAL4) and Ras-WT (UAS-Ras-WT) (GAL80ts;247-GAL4;Ras-WT) or the control group (GAL80ts;247-GAL4;+). (A-B) 24-h memory at permissive temperature (18 °C), only show effect of training, after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min) ($F(1,28) = 29.22$, $p < 0.0001$)(A); and after seven trials of massed training (7M) or seven trials spaced by 15 min (7S-15 min) ($F(1,28) = 5.842$, $p = 0.0224$) (B). (C) 24-h memory at restrictive temperature (29 °C) after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min), only show effect of training ($F(1,28) = 53.02$, $p < 0.0001$). (D) 24-h

memory at restrictive temperature (29 °C) after seven trials of massed training (7M) or seven trials spaced by 15 min (7S-15 min), effect of genotype ($F(1,28) = 33.31$, $p < 0.0001$), training ($F(1,28) = 7.064$, $p = 0.0128$) and interaction ($F(1,28) = 17.88$, $p = 0.0002$). Asterisk indicates significant memory enhancement ($p < 0.0001$ for 7M between genotypes). (E) 24-h memory at permissive (18°C) or restrictive temperature (29 °C) for 6 days after two trials of massed training (2M). Asterisk indicates significant memory enhancement ($p < 0.001$). (A-D) Two-way ANOVA followed by planned multiple comparison Bonferroni test. Bars, mean ± SEM ($n = 8$).

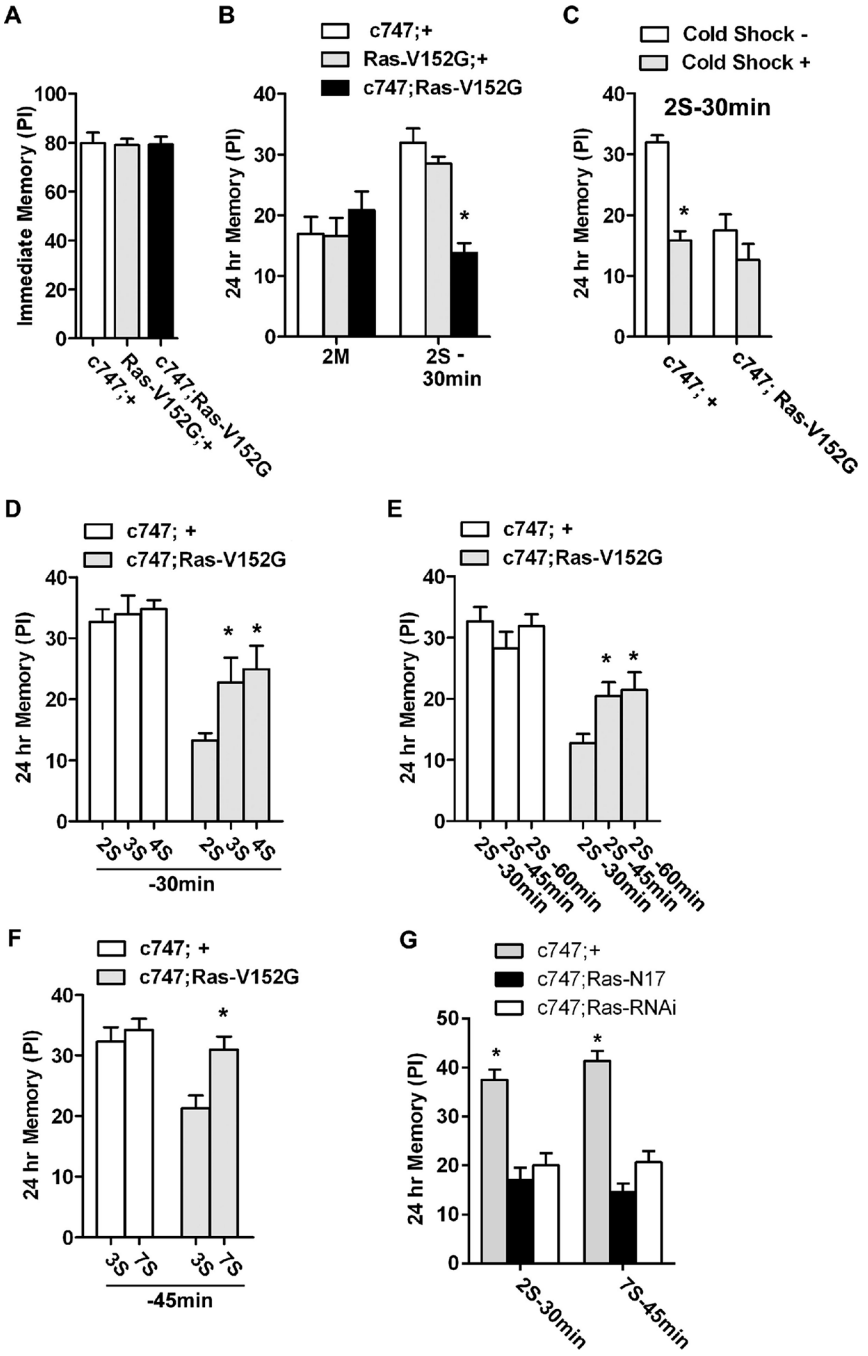

Fig. 7.

Rescuing memory impairment in GOF Ras V152G mutants by additional training or spacing. (A) Immediate memory after 1 trail of training (1T) in flies overexpressing the GOF Ras-V152G mutant in mushroom bodies using the c747-GAL4 driver (c747; Ras-V152G) and parental lines (c747;+ and Ras-V152G;+) ($F(2,21) = 0.0149$, $p = 0.9852$). (B) Effect of genotype (c747;Ras-V152G) and parental lines (c747;+ and Ras-V152G;+) ($F(2,42) = 4.702$, $p = 0.0144$) on 24-h memory after two trials of massed training (2M) or two trials spaced by 30 min (2S-30 min) of training ($F(1,42) = 11.38$, $p = 0.0016$) and the interaction

(F (2,42) = 12.32, p < 0.0001). Asterisk in-dicates significant memory reduction compared with control genotypes (p = 0.0003 for 2S- 30 min c747;Ras-V152G vs. Ras-V152G;+). (C) Effect of anesthetic treatment (Cold shock+ vs. Cold shock-) (F(1,28) = 28.49, p < 0.0001), genotype (c747;Ras-V152G and c747;+) (F (1,28) = 21.21, p < 0.0001) and the interac-tion (F(1,28) = 9.115, p = 0.0054) on 24-h memory after two spaced training trails (2S- 30 min). Asterisk indicates significant memory reduction in control genotype (p < 0.0001 for c747; + Cold shock + vs. Cold shock –). No effect in Ras mutant (p = 0.2248 for c747;Ras-V152G Cold shock + vs. Cold shock –). (D) 24-h memory after two, three or four trials (2S, 3S or 4S) spaced by 30 min (−30 min) in (c747; Ras-V152G) and control genotype (c747;+). Effect of genotype (F(1,14) = 20.58, p = 0.0005), training (F(2,28) = 4.712, p = 0.0172) and in-teraction (F (2,28) = 2.387, p = 0.1103). Asterisk indicates memory rescue (for c747; Ras-V152G, p = 0.0251 2S vs. 3S and p = 0.0048 2S vs. 4S). (E) 24-h memory after two trials (2S) spaced by 30, 45 or 60 min (−30 min, −45 min or −60 min) in (c747; Ras-V152G) and control genotype (c747;+). Effect of genotype (F(1,14 = 36.76, p < 0.0001), training (F (2,28) = 1.678, p = 0.2051) and interaction (F (2,28) = 4.359, p = 0.0225). Asterisk indicates memory rescue (for c747; Ras-V152G, p = 0.0358 30 min vs. 45 min and p = 0.0161 30 min vs. 60 min). (F) 24-h memory after three or seven trials (3S or 7S) spaced by 45 min (−45 min) in (c747; Ras-V152G) and control genotype (c747;+). Effect of genotype (F (1,14) = 11.87, p = 0.0039), training (F (1,14) = 7.266, p = 0.0174) and interaction (F (1,14) = 3.217, p = 0.0945). Asterisk indicates memory rescue (for c747; Ras-V152G, p = 0.0020 3S-45 min vs. 7S-45 min). (G) 24-h memory after two trials spaced by 30 min or seven trials spaced by 45 min in (c747; Ras-N17 and c747; Ras-RNAi) and control genotype (c747;+). (B-G) two-way ANOVA followed by planned multiple comparison Bonferroni test. Bars, mean ± SEM (n = 8).

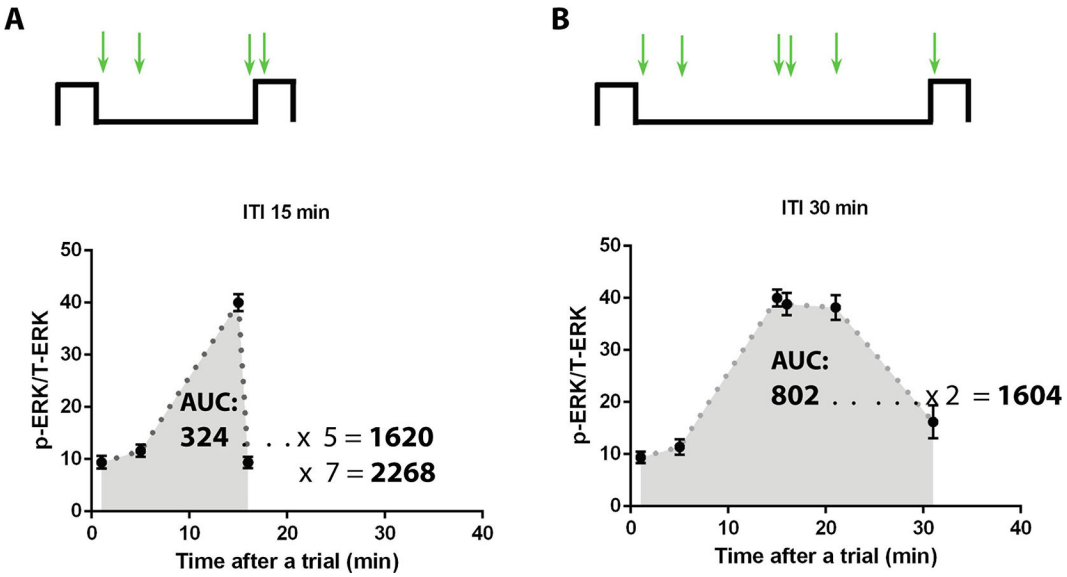

Fig. 8.

The longer inter-trial interval substituted multiple training repetitions by promoting stronger molecular signaling during the inter-trial interval. (A-B)Top: schematic representation of the protocols of training. Square pulses represent a single training trial and arrows the time point where the brain samples were taken. Bottom: quantification of the activated ERK1/2 and the area under the curve (AUC). (A) AUC of activated ERK1/2 by sampling at 1, 5, 15 and 16 min in a inter-trial interval of 15 min. Note that the last time point falls on the second training trial. (B) AUC of activated ERK1/2 by sampling at 1, 5, 15, 16, 25 and 31 min in a inter-trial interval of 30 min.