

Inability to activate Rac1-dependent forgetting contributes to behavioral inflexibility in mutants of multiple autism-risk genes

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The etiology of autism is so complicated because it involves the effects of variants of several hundred risk genes along with the contribution of environmental factors. Therefore, it has been challenging to identify the causal paths that lead to the core autistic symptoms such as social deficit, repetitive behaviors, and behavioral inflexibility. As an alternative approach, extensive efforts have been devoted to identifying the convergence of the targets and functions of the autism-risk genes to facilitate mapping out causal paths. In this study, we used a reversal-learning task to measure behavioral flexibility in *Drosophila* and determined the effects of loss-of-function mutations in multiple autism-risk gene homologs in flies. Mutations of five autism-risk genes with diversified molecular functions all led to a similar phenotype of behavioral inflexibility indicated by impaired reversal-learning. These reversal-learning defects resulted from the inability to forget or rather, specifically, to activate Rac1 (Ras-related C3 botulinum toxin substrate 1)-dependent forgetting. Thus, behavior-evoked activation of Rac1-dependent forgetting has a converging function for autism-risk genes.

autism | forgetting | Rac1 | behavioral flexibility | *Drosophila*

Autism spectrum disorders are common polygenic neurodevelopmental disorders diagnosed by a cluster of core clinical syndromes characterized by impaired social interaction, communication deficits, and behavioral inflexibility (1–3). A large number of autism-risk genes have been identified through different genetic approaches (4–8). These candidate genes play highly diversified roles in synaptic organization, transmission, intracellular signaling pathways, and protein expression regulation, among others (9). How do variants of these risk genes lead to the core symptoms of autism? Are there causal paths that can determine the onset of autism? Answering these questions is difficult because the disorder is multifactorial in nature with multiple genetic variants and environmental factors contributing to the core symptoms (10–13). To face this challenge, extensive efforts have been made to identify the targeting and functional convergence of the autism-risk genes, such as those involved in translation regulation (14–16) and long-range connectivity among different brain regions (17–19). These targets and functions provide a better biological basis for elucidating the causal paths between risk genes and the disorders of autism.

The aim of the this study was to determine whether Rac1 (Ras-related C3 botulinum toxin substrate 1)-dependent forgetting is a functional converging point for autism-risk genes. This idea was inspired by two clues. First, inhibition of Rac1 activity in *Drosophila* leads to the failure to activate Rac1-dependent forgetting, resulting in behavioral inflexibility as assayed through a reversal-learning task (20). Behavioral inflexibility has been observed frequently in the clinical studies of autism patients (21, 22), and children with autism are reported to have impaired reversal-learning, although they can learn new behavioral patterns (23–26). Thus, the inability to evoke Rac1-dependent forgetting causes behavioral inflexibility in transgenic flies, and the inability to forget may cause behavioral

inflexibility in autism patients. Second, a systems biology analysis concludes that Rac1 plays a critical role in the neuropathological events associated with autism based on gene-expression analysis of cerebellar samples from patients with autism (27). Thus, we were led to investigate whether Rac1-dependent forgetting is a converging function of homologs of autism-risk genes.

With a well-defined reversal-learning paradigm in fruit flies, we investigated the effects of five of the most promising or probable autism candidate genes: *Fmr1* (*Fragile X mental retardation 1*), *Ube3a* (*Ubiquitin-protein ligase E3A*), *Nrx-1* (*Neurexin-1*), *Nlg4* (*Neuroigin 4*), and *Tsc1* (*Tuberous sclerosis complex 1*) (28). *Fmr1* encodes an RNA-binding protein that regulates translation and has been extensively characterized in *Drosophila* for its role in synaptic signaling and long-term memory formation (29, 30). *Ube3a* encodes a ubiquitin ligase that has functions in learning and memory, synapse development, and synaptic plasticity (31–34). Importantly, *Drosophila Ube3a* (*dUbe3a*) is highly homologous to human *Ube3a* (*hUbe3a*) (34). *Nrx-1* and *Nlg4* are involved in synapse formation, maturation, and plasticity (35–37). *Tsc1* is a negative regulator in the mTORC1 pathway (38–42) which regulates cell growth and proliferation in flies (43). These five autism-risk genes play vital roles at the synapse to regulate cytoskeleton remodeling via interaction with Rac1 (44–48). Here we report that mutations of all these genes lead to reduced behavioral flexibility as shown by severe reversal-learning impairment, no matter whether learning is affected. This common behavioral phenotype results from the failure to activate Rac1-dependent forgetting.

Significance

Extensive efforts have been devoted to revealing the cognitive and molecular bases of autism spectrum disorders. In this work, by using a reversal-learning paradigm that measures behavioral flexibility in *Drosophila*, we show that flies with mutations of five autism susceptibility genes—*Fragile X mental retardation 1*, *Ubiquitin-protein ligase E3A*, *Neurexin-1*, *Neuroigin 4*, and *Tuberous sclerosis complex 1*—displayed severe reversal-learning defects which were caused by the inability to forget the previously formed memory by the activation of Rac1 (Ras-related C3 botulinum toxin substrate 1). These findings indicate that Rac1-dependent forgetting is a functional converging point for multiple autism-risk genes.

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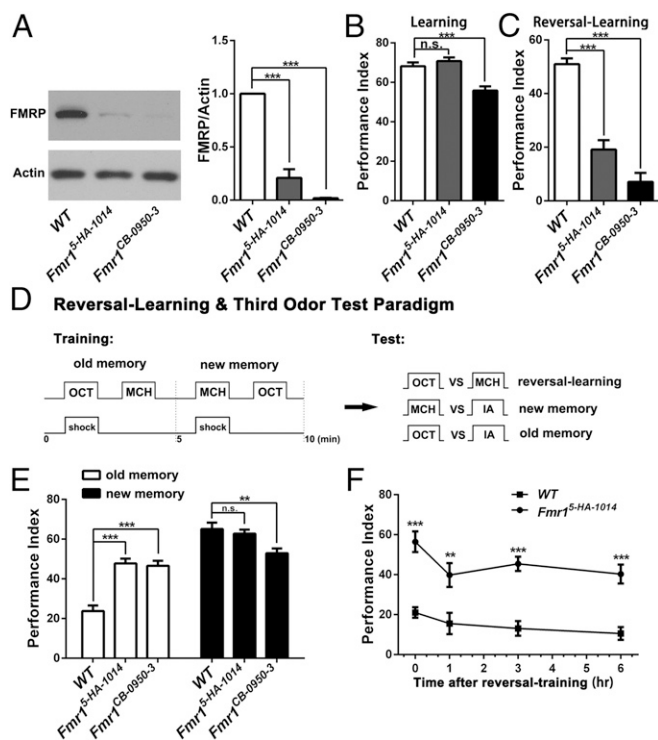


Fig. 1. The inability to forget underlies reduced reversal-learning in *Fmr1* mutant flies. (A) Western blots for different genotypes show significantly reduced FMRP levels in two mutants, *Fmr1^{5-HA-1014}* and *Fmr1^{CB-0950-3}*. $n = 3$. (B) The immediate memory scores for learning ability. Note normal learning in *Fmr1^{5-HA-1014}* flies and a slight defect in *Fmr1^{CB-0950-3}* flies. (C) Immediate memory scores for reversal-learning showing profound defects in both *Fmr1* mutants. (D) Schematic representation of behavioral paradigms for reversal-learning and the third-odor test. (E) Immediate memory scores obtained through the third-odor test after reversal-learning training. The open bars represent the old memory (OCT vs. IA), and the solid bars represent the new memory (MCH vs. IA). The new memory was very similar to the simple learning as shown in B. The old memories were significantly enhanced in both mutants. Numerically, $PI(\text{reversal-learning}) = PI(\text{new memory}) - PI(\text{old memory})$, suggesting that impaired reversal-learning resulted from the inability to erase or forget the old memory. (F) Memory decay curves for the old memory in *Fmr1^{5-HA-1014}* flies was sustained for at least 6 h. $n = 7-8$ for all behavioral data. All data in this and following figures, unless otherwise indicated, are means \pm SEM and were analyzed by ANOVA: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant, $P > 0.05$.

Results

Mutation of *Fmr1* Leads to Impaired Reversal-Learning Through an Inability to Activate Rac1-Dependent Forgetting. First, we screened all mutants of the five susceptibility genes intended for this work and identified *Fmr1* and *Ube3a* mutants with a phenotype that showed impaired reversal-learning without a learning defect. We began our analysis using *Fmr1* mutants and then extended the findings to flies with mutations in *Ube3a* and other three genes. In this study, two *Fmr1* mutant alleles (*Fmr1^{5-HA-1014}* and *Fmr1^{CB-0950-3}*) were used; flies with both mutations showed significantly reduced expression of FMRP (*Fmr1*-encoded protein) on Western blotting, with the protein being nearly undetectable in *Fmr1^{CB-0950-3}* flies (Fig. 1A). The aversive conditioning assay (Fig. 1D) (49) showed that the learning index was normal in flies with the *Fmr1^{5-HA-1014}* mutation (the milder allele) or slightly lower in flies with the *Fmr1^{CB-0950-3}* mutation (the stronger allele) (Fig. 1B). In contrast, both mutants showed severe reversal-learning defects (Fig. 1C). These results are consistent with a previous study of *Fmr1*-knockout mice, which displayed mildly impaired spatial learning ability alongside a significant defect in complex reversal-learning (50).

Because the reversal-learning paradigm used here consists of two opposite aversive conditionings, the reversal-learning performance index (PI) could be perceived as a combination or addition of two opposite memories, referred to as the “old memory,” which is associated with the first conditioning, and the “new memory,” which is associated with the second conditioning. Thus, the defect in reversal-learning could result from a decreased new memory (the inability to learn new behavioral patterns) or an increased old memory (the inability to forget the old memory). These two memories could be dissected through a third-odor test (Fig. 1D) (20). Our third-odor test assay using isopentyl acetate (IA) revealed that *Fmr1* mutants had an enhanced old memory and an unaltered new memory (Fig. 1E). Furthermore, this enhancement was surprisingly persistent (Fig. 1F). Thus, the defective reversal-learning in *Fmr1* mutants is the result of being unable to forget the old memory.

To determine whether forgetting is affected generally, we assayed time-based passive forgetting and interference-based forgetting in the *Fmr1^{5-HA-1014}* mutant with normal learning performance. Indeed, memory decay was slower in the *Fmr1^{5-HA-1014}* mutant (Fig. 2B). This slow-decay phenotype did not result from enhanced memory formation, because acquisition curves were completely normal under various training strengths (Fig. 2A). We also tested both *Fmr1* mutants with an interference-based paradigm, which is also capable of inducing forgetting (Fig. 2C) (20). Again, interference-induced forgetting was blocked in both mutants (Fig. 2D). Thus once the memory has been formed in *Fmr1* mutants, it is so solid that forgetting could not be induced by reversal-learning, time-based decay, or even interference.

Because the activation of the small G protein Rac1 is critical in regulating forgetting and reversal-learning as examined here (20), we compared the activation levels of Rac-GTP in WT and *Fmr1* mutant flies immediately after reversal-learning. In the naive state, total Rac and basal activated Rac were similar in both fly types (Fig. 3A). However, after reversal-learning, but not after 2 \times learning training (Fig. S1), Rac1 activity was significantly evoked in control flies, but no such increase occurred in the *Fmr1* mutant (Fig. 3A). Thus, the inability to activate Rac1 is responsible for the inflexibility in the reversal-learning paradigm in the *Fmr1^{5-HA-1014}* mutant.

If the failure to activate Rac1 contributes to impaired reversal-learning, one would expect to see better reversal-learning performance associated with more Rac1-GTP. To test this possibility, constitutively active Rac1(V12) and dominant-negative Rac1(N17) were expressed under the control of an inducible pan-neuronal driver, *elav* (*Embryonic lethal abnormal vision*) in the Gene-Switch (GS) system (*elav-GS*) (51). Rac1(V12) expression acutely induced by feeding adult flies with the drug RU486 was confirmed by Western blotting (Fig. S2). We showed that reversal-learning was rescued by acute overexpression of Rac1(V12) in the *Fmr1* mutant [*elav-GS/UAS-Drac1(V12);Fmr1^{5-HA-1014}*] (Fig. 3B). However, further inhibition of Rac1 activity through the expression of Rac1(N17) in the *Fmr1* mutant [*elav-GS/UAS-Drac1(N17);Fmr1^{5-HA-1014}*] had no further effect on reversal-learning. In summary, the failure to activate Rac1 contributes to the phenotype of being unable to forget in the *Fmr1* mutant, and that inability in turn leads to behavioral inflexibility.

Acute Down-Regulation of *Fmr1* Expression Leads to Behavioral Inflexibility. One argument for the above phenotypes is that *Fmr1* mutants have a developmental defect, because the *Fmr1* gene is known to play a vital role in regulating neuronal elaboration and the differentiation of dendritic spines (52, 53). To rule out this possibility, we used heat-shock-induced acute expression of *Fmr1-RNAi* to down-regulate FMRP. Western blotting confirmed that heat-shock induction of *UAS-Fmr1-RNAi* was effective, because FMRP was significantly reduced in *hs-Gal4/UAS-Fmr1-RNAi²⁷⁴⁸⁴* transgenic flies 3 h after the heat-shock treatment (Fig. 4A). Consistent with the phenotypes of *Fmr1* mutants, these RNAi flies displayed a reversal-learning defect 3 h after heat shock (Fig. 4B), and this reversal-learning defect also resulted from the inability to forget the old memory (Fig. 4C). In other words, within a short time window of less than 3 h, reduced *Fmr1* expression could result in a defect in

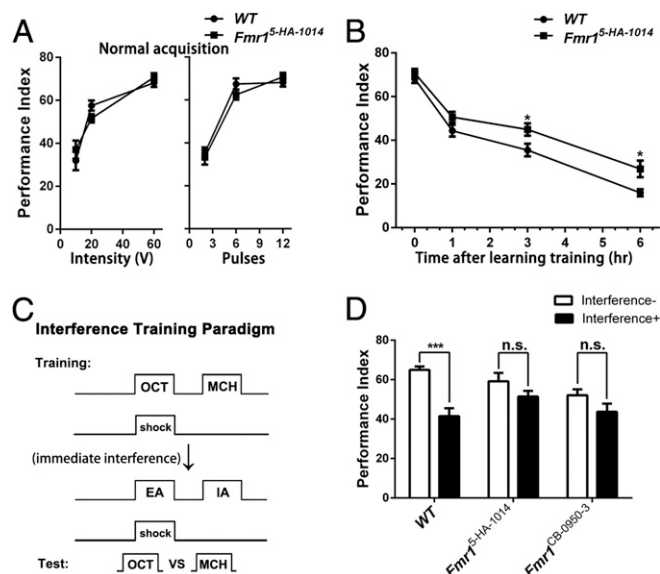


Fig. 2. Slower passive memory decay and weaker interference-induced forgetting in *Fmr1* mutant flies. (A) Normal immediate memory acquisition in response to different training strengths with varied electric shock intensity and number of electric shock pulses in the *Fmr1^{5-HA-1014}* mutant. (B) Passive memory decay curves. Passive decay was slower in the *Fmr1^{5-HA-1014}* mutant. (C) Schematic representation of the interference paradigm. Flies were trained to learn the first odor pairing (OCT vs. MCH), followed immediately by a different odor pairing (EA vs. IA), which represented interference with the first training. The PI was measured immediately after the training procedure. (D) The earlier formed memory was seriously disrupted by a new memory in WT flies but not in the two *Fmr1* mutants ($n = 6-8$ for all behavioral data presented).

forgetting, although the phenotype is milder than that in the mutants. Thus, mutations of the *Fmr1* gene can make an acute physiological contribution to the regulation of forgetting.

Disruption of *Ube3a* Impairs Rac1-Dependent Forgetting. The *Ube3a* mutant flies exhibited normal learning ability (Fig. 5A) but significantly impaired reversal-learning (Fig. 5B). The third-odor test revealed that the reversal-learning phenotype in these flies also resulted from the inability to forget old memory although the ability to learn new behavior patterns was normal (Fig. 5C). Also, spatiotemporal down-regulation of *Ube3a* expression by *elav-GS* induced phenotypes identical to those in the *Ube3a^{EP3214}* mutant (Fig. 5D-F). Consistently, the inability to forget was associated with the failure in reversal-learning-induced activation of Rac1 (Fig. 5G). Thus, the *Ube3a* mutant also showed behavioral inflexibility that resulted from the failure in the activation of Rac1-dependent forgetting.

Impairment of Rac1-Dependent Active Forgetting in Three Other Autism-Susceptibility Gene Mutants. The above findings made us highly interested in knowing whether the observed behavioral inflexibility, resulting from the inability to activate Rac1-dependent forgetting, is a general phenotype for autism-risk genes. We chose three additional autism-risk genes that are functionally unrelated to *Fmr1* and *Ube3a*: *Nrx-1*, *Nlg4*, and *Tsc1*. All mutants used in this study were well characterized (31, 54-56) and were confirmed through PCR and DNA sequencing (Fig. S3).

To our surprise, all three mutants had severe reversal-learning impairments (Fig. 6B) with mild learning defects (Fig. 6A). Using the third-odor test, we found that the *Nlg4* and *Tsc1* mutant flies displayed significantly enhanced old memory; the *Nrx-1* mutant flies also showed an enhanced old memory, but the difference between the *Nrx-1* mutants and WT flies was not statistically significant (Fig. 6C). Also, spatiotemporal down-regulation of *Nrx-1* and *Tsc1* expression by *elav-GS* induced normal learning (Fig. 6D) but reduced

reversal-learning (Fig. 6E) and unforgettable old memory (Fig. 6F). Furthermore, Rac1 could not be activated through reversal training in any of the mutants (Fig. 6G). Thus, the behavioral inflexibility caused by impaired Rac1-dependent forgetting appears to be a common phenotype for autism-risk genes.

***Fmr1*, *Ube3a*, *Nrx-1*, and *Tsc1* Function in the Mushroom Body to Regulate Forgetting.**

Considering the vital roles of the mushroom body in learning, memory, and forgetting (20, 57, 58), we asked whether the autism-risk genes we studied were involved in regulating forgetting in the mushroom body. After acute knockdown of *Fmr1*, *Ube3a*, *Nrx-1*, and *Tsc1* expression in the mushroom body (59), normal learning ability, significant reversal-learning defects, and unforgettable old memory were observed (Fig. 7). The involvement of the mushroom body in regulating forgetting was confirmed further by knockdown of *Fmr1* with the *OK107* driver (Fig. S4). Thus, the autism-susceptibility genes *Fmr1*, *Ube3a*, *Nrx-1*, and *Tsc1* are critical for regulating forgetting in *Drosophila* mushroom body.

Discussion

This study investigates whether forgetting-dependent behavioral inflexibility is a common phenotype for autism-risk genes and whether behavior-dependent activation of Rac1 is the convergent molecular target for these genes. To minimize genetic and

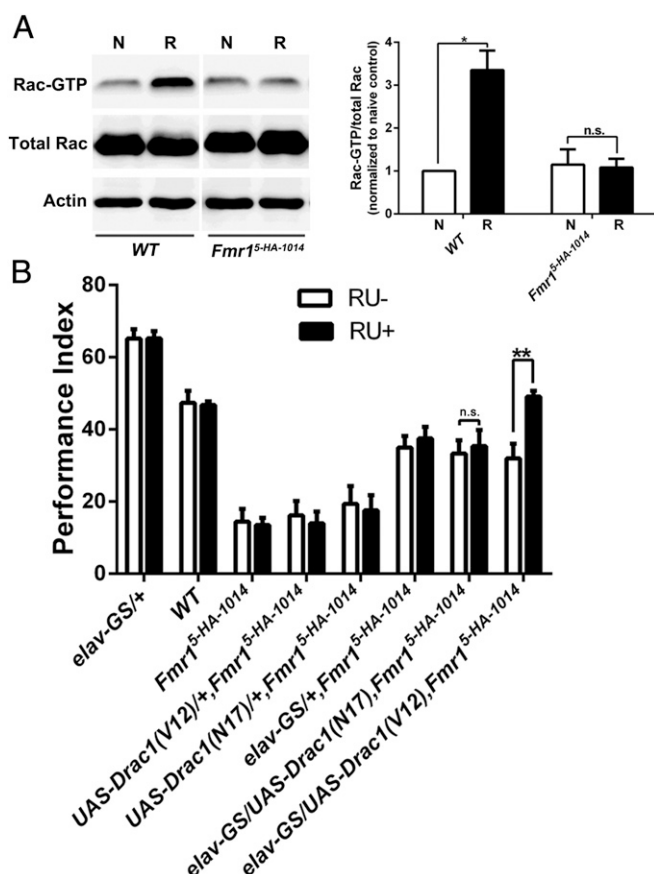


Fig. 3. The inability to activate Rac1 in the *Fmr1^{5-HA-1014}* mutant. (A) Western blots showing Rac1 activation in heads of naive (N) and reversal-learning (R)-trained flies. Reversal-learning activates Rac1 immediately in WT flies but not in the *Fmr1^{5-HA-1014}* mutant. $n = 3$. (B) Rescue of the reversal-learning defect by acute overexpression of constitutively active Rac1(V12). The pan-neuronal Gene-Switch (GS) expression system was used to manipulate the expression of mutant Rac1(V12) acutely. In the RU486 feeding group (RU+), the reversal-learning defect of *elav-GS/UAS-Drac1(V12)*, *Fmr1^{5-HA-1014}* flies was partially rescued by acutely induced overexpression of Rac1(V12) ($n = 6-8$).

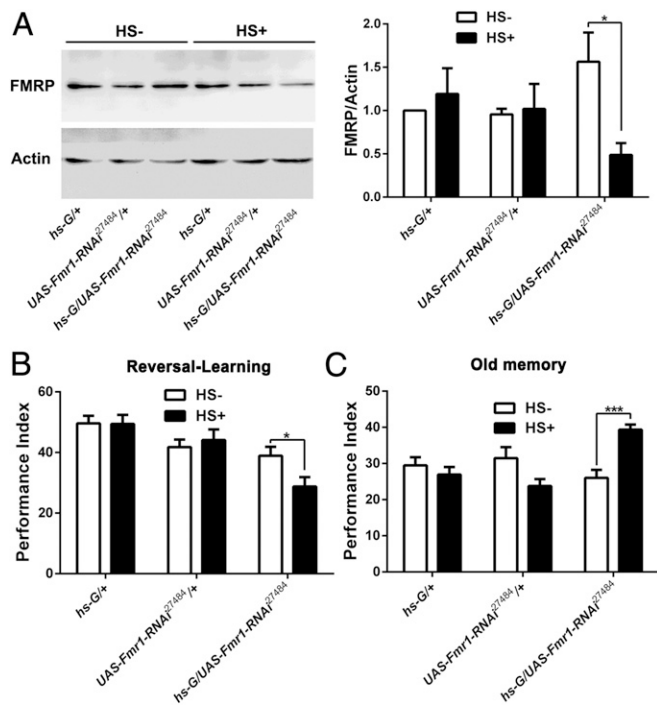


Fig. 4. Acute knockdown of *Fmr1* expression leads to diminished forgetting. (A) Western blots and statistical analysis confirm that the level of FMRP is reduced by acutely heat-shock-induced expression of *Fmr1-RNAi*. $n = 3$. (B) Reduced reversal-learning in transgenic flies with down-regulation of FMRP expression. (C) Enhanced old memory in transgenic flies subjected to reversal-learning training. Acute down-regulation of FMRP expression led to enhanced old memory in the third-odor test after reversal-learning training ($n = 6-8$ for all behavioral data presented).

behavioral variations, flies for all genotypes had the same isogenic background, and behavioral assays were performed using the well-established balanced protocol (*Methods*). We used aversive conditioning and its associated reversal-learning task to investigate the functions of five autism-susceptibility genes: *Fmr1*, *Ube3a*, *Nrx-1*, *Nlg4*, and *Tsc1*. Four major findings emerge from the current study. First, mutation and RNAi-induced knockdown of these five autism-risk genes result in strong reversal-learning defects independent of learning ability. Second, these reversal-learning phenotypes are all caused by the inability to forget the old memory. Third, all the autism-risk gene mutants studied failed to trigger the behavior-dependent activation of Rac1, a reported regulator of forgetting. Fourth, the effects of these genes are confined within the mushroom body, in which Rac1-dependent forgetting is involved. These results suggest that the inability to activate Rac1-dependent forgetting is a converging mechanism for multiple autism-risk genes.

It is interesting that all five of the autism-risk genes with diverse functions funnel to a deficit in Rac1 activation. These five autism-risk genes have been reported to be involved in the Rac1 signaling pathway. The cytoplasmic FMRP-interacting protein (CYFIP) directly links Rac1 and FMRP to modulate cytoskeleton remodeling (45); *Tsc1* functionally regulates Rac1 activity (46, 60); *Ube3a* promotes Rho-GEF Pbl degradation via ubiquitination to affect Rac1 activation (47); and upon synaptic activation Rho-GEF Kal-7 disassembles from the *Nrx-1/Nlg4/DISC1* complex to modulate the Rac1 pathway (48). Several other autism-risk genes, such as *Nlg1*, *Nrx-4*, *P-Rex1*, and *Shank-3*, have also been reported to participate in the Rac1-signaling pathway (61–64). In addition, when the gene–environment interactions of 122 genes and 191 factors in the autistic context were analyzed by systems biology, Rac1 was predicted to be a converging node that genetically links to the neurobiology of autism

(27, 65). Taken together, these findings indicate that Rac1 is a functional converging site for autism-risk genes.

Although autism is considered to be a developmental disorder, emerging evidence points to the postdevelopmental effects of autism-risk genes in adults (66–68). In this study, acute down-regulation of these five autism-risk genes at the adult stage led to impaired behavioral flexibility with reduced reversal-learning and resistant old memory. Thus, all five autism-risk genes in our study are physiologically involved in regulating behavioral flexibility.

In addition to behavioral flexibility, the autism genes in our study participate in some other cognitive processes such as learning and long-term memory (29, 33, 69, 70). For instance, as a general translational repressor, *Fmr1* interacts with Staufen and the RNAi pathway to regulate long-term memory formation. However, Rac1-dependent forgetting has been reported to be independent of protein synthesis and long-term memory (20). These findings indicate that *Fmr1* plays a dual role in regulating long-term memory and forgetting. Taken together, the data presented here indicate that the inability to forget is a cognitive mechanism in autism.

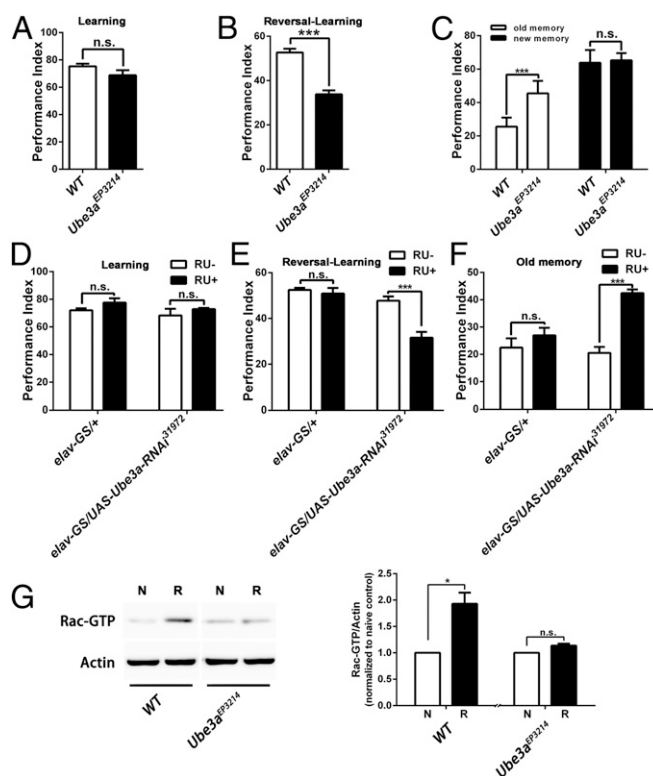


Fig. 5. Disrupted expression of *Ube3a* produces the inability to forget. (A) Immediate memory scores for learning ability show normal learning in the *Ube3a*^{EP3214} mutant. (B) Immediate memory scores for reversal-learning show a profound defect in the *Ube3a*^{EP3214} mutant. (C) Immediate memory scores obtained through the third-odor test after reversal-learning training. The new memory was normal in the *Ube3a*^{EP3214} mutant, whereas the old memory was significantly enhanced in mutant flies. (D) Normal learning ability in transgenic flies with acute down-regulation of *Ube3a* by the *elav-GS* system. (E) Reduced reversal-learning in transgenic flies with down-regulation of *Ube3a* by the *elav-GS* system. (F) Enhanced old memory in transgenic flies subjected to reversal-learning training. Acute down-regulation of *Ube3a* expression led to enhanced old memory in the third-odor test after reversal-learning training. $n = 6-8$ for all behavioral data. (G) The inability to activate Rac1 in the *Ube3a*^{EP3214} mutant. Rac-GTP was significantly activated in WT flies, whereas there was little activation in the *Ube3a*^{EP3214} mutant ($n = 3$).

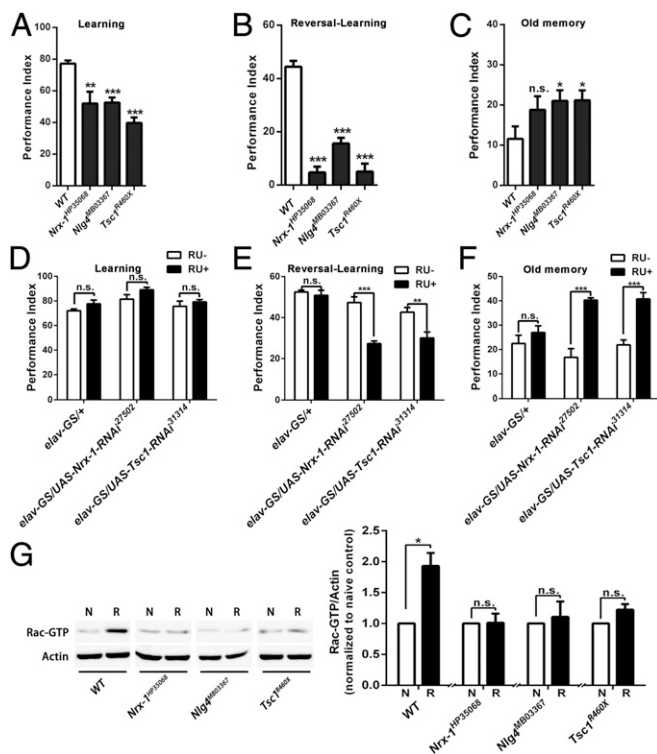


Fig. 6. Impairment of Rac1-dependent forgetting results in behavioral inflexibility in mutants of autism candidate genes. (A) Flies with mutations of the autism candidate genes *Nrx-1*, *Nlg4*, and *Tsc1* displayed mild learning defects. (B) Flies with mutations of these autism candidate genes displayed severe reversal-learning deficiencies. (C) The old memories were significantly enhanced in the *Nlg4^{MB03367}* and *Tsc1^{R460X}* mutant flies. (D) Transgenic flies with acute down-regulation of *Nrx-1* and *Tsc1* by the *elav-GS* system had normal learning ability. (E) Reversal-learning was reduced in transgenic flies with down-regulation of *Nrx-1* and *Tsc1* by the *elav-GS* system. (F) Old memory was enhanced in transgenic flies with down-regulation of *Nrx-1* and *Tsc1* by the *elav-GS* system. $n = 6-8$ for all behavioral data. (G) The inability to activate Rac1 after reversal-learning in flies with mutations in the autism candidate genes. N, naive; R, reversal-learning training ($n = 3$).

Materials and Methods

Drosophila Stock. Flies were raised at the room temperature of 23 °C, with 70% relative humidity. The following strains were obtained from the Bloomington *Drosophila* Stock Center: *Fmr1^{CB-0950-3}* (no. 123391), *Fmr1^{5-HA-1014}* (no. 125009), *Ube3a^{EP3214}* (no. 17099), *Nrx-1^{HP35068}* (no. 21977), *Nlg4^{MB03367}* (no. 23608), *UAS-Fmr1-RNAi* (no. 27484), *UAS-Drac1(V12)* (no. 6291), *UAS-Drac1(N17)* (no. 6292), *UAS-Ube3a-RNAi* (no. 31972), *UAS-Nrx1-RNAi* (no. 27502), and *UAS-Tsc1-RNAi* (no. 31314). *OK107* and *hs-GAL4* are laboratory stocks. *elav-GS* and mushroom body (*MB*)-*GS* were kindly provided by Ronald L. Davis, The Scripps Research Institute, Jupiter, FL. *UAS-Fmr1-RNAi* (no. 34933) was kindly provided by Jianquan Ni, Tsinghua Fly Center, Beijing. *Tsc1^{R460X}* was kindly provided by Rongwen Xi, National Institute of Biological Sciences, Beijing. All flies with autism-susceptibility gene mutations were backcrossed with the *w¹¹¹⁸* flies (stock in our laboratory) for five generations.

Heat-Shock Regimen. When the *hs-GAL4* and *UAS-Fmr1-RNAi* strains were crossed, flies were raised at 23 °C. Flies for our studies were collected 2 to 4 d after eclosion. Before the behavioral and Western-blotting experiments, the heat-induced group was transferred to a 37 °C incubator for 40 min and then was placed at 23 °C for 3 h to induce the expression of RNAi. The uninduced group was kept at 23 °C.

Drug Feeding. The procedures were described previously (71). For RU486 feeding (RU⁺), flies were fed with 500 μ M RU486 (mifepristone; J&K Chemicals) dissolved in control solution.

Pavlovian Olfactory Associative Aversive Learning. During one training session, about 100 flies were loaded in a training tube and then were sequentially exposed to two odors, 3-octanol (OCT) (1.5×10^{-3} dilution in heavy mineral oil; Fluka) and

4-methylcyclohexanol (MCH) (1.0×10^{-3} dilution in heavy mineral oil; Fluka), for 60 s each, with 45 s of fresh air between the two odor presentations. Foot shock (60 V, 12 1.5-s pulses) was presented during the presentation of the first odor [positive conditioned stimulus (CS⁺), but not during the second [negative conditioned stimulus (CS⁻)]. To test the learning (immediate memory) index, the flies were transferred to a T-maze and allowed to choose between the CS⁺ and the CS⁻ arms for 60 s. The PI was calculated from the distribution of the number of flies in the two arms of the T-maze, according to the equation $(CS^- - CS^+) / (CS^- + CS^+) \times 100$. To eliminate any odor-preference bias, two reciprocal groups of flies were trained using OCT and MCH as the CS⁺ respectively. Each PI ($n = 1$) is the average of PI (OCT) and PI (MCH). A PI of 0 suggests no learning, whereas a PI of 100 suggests perfect learning.

Reversal-Learning and Third-Odor Test. In the reversal-learning paradigm, following a regular learning session, flies were retrained immediately by another session in which the conditioned stimulus–unconditioned stimulus contingency was reversed. In the third-odor test, after reversal-learning training (note that OCT was paired with shock in the initial session), flies were required to choose between OCT and a previously unexposed odor, IA (7.0×10^{-3} dilution; Avocado Research Chemicals) to measure their old memory and to choose between MCH and IA (1.0×10^{-3} dilution) to measure their new memory. In the end, reciprocal groups were averaged to get $n = 1$. Before the behavioral test, untrained flies were used to balance the odor preference, and the score of the untrained group was subtracted from that of the trained group to yield a final PI.

Interference Learning. After a regular learning session (OCT/MCH), trained flies immediately received another learning session with a novel pair of odors: ethyl acetate (EA) (2.0×10^{-3} dilution; Alfa Aesar) and IA (2.0×10^{-3} dilution; Avocado Research Chemicals) as CS⁺ and CS⁻ (referred to as “interference+”), respectively. The control group (“interference–”) received only the initial learning session. After the interference training session, flies were required to choose between OCT and MCH to measure their previous memory.

Rac1 Activity Assay and Western Blotting. The heads of approximately 400 flies were isolated to detect the relative levels of Rac1-GTP by PBD pull-down assay (no. 17-441; Millipore) according to the manufacturer’s procedure. The anti-human Rac1 monoclonal antibody (1:3,000 dilution; BD Transduction Laboratories) was used to detect total and activated Rac1 levels. For the detection of FMRP levels, the heads of 50 flies were homogenized in 100 μ l of radioimmunoprecipitation assay lysis buffer (no. P0013; Beyotime). The anti-FMRP antibody (no. 05-1512; Millipore) was diluted 1:3,000.

Statistics. The statistical results are shown as means \pm SEM and were analyzed by ANOVA [$*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s., nonsignificant ($P > 0.05$)].

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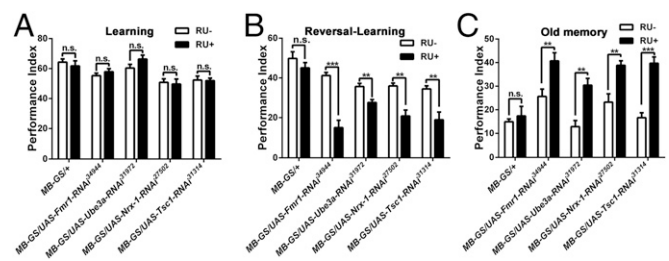


Fig. 7. Forgetting cannot be produced by acute down-regulation of *Fmr1*, *Ube3a*, *Nrx-1*, and *Tsc1* expression in the mushroom body. (A) Transgenic flies with acute down-regulation of *Fmr1*, *Ube3a*, *Nrx-1*, and *Tsc1* in the mushroom body by the *MB-GS* system have normal learning ability. (B) Transgenic flies with acute down-regulation of *Fmr1*, *Ube3a*, *Nrx-1*, and *Tsc1* in the mushroom body have reduced reversal-learning ability. (C) Old memory is enhanced in transgenic flies with acute down-regulation of *Fmr1*, *Ube3a*, *Nrx-1*, and *Tsc1* in mushroom body neurons ($n = 6$ for all behavioral data presented).

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Supporting Information

Dong et al. 10.1073/pnas.1602152113

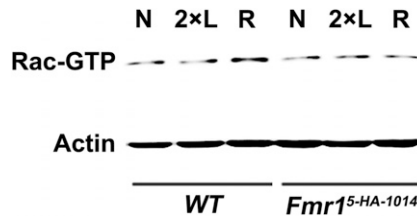


Fig. S1. The inability to activate Rac after 2xlearning training in WT and *Fmr1*^{5-HA-1014} flies. Western blots show an increase in Rac activity by reversal-learning (R) rather than 2xlearning (2xL) in WT flies. However, in *Fmr1*^{5-HA-1014} flies, the Rac activity displays no alteration after either 2xlearning or reversal-learning training.

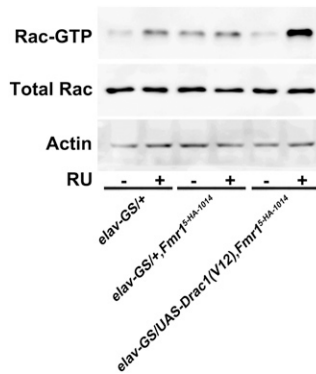


Fig. S2. Acute overexpression of constitutively active Rac1(V12) by the GS system. Western blots show induced Rac1 activity in *elav-GS/UAS-Drac1(V12)*, *Fmr1*^{5-HA-1014} flies after RU486 feeding.

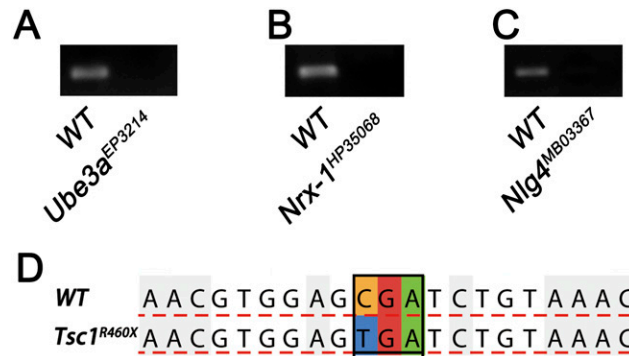


Fig. S3. Verification of autism-susceptibility gene mutations. (A–C) PCR primers were designed for amplification near the upstream and downstream of the P-element insertion point for *Ube3a*^{EP3214} (A) *Nrx-1*^{HP35068} (B), and *Nlg4*^{MB03367} (C) mutant flies. Primers for *Ube3a*^{EP3214} are: sense (S): GCCACCGTTCATCTTCGC, antisense (A): ATCCCTTGGAGTTTCAATCA; primers for *Nrx-1*^{HP35068} are S: CCAAACCCATAGCACAA, A: ATACGCACTCGGACAAGG; and primers for *Nlg4*^{MB03367} are S: TCAACAGAACGGTTCC, A: TGTGGTGTCTTGTAGCG. (D) DNA sequencing for testing the mutation in *Tsc1*^{R460X} from 460 CGA (Arg) to TGA (stop). Primers for *Tsc1*^{R460X} sequencing are S: AACGAGATTGCCACGACC, A: CTGTAGCTTGCCATACGATT.

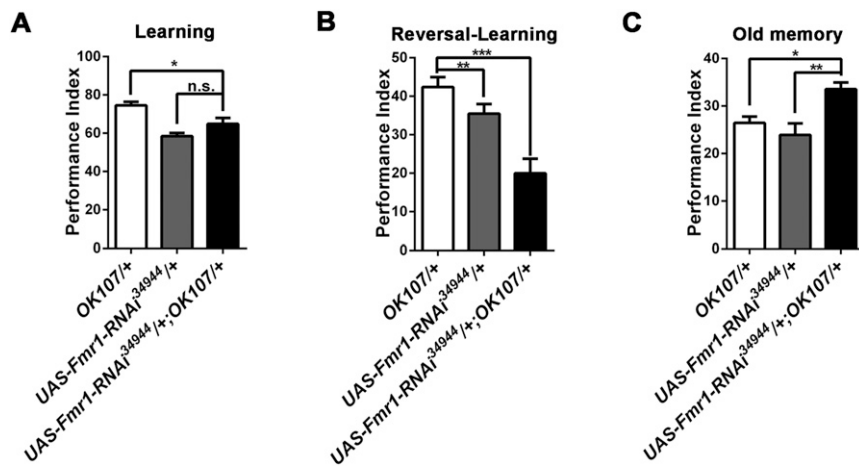


Fig. S4. Down-regulation of *Fmr1* expression in the mushroom body leads to diminished forgetting. *UAS-Fmr1-RNAi³⁴⁹⁴⁴* flies were crossed with WT flies (+) and the MB Gal4, *OK107*. (A) No effect on learning was found when *Fmr1* was down-regulated in the mushroom body. (B) Reversal-learning was reduced in *UAS-Fmr1-RNAi³⁴⁹⁴⁴/+; OK107/+* flies with down-regulation of FMRP in the mushroom body. (C) Down-regulation of FMRP expression led to enhanced old memory in transgenic flies subjected to reversal-learning training.