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Archival Report

SAR405, a Highly Specific VPS34 Inhibitor, Disrupts Auditory Fear Memory Consolidation of Mice via Facilitation of Inhibitory Neurotransmission in Basolateral Amygdala

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ABSTRACT

BACKGROUND: Autophagy has been demonstrated to play an important role in memory deficits as well as the degradation of neurotransmitter receptors. SAR405 is a newly discovered inhibitor that can specifically inhibit vacuolar sorting protein 34 and prevent autophagosome biogenesis. However, the effects of SAR405 on memory processes remain largely unknown.

METHODS: Western blotting, immunofluorescence, and transmission electron microscopy were used to assess the level of autophagy after fear conditioning and SAR405 treatment. Behavioral tests, biotinylation assay, electrophysiology, and co-immunoprecipitation were used to unravel the mechanisms of SAR405 in memory consolidation.

RESULTS: SAR405 infusion into the basolateral amygdala impaired long-term memory through autophagy inhibition. Furthermore, the trafficking of gamma-aminobutyric acid type A receptors ($GABA_AR$s) following fear conditioning was disrupted by SAR405, and the decreased frequency and amplitude of miniature inhibitory postsynaptic currents induced by fear conditioning were also reversed by SAR405, suggesting that SAR405 disrupted memory consolidation through blockade of the downregulated inhibitory neurotransmission in basolateral amygdala. $GABA_AR$-associated protein (GABARAP) and its interaction with $GABA_AR$ $\gamma 2$ subunit were found to be upregulated after fear conditioning, and SAR405 could suppress this increased interaction. Moreover, disruption of the GABARAP-$GABA_AR$ binding by a trans-activating transcriptional activator–GABARAP inhibitory peptide blocked the decrease in surface expression of $GABA_AR$s and attenuated long-term memory.

CONCLUSIONS: The present study suggests that SAR405 can prevent the memory consolidation via intervening autophagy and $GABA_AR$ trafficking and has a potential therapeutic value for disorders characterized by exaggerated fear memories, such as posttraumatic stress disorder.

Keywords: Amygdala, Fear conditioning, $GABA_A$ receptor, GABARAP, Memory consolidation, SAR405

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Macroautophagy (hereafter referred to as autophagy) is an essential process for the maintenance of cellular homeostasis by degrading diverse metabolic products (1). It has been demonstrated that autophagy plays an important role in neurodegenerative diseases and relative memory deficits, especially in Alzheimer's disease (2,3). Autophagy was also reported to be involved in the impairment of learning and memory induced by aging, morphine, hypoxic-ischemic brain injury, and manganese (4–7). In addition, a few studies have found that autophagy is involved in the degradation of neurotransmitter receptors. In a model of chemical long-term depression, low-dose *N*-methyl-D-aspartate (NMDA)–induced autophagy functioned in alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor degradation, suggesting that autophagy plays a role in NMDA receptor–dependent synaptic remodeling (8). Also, Rowland *et al.* (9) reported that the gamma-aminobutyric acid type A receptors ($GABA_AR$s) trafficked to autophagosomes after endocytic removal from the cell surface, indicating that autophagy can degrade cell-surface $GABA_AR$s.

Vacuolar sorting protein 34 (VPS34), also known as class III phosphatidylinositol-3, can specifically phosphorylate the 3-position of phosphatidylinositol to produce phosphatidylinositol-3-phosphate, which is involved in autophagosomes biogenesis (10). In mammals, beclin 1-VPS34 complexes are essential for vesicle trafficking and autophagosomes formation (11). SAR405, a selective inhibitor of VPS34, has been recently developed by a high-throughput screening that intended to identify novel autophagy inhibitors. It can inhibit vesicle trafficking from late endosomes to lysosomes and can prevent

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autophagy induced by multiple stimulus (12). Recent studies have proved SAR405 to be an effective autophagy inhibitor in several cell types (13,14). Therefore, SAR405 is a unique and specific kinase inhibitor for clarifying the potential role of autophagy under pathophysiological condition.

Auditory fear conditioning presents a framework that is perfectly suited for studying the mechanisms of learning and memory formation (15), and it is also an animal model for some anxiety disorders such as posttraumatic stress disorder (16). A substantial body of studies implicated that the GABAergic activity in amygdala is critical in controlling the acquisition and consolidation of conditioned fear memory (17–19). Systemic or amygdala treatment of muscimol, a classic agonist of the $GABA_A$Rs, impaired the consolidation processes; consistently, administration of $GABA_A$R antagonist bicuculline improved the consolidation processes (20).

$GABA_A$R-associated protein (GABARAP), the first isolated $GABA_A$R interacting protein, was found to recognize and bind the γ2 subunit of $GABA_A$R and was proposed to play a key role in $GABA_A$R trafficking and postsynaptic localization (21–23). Meanwhile, GABARAP is also a member of the autophagy-related protein (ATG) 8 family that is essential for the autophagic process and promotes the autophagosome biogenesis at a later stage (24). However, whether GABARAP plays a role in the autophagic degradation of $GABA_A$Rs remains unknown.

Autophagy has been proposed as a new player in the memory process and synaptic plasticity; however, the impact of autophagic degradation of neurotransmitter receptors and related changes in synaptic plasticity in learning and memory is poorly understood. Because SAR405 is a specific autophagy inhibitor and may be an ideal tool drug to investigate the role of autophagy in memory, we thus employed it to explore the mechanism of autophagy in memory process using auditory fear conditioning as a model of learning and memory.

METHODS AND MATERIALS

Animals

Male C57BL/6 mice used in the present study were obtained from Hunan Silaike Jingda Laboratory Animal Corporation Ltd (Changsha, China). They were maintained on a 12-hour light/dark cycle at a constant temperature (22 ± 2°C) and humidity of 50 ± 10% with food and water available ad libitum. Five mice were housed in a cage, and mice 8 to 11 weeks of age were used in the experiment. All experimental procedures were approved by the Animal Welfare Committee of Huazhong University of Science and Technology.

Auditory Fear Conditioning

According to a previous study with some modifications (25), auditory fear conditioning was carried out in two contexts: context A and context B. We describe it in detail in the Supplement.

Western Blotting

The procedures were processed according to previous protocol with minor modifications (26,27) and are described in the Supplement.

Monitoring Autophagy by Lentiviral-Green Fluorescent Protein-Light Chain 3 and Transmission Electron Microscopy

Immunohistochemical and transmission electron microscopy analyses of autophagy are described in the Supplement.

Intra-amygdala Microinjections

Intra-amygdala microinjections of pharmacological inhibitors and virus are described in the Supplement.

Biotinylation Assay of Surface Receptor

Biotinylation assays of surface expression of $GABA_A$Rs are described in the Supplement.

Electrophysiological Recordings In Vitro

The miniature inhibitory postsynaptic currents (mIPSCs) were recorded in the whole-cell patch-clamp technique. Mice were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneal) and perfused with ice-cold cutting solution (209-mM sucrose, 22-mM glucose, 3.1-mM sodium pyruvate; 12-mM sodium L-ascorbate, 1.25-mM NaH_2PO_4, 4.9-mM $MgSO_4$-$7H_2O$, and 26-mM $NaHCO_3$; oxygenated with 95% O_2 and 5% CO_2, pH 7.2–7.4). Slices (300-μm thick) containing the basolateral amygdala (BLA) region were cut by a microslicer (Leica VT1000 S; Leica Biosystems, Nussloch, Germany) and incubated in artificial cerebrospinal fluid for at least 1 hour at 27°C. Patch pipettes (3–5 MΩ) for whole-cell voltage-clamp recordings were filled with internal solution (153.3-mM CsCl, 1-mM $MgCl_2$-$6H_2O$, 4-mM Mg-ATP, 10-mM HEPES, and 5-mM EGTA; pH 7.2, 290 mOsm). mIPSCs were recorded in voltage-clamp mode (Vh: −65 mV) and in the presence of tetrodotoxin (10 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM), and D-2-amino-5-phosphonovalerate (50 μM). All data were acquired from a Multiclamp 700B amplifier and pCLAMP 10 software (Axon Instruments, Molecular Devices, San Jose, CA). Records were low-pass filtered at 2 to 20 kHz and digitized at 5 to 50 kHz.

Co-immunoprecipitation

Tissue from BLA was homogenized with ice-cold radioimmunoprecipitation assay buffer. Protein extracts (500 μg) were then incubated with 2 μg of $GABA_A$R γ2 subunit antibodies (sc-131935; Santa Cruz Biotechnology, Dallas, TX) or 2 μg of immunoglobulin overnight at 4°C with constant shaking. The antibody-bound complexes were incubated with Protein A/G agarose (sc-2003; Santa Cruz Biotechnology) at 4°C for 3 hours. The protein-bound beads were washed in radioimmunoprecipitation assay buffer three times. The beads were resuspended in 2× sodium dodecyl sulfate loading buffer, and the immune complexes were eluted by boiling at 95°C for 5 minutes. Total and immunoprecipitated proteins were separated by electrophoresis on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels. The blots were probed with antibodies to GABARAP (1:1000, NBP1-95588; Novus Biologicals, Littleton, CO).

Trans-activating Transcriptional Activator–GABARAP Peptide

Trans-activating transcriptional activator (TAT)-GABARAP peptide is described in the Supplement.

Statistical Analysis

Animals were randomly assigned to experimental groups. Data were analyzed with the statistical program SPSS, version 18.0 (IBM Corp, Armonk, NY) and no data points were excluded. The results from fear conditioning training and memory test were statistically evaluated using repeated-measures analysis of variance with Bonferroni's post hoc test. Between-group comparisons of data were made using either a two-sided unpaired *t* test or analysis of variance with subsequent post hoc test. Statistical tests are shown in each figure's legend. Differences between experimental conditions were considered statistically significant when $p < .05$. Data are presented as mean ± SEM.

RESULTS

Infusion of SAR405 Into BLA Impairs Long-Term Memory and Inhibits Autophagy Induced by Auditory Fear Conditioning

First, we explored whether SAR405 had an effect on auditory fear conditioning. Mice implanted with a bilateral guide cannula aimed at the BLA were administered with SAR405 (1 μM) after recovering from surgery, as shown in Figure 1A. These mice were subjected to auditory fear conditioning 24 hours after administration. Fear memory was evaluated by measuring the percentage of freezing behavior at 3 hours (short-term memory [STM]) and 24 hours (long-term memory [LTM]) after training to investigate whether memory acquisition or consolidation were affected (28,29). The results showed that local delivery of SAR405 to the BLA impaired freezing behaviors at 24 hours, with no difference was observed at 3 hours (Figure 1B) ($F_{2,27}$ = 5.090, p = .001). In addition, we found that SAR405 administation had no effect on memory retrival test (Supplemental Figure S1). Memory consolidation is the process that converts STM into a persistent LTM. It has been demonstrated that when a drug was applied before or after fear training, the drug caused the impairment of LTM, but without effect on STM, indicating that the drug could disrupt memory consolidation (18,28,29). Thus, our results suggest that SAR405 pretreatment specifically attenuates fear memory consolidation while leaving memory acquisition unaffected.

Because memory consolidation is a time-dependent process occurring within a few hours following acquisition (29), we monitored the autophagy status after fear conditioning by detecting the time course of the light chain 3 (LC3) levels in the BLA. LC3 is a member of mammalian ATG8 homologs that is involved in phagophore formation. The nonlipidated and lipidated forms are usually referred to as LC3-I and LC3-II, respectively, and LC3-II is the only protein marker that is reliably associated with completed autophagosomes and also localized to phagophore (30). We found that the levels of LC3-II were increased significantly at 1 hour after training when compared with control mice, which were exposed to the box without foot shock, and recovered to the baseline at 6 hours after acquisition (Figure 1C) ($F_{5,35}$ = 2.698, p = .037). To verify the LC3-II increase detected by Western blotting, lentiviral-green fluorescent protein-LC3 (LV-GFP-LC3) and transmission electron microscopy were used to further validate the increased autophagy. The GFP-LC3 puncta of cells in the BLA were significantly increased at 1 hour after training (Figure 1D) (t_{169} = 8.029, $p < .001$), and an accumulation of autophagic vacuoles, including double-membrane autophagosomes as well as autophagic vacuoles at various stages of degradation, were observed in the neurons and synapses in amygdala at 1 hour following fear conditioning, while few autophagic structures were found in control group (Figure 1E) (t_{26} = 3.588, p = .001). p62 is one of the autophagic substrates that is degraded in autolysosomes, and accelerated degradation or decreased levels of p62 protein thus served as an indicator of increased autophagy (30). Consistent with the changes in LC3, p62 levels were time-dependently decreased and reached the lowest level at 1.5 hours after fear conditioning (Supplemental Figure S2). The degradation of autophagosomes was further monitored with a tandem GFP-monomeric red fluorescent protein-LC3 construct (31). By confocal imaging, the BLA neurons of mice transfected with GFP-monomeric red fluorescent protein-LC3 displayed a significant increase in both autophagosomes and autolysosomes at 1 hour after training (Supplemental Figure S3), suggesting that autophagy in the BLA is activated at 1 to 1.5 hours following fear conditioning.

The effect of SAR405 on fear training–induced autophagy was then investigated. We found that SAR405 significantly inhibited the increase in LC3-II at 1 hour after fear conditioning (Figure 1F) ($F_{3,38}$ = 4.105, p = .013). Correspondingly, the increase in p62 level at 1.5 hours after fear conditioning was also suppressed by SAR405 (Supplemental Figure S4). Taken together, these results demonstrate that SAR405 infusion into the BLA impaired the memory consolidation by inhibiting the autophagy elicited by fear conditioning.

Inhibition of Autophagy With 3-Methyladenine and LV-ATG5 Short Hairpin RNA Impairs LTM

We also used other methods to inhibit the autophagy to confirm the effect of SAR405 on memory consolidation was mediated through the autophagy pathway; 3-methyladenine (3-MA) is an inhibitor of phosphatidylinositol-3-phosphate I and VPS34/class III phosphatidylinositol-3 kinase and thus commonly used as a nonspecific autophagy inhibitor (30). We found that local delivery of 3-MA (500 μM) to the BLA inhibited the increased LC3-II level elicited by training (Figure 2A) ($F_{3,17}$ = 7.731, p = .002), and the freezing behaviors at 24 hours were decreased, with no differences detected at 3 hours (Figure 2B) ($F_{2,43}$ = 3.429, p = .035). Considering that both SAR405 and 3-MA are pharmlogical inhibitors of autophagy, LV short hairpin RNA (shRNA) targeted to ATG5, one of the key mediators for autophagic activity, was employed to specifically interfere with autophagy. Western blot analysis confirmed that LV-ATG5 shRNA transfection (Figure 2C, left panel) produced a 40% to 50% knockdown of LV-ATG5 (Supplemental Figure S5), in parallel with the significant reduction of LC3-II levels at 1 hour after fear conditioning (Figure 2C, right panel) (t_8 = 3.618, p = .007). Consistent with the pharmacological inhibitors of autophagy, knockdown of ATG5 in the amygdala significantly decreased the LTM, with no influence on STM

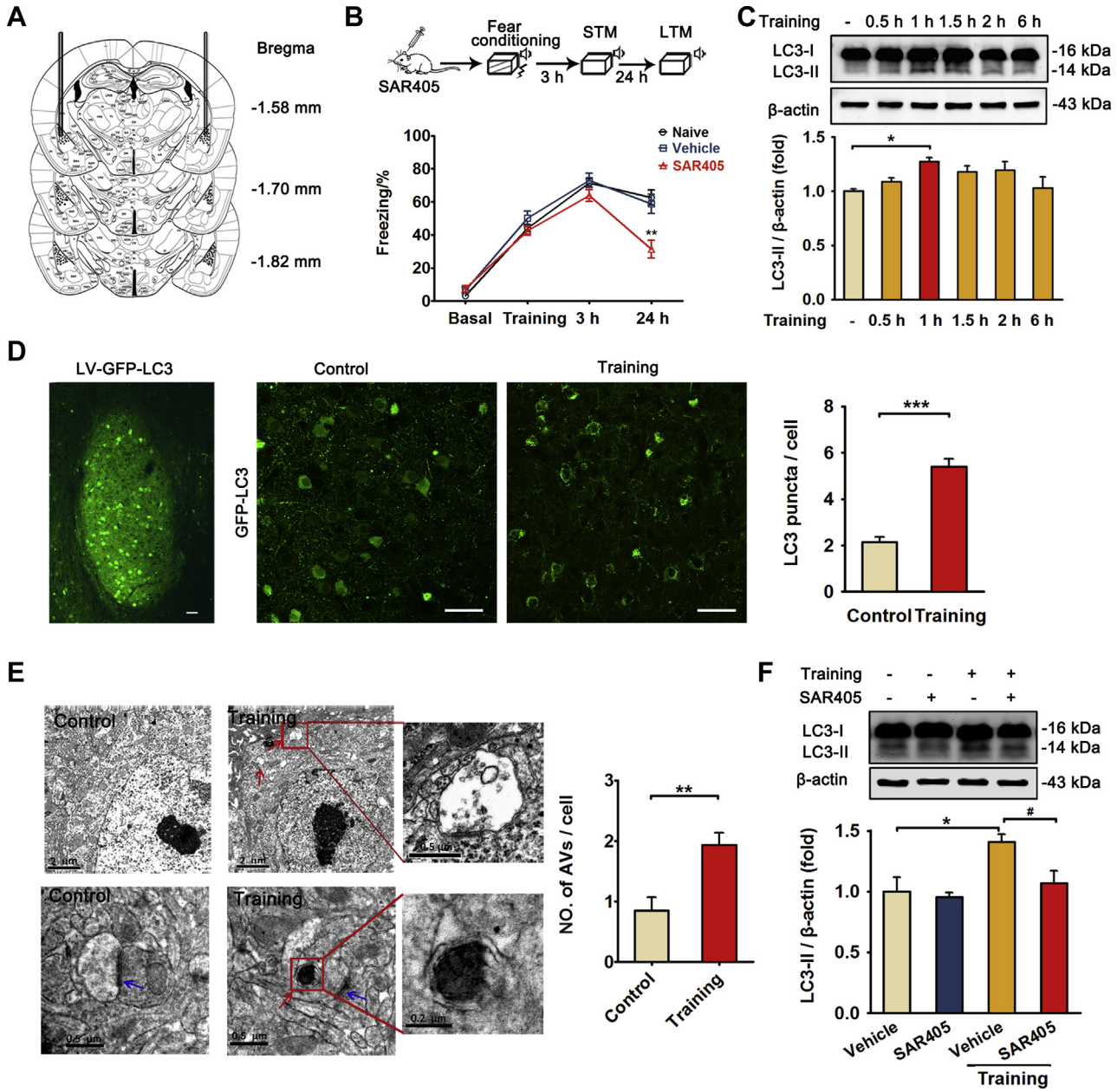

Figure 1. SAR405 attenuates long-term fear memory through the inhibition of autophagy. **(A)** Representative schematic drawings of guide cannula tip positions (black dots) in the basolateral amygdala (BLA). **(B)** Interference with freezing behavior by SAR405 (1 μM) administration. Short-term memory (STM) (3 hours) and long-term memory (LTM) (24 hours) were tested. n = 10 mice per group; repeated-measures two-way analysis of variance (ANOVA) with Bonferroni's post hoc test ($F_{2,27}$ = 5.090, p = .001). Post hoc: $^{**}p < .01$ vs. vehicle group. **(C)** Western blot analysis of light chain 3 (LC3) in the BLA after fear conditioning at 0.5, 1, 1.5, 2, and 6 hours. n = 7, 7, 7, 7, 7, 6 mice, respectively; one-way ANOVA with Tukey's test ($F_{5,35}$ = 2.698, p = .037). Post hoc: $^{*}p < .05$. **(D)** Representative image of green fluorescent protein (GFP) expression of BLA in lentiviral-GFP-LC3 (LV-GFP-LC3)–infected mice (left panel) and GFP-LC3 puncta of cells at 1 hour after fear conditioning (middle panel). Scale bar = 50 μm. The right panel was quantification of LC3 puncta. n = 82, 89 cells from three slices of 3 mice; unpaired t test (t_{169} = 8.029). $^{***}p < .0001$. **(E)** Transmission electron microscopy images from BLA tissues in fear conditioning mice (left panel) and quantification of autophagic vacuoles (AVs) (right panel). AVs are marked by red arrowheads, and postsynaptic active zones are marked by blue arrowheads. The red boxed area contains representative AVs. Scale bars = 2.0, 0.5, and 0.2 μm. n = 13, 15 cells from 6 mice; unpaired t test (t_{26} = 3.588). $^{**}p$ = .00. **(F)** Western blotting analysis of lipdated LC3 (LC3-II) levels in mice receiving intra-BLA SAR405 infusions. n = 10, 10, 11, 11 mice; one-way ANOVA with Tukey's test ($F_{3,38}$ = 4.105, p = .013). Post hoc: $^{*}p < .05$ vs. vehicle group, $^{\#}p < 0.05$ vs. training + vehicle group. LC3-I, nonlipidated light chain 3.

(Figure 2D) ($F_{2,37}$ = 3.693, p = .039). Collectively, these results suggest that inhibition of autophagy in the BLA impaired fear memory consolidation without affecting the acquisition.

In addition, we performed the open field test, which is a common measure of anxiety behavior and general activity in mice. The results showed that mice treated with SAR405 and

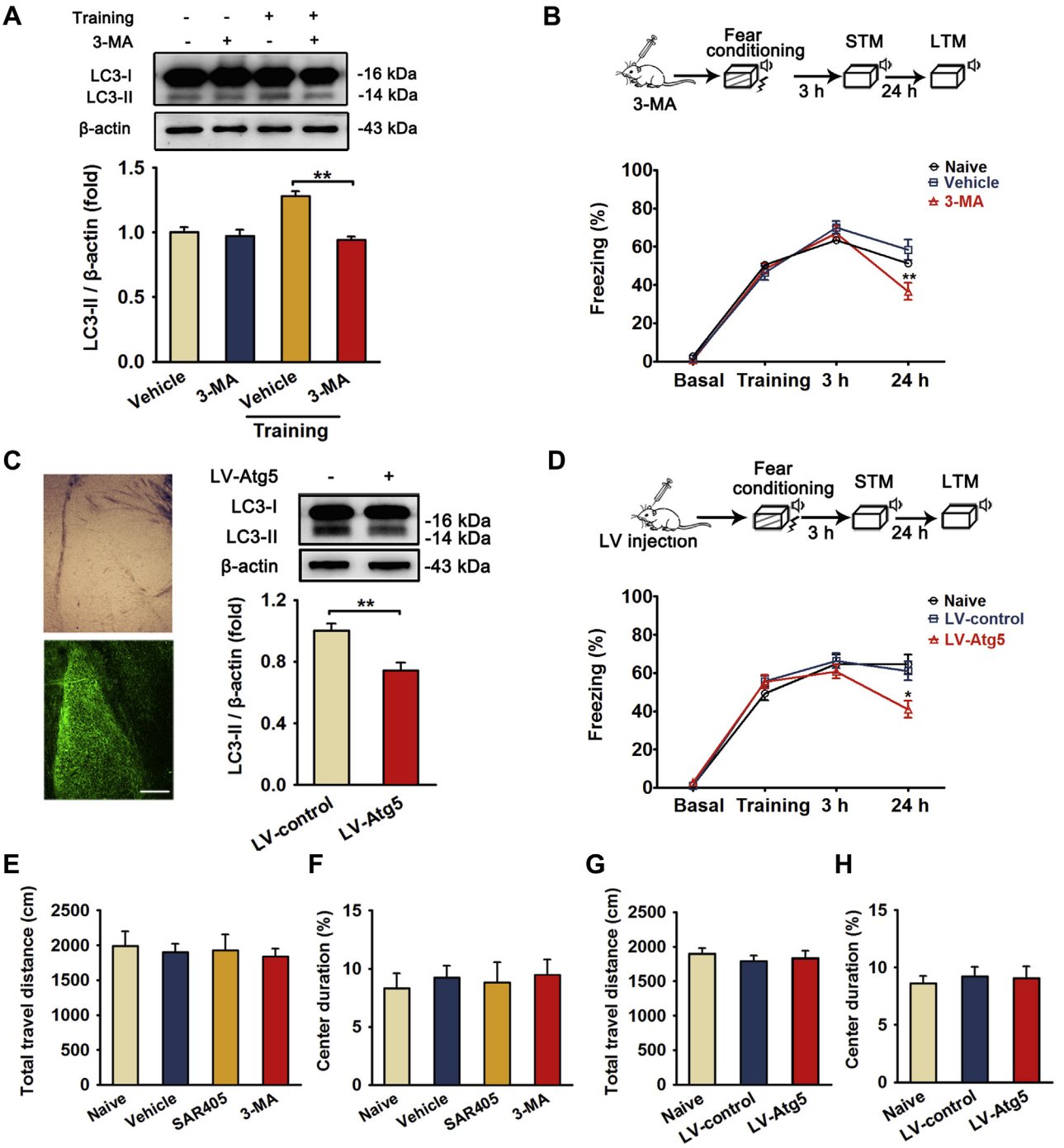

Figure 2. Inhibition of autophagy by 3-methyladenine (3-MA) and lentiviral–autophagy-related protein 5 (LV-ATG5) short hairpin RNA (shRNA) also impairs long-term fear memory with no effect on anxiety-like behaviors. **(A)** Western blotting analysis of LC3 levels in mice receiving intrabasolateral amygdala (intra-BLA) 3-MA infusions after fear conditioning. n = 5, 5, 5, 6 mice; one-way analysis of variance (ANOVA) with Tukey's test ($F_{3,17}$ = 7.731, p = .002). Post hoc: $^{**}p < .01$. **(B)** Interference with freezing behavior by 3-MA (500 μM) injection immediately after fear conditioning. n = 14, 14, 18 mice; repeat two-way ANOVA with Bonferroni's post hoc test ($F_{2,43}$ = 3.429, p = .035). Post hoc: $^{**}p < .01$. **(C)** Representative image of green fluorescent protein expression in LV-ATG5 shRNA–infected cells in the BLA (left panel). Scale bar = 200 μm. Western blotting analysis showed that LV-ATG5 shRNA led to a remarkable reduction in the expression of lipidated light chain 3 (LC3-II) protein 1 hour after fear conditioning (right panel). n = 5 mice per group, unpaired t test (t_8 = 3.618). $^{**}p$ = .007. **(D)** Interference with fear memory by LV-ATG5 injection. n = 12, 14, 14 mice, repeat two-way ANOVA with Bonferroni's post hoc test ($F_{2,37}$ = 3.693, p = .039). Post hoc: $^{*}p < .05$. **(E)** Total distance moved (n = 9 mice per group; one-way ANOVA [$F_{3,32}$ = 0.194, p = .900]) and **(F)** the percentage of time spent in central area (n = 9 mice per group; one-way ANOVA [$F_{3,32}$ = 0.149, p = .930]) of mice administrated with 3-MA and SAR405 during 15-minute intervals in the open field test. **(G)** Total distance moved (n = 9 mice/group, one-way ANOVA [$F_{2,24}$ = 0.335, p = .719]) and **(H)** the percentage of time spent in central area (n = 9 mice per group; one-way ANOVA [$F_{2,24}$ = 0.142, p = .869]) of mice injected with LV-ATG5 shRNA during 15-minute intervals in the open field test. LC3-I, nonlipidated light chain 3; LTM, long-term memory; STM, short-term memory.

3-MA exhibited no differences in either total distance (Figure 2E) ($F_{3,32}$ = 0.194, p = .900) or center duration (Figure 2F) ($F_{3,32}$ = 0.149, p = .930) in the open field test. Similar results were observed with LV-ATG5 shRNA (Figure 2G, H) (total distance [$F_{2,24}$ = 0.335, p = .719] and center duration [$F_{2,24}$ = 0.142, p = .869]). These results ruled out the possibility that the alterations in freezing behaviors were due to different locomotive activity or anxiety-related behavior.

SAR405 Reverses the Decreased Surface Expression of GABA$_A$R γ2 Subunit After Fear Conditioning Through Autophagy Inhibition

Alterations in synaptic function and plasticity have been identified as key components of the finely tuned machinery underlying memory consolidation. It is known that the surface expression of GABA$_A$Rs is decreased after fear conditioning (32), and the decreased GABAergic inhibitory transmission is necessary for memory consolidation (18). On the other hand, GABA$_A$Rs have been reported to traffic from cell surface to autophagosome in *Caenorhabditis elegans* (9), indicating a novel function for autophagy in GABA$_A$R degradation. Therefore, we proposed a hypothesis that the autophagic degradation of GABA$_A$Rs may contribute to SAR405-mediated impairment of memory consolidation. Over 90% of GABA$_A$Rs include the γ2 subunit, which is the most abundant GABA$_A$R subunit in the central nervous system (33). Thus, we observed the levels of GABA$_A$Rs using an antibody directed against the extracellular region of the γ2 subunit. First, we confirmed that the surface expression of GABA$_A$R γ2 subunit was downregulated after fear conditioning in biotinylation experiments (Figure 3A) (t_{12} = 3.952, p = .002), whereas no difference was found in total expression of the GABA$_A$R γ2 subunit (Figure 3B) (t_{12} = 0.389, p = .704). Next, the effects of SAR405 administration on GABA$_A$Rs were detected using biotinylation analysis. The results showed that SAR405 reversed the decreased surface expression of the GABA$_A$R γ2 subunit after training, and the surface expression of the GABA$_A$R γ2 subunit in untrained mice were not affected (Figure 3C) ($F_{4,40}$ = 4.254, p = .006). The total expression of the GABA$_A$R γ2 subunit remained unchanged after SAR405 administration (Supplemental Figure S4). We also investigated the influences of additional inhibition methods of autophagy on GABA$_A$R γ2 subunits. Consistent with SAR405, 3-MA and LV-ATG5 shRNA reversed the training-induced decrease in the surface level of the GABA$_A$R γ2 subunit, and the alteration of surface GABA$_A$R γ2 subunit level was not seen in untrained mice (Figure 3D, E) (3-MA [$F_{4,30}$ = 4.303, p = .007] and LV-ATG5 [$F_{3,23}$ = 9.998, $p < .001$]). To directly determine the trafficking of GABA$_A$R γ2 subunit between autophagosome and cell membrane, the colocalization of GFP-LC3 and GABA$_A$Rs in HT22 cells (a hippocampal neuronal cell line) was investigated. The results showed that the GFP-LC3 puncta were colocalized with GABA$_A$R γ2 subunits after autophagy was induced by starvation, and pretreatment with SAR405 significantly prevented this colocalization (Supplementary Figure S7). These findings indicate that the surface GABA$_A$R γ2 subunit was degraded by autophagy during memory consolidation, and inhibition of autophagy by SAR405 blocks the downregulation of the surface GABA$_A$R γ2 subunit after training.

SAR405 Enhances GABAergic Activity in the BLA After Fear Conditioning

Considering that SAR405 blocked the fear conditioning-induced decrease in the surface expression of the GABA$_A$R γ2 subunit, we examined whether the inhibitory synaptic transmission during memory consolidation was affected by SAR405. In line with the changes in surface expression of GABA$_A$Rs, the significant longer interevent intervals were observed in both cumulative frequency distribution and mean frequency of mIPSCs in slices from the paired animals compared with those from the untrained mice (Figure 4A, B) (t_{18} = 3.586, p = .002), the mean amplitude of mIPSCs and the cumulative data were also lower in conditioned mice (Figure 4C) (t_{18} = 3.875, p = .001), suggesting that GABAergic transmission is reduced in the BLA after fear conditioning. It is noteworthy that both the frequency and amplitude of mIPSCs in conditioned mice were increased by SAR405 infusion (Figure 4D), with a significant shift in the cumulative frequency (Figure 4E) (t_{10} = 3.681, p = .004) and amplitude distribution (Figure 4F) (t_{10} = 2.276, p = .046), demonstrating that SAR405 reversed the fear training–induced downregulation of GABAergic activity. Influences of LV-ATG5 shRNA on mIPSCs were also measured, and similar results with SAR405 were observed (Supplemental Figure S8). Taken together, these results support the speculations that SAR405 impaired memory consolidation through enhancing the GABA$_A$R-mediated synaptic transmission, which was downregulated during the consolidation process.

GABARAP and Its Interaction With the GABA$_A$R γ2 Subunit Contribute to the Enhancement of Inhibitory Neurotransmission Produced by SAR405

Given that GABARAP, a critical protein for autophagosome biogenesis and maturation (24), plays an important role in GABA$_A$Rs trafficking through binding to the γ2 subunit (22,34), we asked whether GABARAP might be involved in SAR405-mediated increase in the surface expression of the GABA$_A$R γ2 subunit. First, we detected the GABARAP levels by Western blotting and found that the expression of GABARAP was also increased at 1 hour after fear conditioning, consistent with the changes in LC3-II expression (Figure 5A) (t_{21} = 3.465, p = .002). Meanwhile, the co-immunoprecipitation assay was conducted and revealed an increased interaction between the GABA$_A$R γ2 subunit and GABARAP following fear conditioning (Figure 5B) (t_{14} = 3.873, p = .002). The effect of SAR405 administration on the GABARAP expression and its interaction with the GABA$_A$R γ2 subunit were further addressed. We found that SAR405 treatment significantly suppressed the increased GABARAP level (Figure 5C) ($F_{4,27}$ = 6.505, p = .001) and its interaction with the GABA$_A$R γ2 subunit (Figure 5D) ($F_{4,26}$ = 3.852, p = .014) after fear conditioning.

To test the involvement of the GABARAP-GABA$_A$Rs binding in SAR405-induced impairment of memory consolidation, a cell-permeable TAT-conjugated peptide mimicking the GABARAP-binding domain of the GABA$_A$R γ2 subunit was constructed to disrupt the interaction between GABARAP and GABA$_A$Rs (32). As shown in Supplemental Figure S9, local delivery of TAT-GABARAP inhibitory peptide into the BLA disrupted the GABARAP-GABA$_A$R interaction when compared

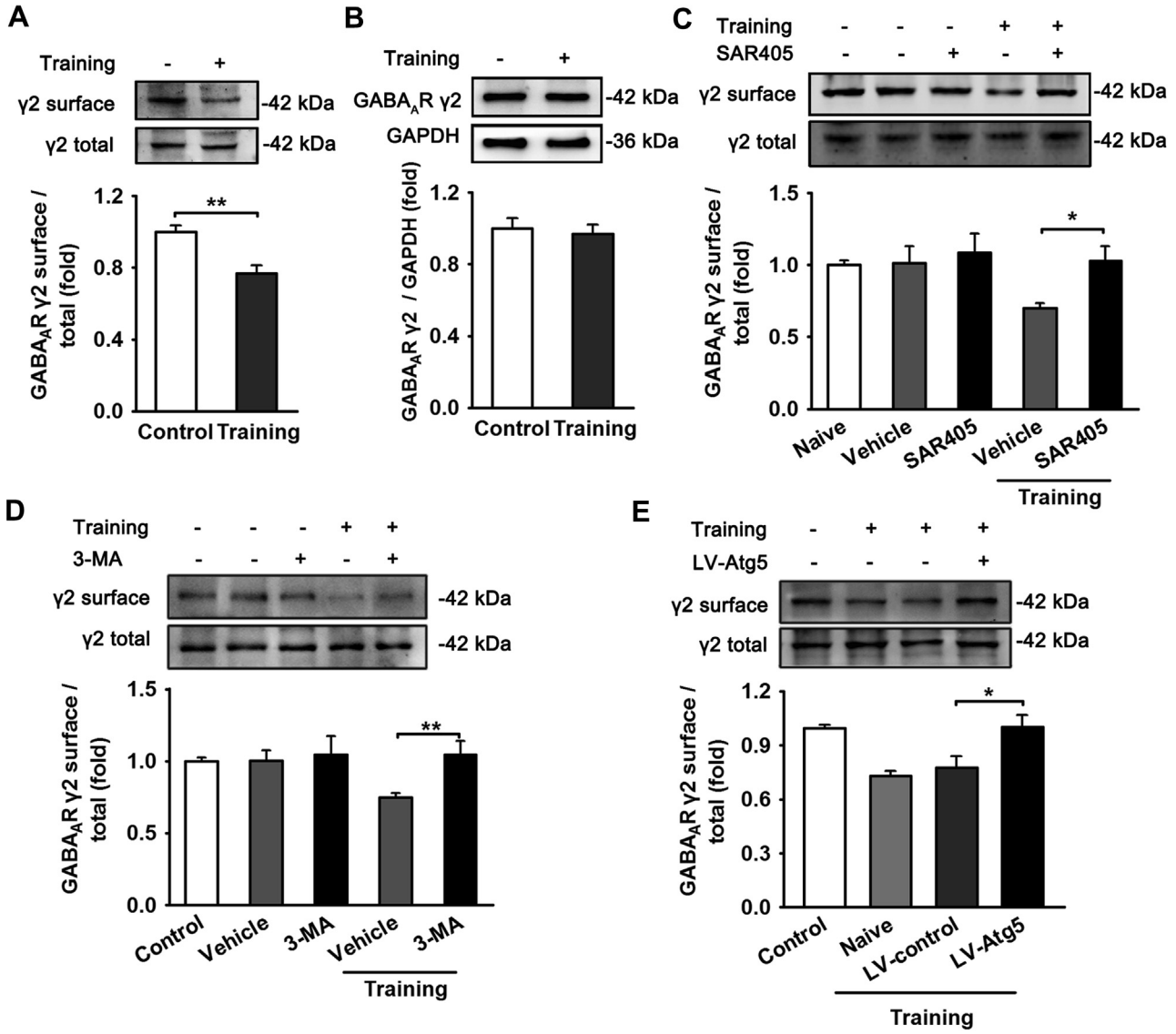

Figure 3. Inhibition of autophagy by SAR405 reverses the decrease in surface gamma-aminobutyric acid type A receptor ($GABA_AR$) γ2 subunit induced by fear conditioning. **(A)** Immunoblots and quantification data of the surface expression of $GABA_AR$ γ2 subunit following fear conditioning. $n = 7$ mice per group; unpaired t test ($t_{12} = 3.952$). $^{**}p = .002$. **(B)** Western blotting analysis of the total expression of $GABA_AR$ γ2 subunit. $n = 7$ mice per group; unpaired t test ($t_{12} = 0.389$, $p = .704$). Immunoblots and quantification data of the surface expression of $GABA_AR$ γ2 subunit by **(C)** SAR405 ($n = 9$ mice per group; one-way analysis of variance with Tukey's test [$F_{4,40} = 4.254$, $p = .006$]; post hoc: $^{*}p < .05$) and **(D)** 3-methyladenine (3-MA) ($n = 7$ mice per group; one-way analysis of variance with Tukey's test [$F_{4,30} = 4.303$, $p = .007$]; post hoc: $^{**}p < .01$) administration after fear conditioning. **(E)** Immunoblots and quantification data of the surface expression of $GABA_AR$ γ2 subunit by lentiviral–autophagy-related protein 5 (LV-ATG5) short hairpin RNA injection after fear conditioning. $n = 7, 7, 7, 6$ mice per group; one-way analysis of variance with Tukey's test ($F_{3,23} = 9.998$, $p < .001$). Post hoc: $^{*}p < .05$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

with TAT-Scramble peptide. In the fear conditioning paradigm, mice injected with TAT-GABARAP peptide exhibited reduced LTM with no influence on STM (Figure 5E) ($F_{2,35} = 3.549$, $p = .031$), indicating that the interaction between GABARAP and $GABA_ARs$ was required for memory consolidation. Moreover, the results of biotinylation analysis showed that the decrease in surface expression of $GABA_AR$ γ2 subunits after fear conditioning was reversed by TAT-GABARAP inhibitory peptide (Figure 5F) ($F_{3,25} = 5.505$, $p = .005$). These results demonstrated that the GABARAP-$GABA_AR$ binding was required for the degradation of surface $GABA_ARs$ during fear memory condsolidation. Thus, the disrupted interaction between $GABA_ARs$ and GABARAP was speculated to be the underlying mechanisim of SAR405-mediated memory consolidation impairment.

Both TAT-GABARAP peptide and TAT-Scramble peptide appeared to have no significant effect on the surface expression of $GABA_ARs$ in unconditioned mice (Supplemental Figure S10), suggesting that GABARAP-$GABA_AR$ binding may not be essential for the surface expression of $GABA_ARs$

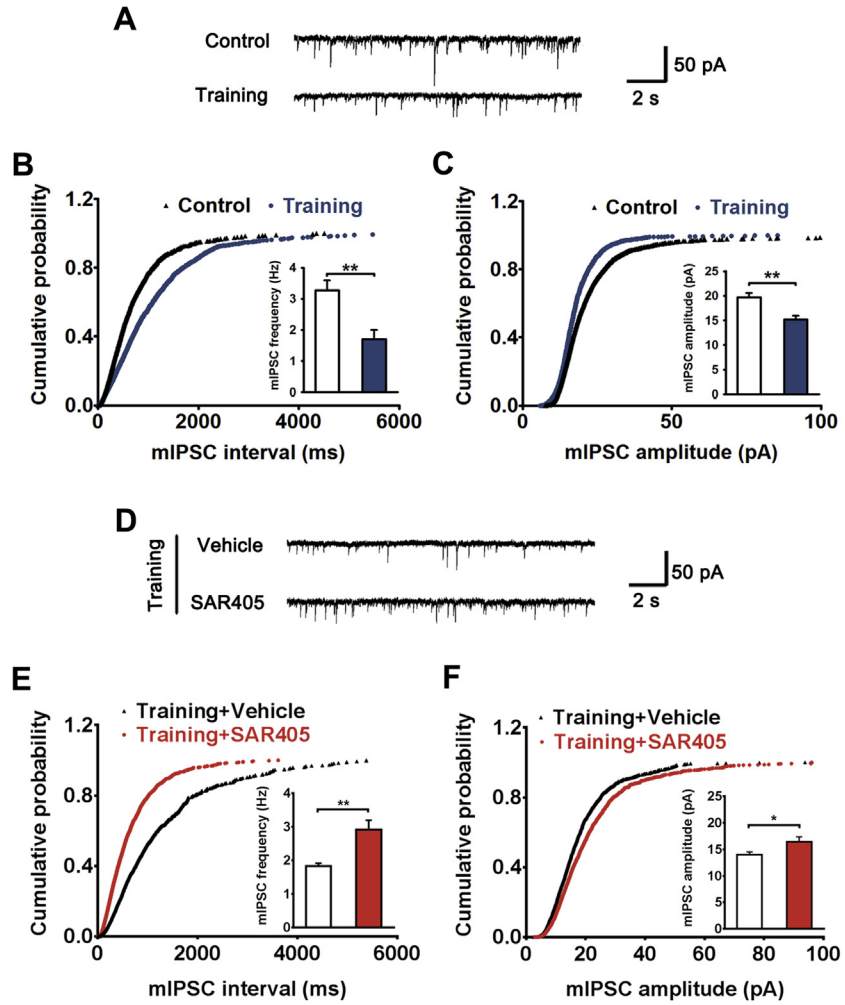

Figure 4. SAR405 prevents the decrease in inhibitory synaptic transmission following fear conditioning. **(A)** Representative traces of miniature inhibitory postsynaptic currents (mIPSCs) before (control) and after training. **(B)** Cumulative plots of mIPSC interevent intervals and mIPSC frequency (inside) following fear conditioning. n = 10, 10 cells from 7 mice; unpaired t test (t_{18} = 3.586). $^{**}p$ = .002. **(C)** Cumulative plots and bar plot (inside) of mIPSC amplitude following fear conditioning. n = 10, 10 cells from 7 mice; unpaired t test (t_{18} = 3.875). $^{**}p$ = .001. **(D)** Representative traces of mIPSCs in SAR405-treated mice. **(E)** Effect of SAR405 on cumulative plots of mIPSC interevent intervals and mIPSC frequency (inside) following fear conditioning. n = 6, 6 cells from 5 mice; unpaired t test (t_{10} = 3.681). $^{**}p$ = .004. **(F)** Effect of SAR405 on cumulative plots and bar plot (inside) of mIPSC amplitude following fear conditioning. n = 6, 6 cells from 5 mice; unpaired t test (t_{10} = 2.276). $^{*}p$ = .046.

under physiological conditions. This result was consistent with the findings that GABARAP knockout mice were phenotypically normal and exhibits no loss of punctate immunoreactivity for synaptic GABARs (35).

DISCUSSION

The present study demonstrated that SAR405, as a specific autophagy inhibitor, impaired memory consolidation through blocking the autophagy-dependent downregulation of the surface $GABA_AR$ $\gamma2$ subunit, and the increased GABARAP-$GABA_AR$ binding was required for autophagic degradation of surface $GABA_ARs$ (Figure 6). These observations uncovered a novel role of autophagy pathway in learning and memory, and propose SAR405 as a new candidate for prevention of psychiatric disorders with altered fear learning, such as posttraumatic stress disorder.

Previous studies have reported that autophagy is activated in the hippocampus after hypoxic-ischemic brain injury (5) or morphine treatment (7), and 3-MA administration improved the memory deficits caused by hypoxic-ischemic brain injury or morphine treatment in the Morris water maze test. In contrast, a recent study has demonstrated that training in the Morris water maze task increases the number of autophagosomes and 3-MA infusion into the hippocampus before training impaired the LTM at 24 hours, leaving the learning process unaffected (36). Consistent with this result, our current findings demonstrated that SAR405 impaired memory consolidation; moreover, we employed multiple methods, including 3-MA and ATG5 shRNA to inhibit autophagy, and all of them were demonstrated to impair the LTM. Those opposite effects of autophagy inhibition on memory suggest that the role of autophagy in memory process can be completely opposite under different conditions, and the stimulus that elicited autophagy may be the key factor.

Numerous studies have shown that pre- and posttraining administration of $GABA_AR$ agonists leads to impaired fear consolidation (18), and fear conditioning was found to decrease the frequency and amplitude of mIPSCs and the surface levels of $GABA_ARs$ (32,37). Consistent with these findings, we found that pretraining administration of SAR405

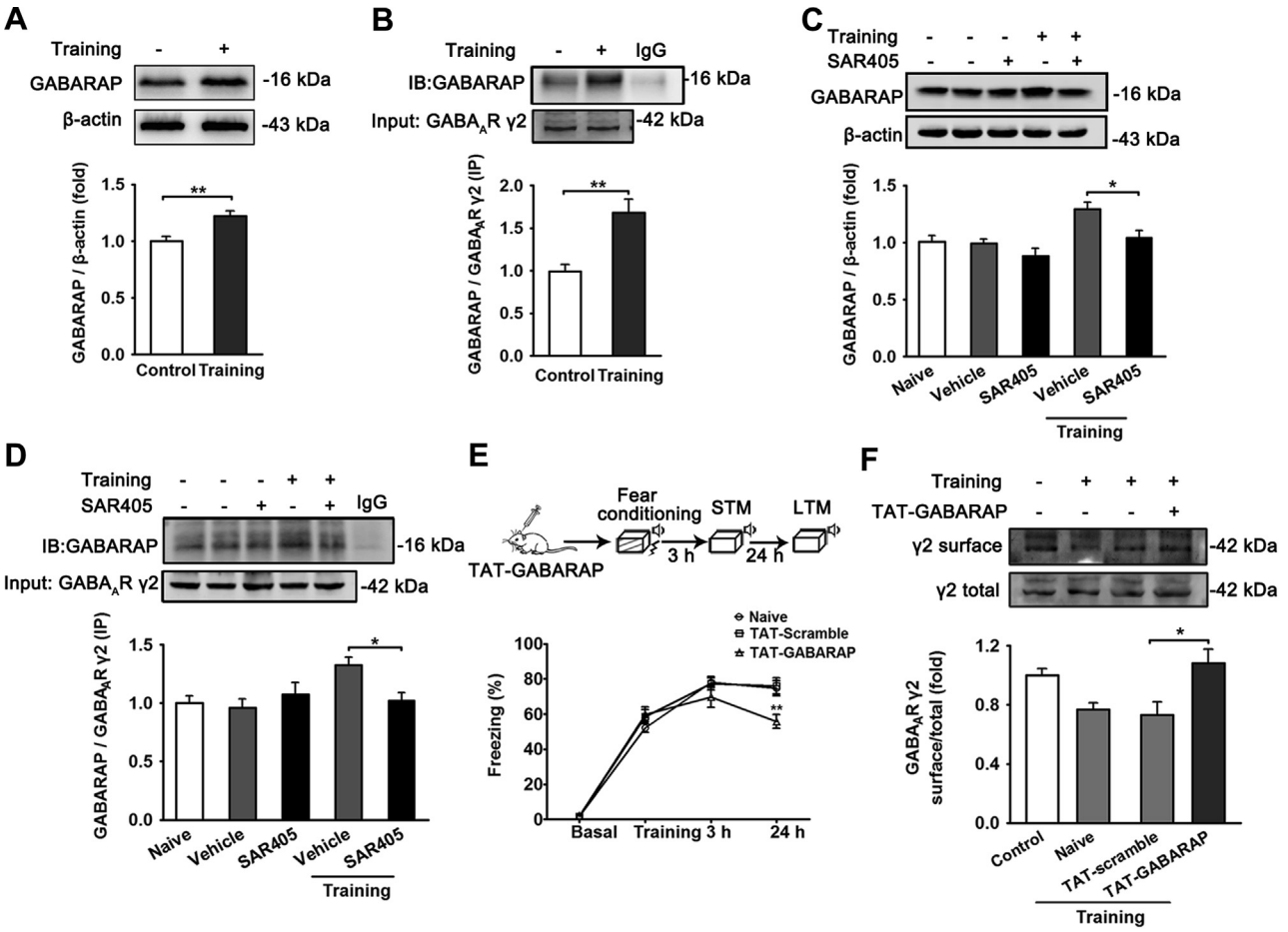

Figure 5. The SAR405-induced impairment of memory consolidation depends on the blockade of the interaction between gamma-aminobutyric acid type A receptor ($GABA_AR$)–associated protein (GABARAP) and $GABA_ARs$. **(A)** Immunoblots assessing the expression of GABARAP in the basolateral amygdala (BLA) 1 hour after fear conditioning. $n = 11, 12$ mice, unpaired t test ($t_{21} = 3.465$). $^{**}p = .002$. **(B)** Protein homogenates of BLA tissues were immunoprecipitated with anti-$GABA_AR$ γ2 subunit, and the immunocomplex was immunoblotted for GABARAP. The quantification data assessed the binding of GABARAP to the $GABA_AR$ γ2 subunit after fear conditioning. $n = 8$ mice per group; unpaired t test ($t_{14} = 3.873$). $^{**}p = .002$. **(C)** Immunoblots assessing the expression of GABARAP with SAR405 infusion in BLA. $n = 7, 7, 6, 6, 6$ mice; one-way analysis of variance (ANOVA) with Tukey's test ($F_{4,27} = 6.505$, $p = .001$). Post hoc: $^{*}p < .05$. **(D)** Co-immunoprecipitation followed by Western blotting analysis assessed the interaction of GABARAP and $GABA_AR$ γ2 subunit after SAR405 treatment. $n = 7, 6, 6, 6, 6$ mice; one-way ANOVA with Tukey's test ($F_{4,26} = 3.852$, $p = .014$). Post hoc: $^{*}p < .05$. **(E)** Interference with fear memory by TAT-GABARAP peptide injection immediately following fear conditioning. $n = 13, 12, 13$ mice; repeated-measures two-way ANOVA with Bonferroni's post hoc test ($F_{2,35} = 3.549$, $p = .031$). Post hoc: $^{**}p < .01$. **(F)** Representative immunoblots and quantification data of the surface expression of $GABA_AR$ γ2 subunits in mice receiving intra-BLA TAT-GABARAP inhibitory peptide infusions after fear conditioning. $n = 7, 7, 8, 7$ mice; one-way ANOVA with Tukey's test ($F_{3,25} = 5.505$, $p = .005$). Post hoc: $^{*}p < .05$. IB, immunoblot; IP, immunoprecipitation; LTM, long-term memory; STM, short-term memory.

and genetic inhibition of autophagy by LV-ATG5 shRNA inpaired memory consolidation by increasing the surface expression of $GABA_AR$ γ2 subunit and enhancing the frequency and amplitude of mIPSCs. Autophagy was reported to take part in alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor degradation after induction of NMDA receptor–dependent chemical long-term depression (8) and selective degradation of surface $GABA_ARs$ in *Caenorhabditis elegans* (9). Our results, including the direct evidence that GFP-LC3 puncta were colocalized with anti-$GABA_AR$ γ2 subunit immunoreactivity in HT22 cells after autophagy was induced, further demonstrated that the surface $GABA_ARs$ were degraded through the autophagy pathway during the memory consolidation process. Notably, total expression of the $GABA_AR$ γ2 subunit remained unchanged after SAR405 administration. This may be explained by the dynamic alterations in assembly, exocytosis, and/or recycling of the $GABA_AR$ γ2 subunit during memory consolidation. Previous studies have reported that the messenger RNA levels of various GABA-related proteins (i.e., gephyrin and GABA transporter 1) and the protein level of gephyrin are decreased after fear conditioning (32,38), suggesting that the regulation of $GABA_ARs$ after fear acquisition involves multiple signaling pathways. In addition, autophagy has been reported to modulate presynaptic neurotransmission (39) and participate in the decrease in mIPSC frequency induced by amyloid-β protein 42 (40). Our results also found that both the frequency and amplitude of mIPSCs were

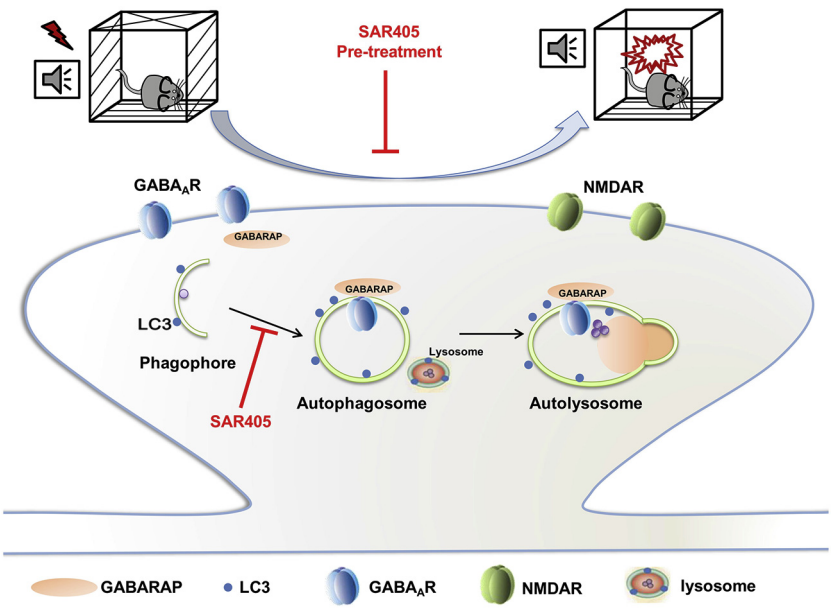

Figure 6. The schematic for the effect and underlying mechanism of SAR405 on fear memory consolidation. Pretreatment with SAR405 into basolateral amygdala attenuates long-term fear memory by inhibiting the autophagy elicited by fear conditioning. SAR405 treatment blocks the autophagy-dependent translocation of gamma-aminobutyric acid type A receptor (GABA$_A$R) γ2 subunit from the cell surface during memory consolidation process, and the interaction between GABA$_A$R–associated protein (GABARAP) and the GABA$_A$R γ2 subunit is required for its autophagic degradation, which is also suppressed by SAR405. LC3, light chain 3; NMDAR, *N*-methyl-D-aspartate receptor.

affected by fear conditioning and SAR405 treatment, suggesting that both pre- and postsynaptic effects are involved in this process. Further study is necessary to determine the role of autophagy in presynaptic terminals.

Rowland *et al.* (9) reported that GABA$_A$Rs traffic to autophagosomes after endocytic removal from the cell surface in *C. elegans*. Moreover, reduced endocytosis of GABA$_A$Rs caused by mutation of tyrosine phosphorylation residues of the γ2 subunit can lead to enhanced GABA$_A$R accumulation at synapse, resulting in impaired spatial memory (41). These results suggest that endocytosis of GABA$_A$Rs from the cell surface may contribute to its autophagic degradation and memory process in mice. We found similar effects of SAR405 and ATG5 shRNA on mIPSCs and fear memory, suggesting that autophagy is the major signaling pathway for SAR405 in disrupting memory consolidation. However, considering that SAR405 also affected vesicle trafficking from late endosomes to lysosomes besides autophagy (12), it is difficult to rule out the possibility that the endocytic pathway contribute to the alterations in surface expression of GABA$_A$Rs and fear behaviors caused by SAR405.

GABARAP was reported to bind the GABA$_A$R through interacting specifically with the γ2 subunit (42). Several studies have shown that GABARAP enhanced the surface expression of γ2-containing GABA$_A$Rs in both heterologous expression systems and cultured hippocampal neurons (34,43). However, in the present study, we showed that SAR405 disrupted the interaction between GABARAP and GABA$_A$R and showed that a TAT-conjugated peptide that was designed to disrupt GABARAP-GABA$_A$R binding effectively attenuated memory consolidation and reversed the decrease in surface expression of GABA$_A$Rs. These results suggest that GABARAP-GABA$_A$R binding may play a role in the autophagic degradation of surface GABA$_A$Rs. In contrast to prior studies, this novel role of GABARAP in GABA$_A$R trafficking may be related to its function in autophagy. Further investigations on the localization and trafficking of GABARAP-GABA$_A$R complex are required to address the mechanism.

In our study, the effect of LV shRNA that targeted to ATG5 was similar to the effects of SAR405 and 3-MA. It can infect both neurons and glial cells, which are involved in learning and memory (44). It has been reported that disruption of autophagy in glial cells may have a profound non–cell-autonomous influence on neuronal health and development (45). Thus, there is a possibility that disruption of autophagy in glial cells of the BLA can affect the synaptic plasticity during fear conditioning.

Being a newly developed kinase inhibitor, SAR405 is mainly used as a pharmacological approach for specific VPS34 inhibition to explore the role of VPS34 or autophagy (14,46). Our study provided evidence that inhibition of autophagy by SAR405 impaired memory consolidation through blocking the degradation of the surface GABA$_A$R γ2 subunit, highlighting the role of autophagy in GABA$_A$R trafficking and memory consolidation and proposing SAR405 as a novel pharmacological treatment strategy for psychiatric disorders characterized by exaggerated fear memories such as that observed in posttraumatic stress disorder.

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ARTICLE INFORMATION

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REFERENCES

1. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008): Autophagy fights disease through cellular self-digestion. Nature 451:1069–1075.
2. Yang DS, Stavrides P, Mohan PS, Kaushik S, Kumar A, Ohno M, *et al.* (2011): Reversal of autophagy dysfunction in the TgCRND8 mouse model of Alzheimer's disease ameliorates amyloid pathologies and memory deficits. Brain 134:258–277.
3. Nixon RA (2013): The role of autophagy in neurodegenerative disease. Nat Med 19:983–997.
4. Gupta VK, Scheunemann L, Eisenberg T, Mertel S, Bhukel A, Koemans TS, *et al.* (2013): Restoring polyamines protects from age-induced memory impairment in an autophagy-dependent manner. Nat Neurosci 16:1453–1460.
5. Xu Y, Tian Y, Tian Y, Li X, Zhao P (2016): Autophagy activation involved in hypoxic-ischemic brain injury induces cognitive and memory impairment in neonatal rats. J Neurochem 139:795–805.
6. Wang D, Zhang J, Jiang W, Cao Z, Zhao F, Cai T, *et al.* (2017): The role of NLRP3-CASP1 in inflammasome-mediated neuroinflammation and autophagy dysfunction in manganese-induced, hippocampal-dependent impairment of learning and memory ability. Autophagy 13:914–927.
7. Pan J, He L, Li X, Li M, Zhang X, Venesky J, *et al.* (2017): Activating autophagy in hippocampal cells alleviates the morphine-induced memory impairment. Mol Neurobiol 54:1710–1724.
8. Shehata M, Matsumura H, Okubo-Suzuki R, Ohkawa N, Inokuchi K (2012): Neuronal stimulation induces autophagy in hippocampal neurons that is involved in AMPA receptor degradation after chemical long-term depression. J Neurosci 32:10413–10422.
9. Rowland AM, Richmond JE, Olsen JG, Hall DH, Bamber BA (2006): Presynaptic terminals independently regulate synaptic clustering and autophagy of GABAA receptors in Caenorhabditis elegans. J Neurosci 26:1711–1720.
10. Backer JM (2008): The regulation and function of Class III PI3Ks: Novel roles for Vps34. Biochem J 410:1–17.
11. Funderburk SF, Wang QJ, Yue Z (2010): The Beclin 1-VPS34 complex–at the crossroads of autophagy and beyond. Trends Cell Biol 20:355–362.
12. Ronan B, Flamand O, Vescovi L, Dureuil C, Durand L, Fassy F, *et al.* (2014): A highly potent and selective Vps34 inhibitor alters vesicle trafficking and autophagy. Nat Chem Biol 10:1013–1019.
13. Schlutermann D, Skowron MA, Berleth N, Bohler P, Deitersen J, Stuhldreier F, *et al.* (2018): Targeting urothelial carcinoma cells by combining cisplatin with a specific inhibitor of the autophagy-inducing class III PtdIns3K complex. Urol Oncol 36:e1-160.e13.
14. An H, Harper JW (2018): Systematic analysis of ribophagy in human cells reveals bystander flux during selective autophagy. Nat Cell Biol 20:135–143.
15. Kandel ER, Dudai Y, Mayford MR (2014): The molecular and systems biology of memory. Cell 157:163–186.
16. Mahan AL, Ressler KJ (2012): Fear conditioning, synaptic plasticity and the amygdala: Implications for posttraumatic stress disorder. Trends Neurosci 35:24–35.
17. Carbo Tano M, Molina VA, Maldonado H, Pedreira ME (2009): Memory consolidation and reconsolidation in an invertebrate model: The role of the GABAergic system. Neuroscience 158:387–401.
18. Makkar SR, Zhang SQ, Cranney J (2010): Behavioral and neural analysis of GABA in the acquisition, consolidation, reconsolidation, and extinction of fear memory. Neuropsychopharmacology 35:1625–1652.
19. Lu Y, Sun XD, Hou FQ, Bi LL, Yin DM, Liu F, *et al.* (2014): Maintenance of GABAergic activity by neuregulin 1-ErbB4 in amygdala for fear memory. Neuron 84:835–846.
20. Misane I, Kruis A, Pieneman AW, Ogren SO, Stiedl O (2013): GABA(A) receptor activation in the CA1 area of the dorsal hippocampus impairs consolidation of conditioned contextual fear in C57BL/6J mice. Behav Brain Res 238:160–169.
21. Coyle JE, Qamar S, Rajashankar KR, Nikolov DB (2002): Structure of GABARAP in two conformations: Implications for GABA(A) receptor localization and tubulin binding. Neuron 33:63–74.
22. Chen ZW, Olsen RW (2007): GABAA receptor associated proteins: A key factor regulating GABAA receptor function. J Neurochem 100:279–294.
23. Luscher B, Fuchs T, Kilpatrick CL (2011): GABAA receptor trafficking-mediated plasticity of inhibitory synapses. Neuron 70:385–409.
24. Weidberg H, Shvets E, Shpilka T, Shimron F, Shinder V, Elazar Z (2010): LC3 and GATE-16/GABARAP subfamilies are both essential yet act differently in autophagosome biogenesis. EMBO J 29:1792–1802.
25. Wang CM, Yang YJ, Zhang JT, Liu J, Guan XL, Li MX, *et al.* (2015): Regulation of emotional memory by hydrogen sulfide: Role of GluN2B-containing NMDA receptor in the amygdala. J Neurochem 132:124–134.
26. Zhou J, Luo Y, Zhang JT, Li MX, Wang CM, Guan XL, *et al.* (2015): Propranolol decreases retention of fear memory by modulating the stability of surface glutamate receptor GluA1 subunits in the lateral amygdala. Br J Pharmacol 172:5068–5082.
27. Wu X, Zhang JT, Li D, Zhou J, Yang J, Zheng HL, *et al.* (2017): Aquaporin-4 deficiency facilitates fear memory extinction in the hippocampus through excessive activation of extrasynaptic GluN2B-containing NMDA receptors. Neuropharmacology 112:124–134.
28. Johansen JP, Cain CK, Ostroff LE, LeDoux JE (2011): Molecular mechanisms of fear learning and memory. Cell 147:509–524.
29. Nader K, Hardt O (2009): A single standard for memory: The case for reconsolidation. Nat Rev Neurosci 10:224–234.
30. Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, *et al.* (2016): Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12:1–222.
31. Kimura S, Noda T, Yoshimori T (2007): Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 3:452–460.
32. Lin HC, Mao SC, Gean PW (2009): Block of gamma-aminobutyric acid-A receptor insertion in the amygdala impairs extinction of conditioned fear. Biol Psychiatry 66:665–673.
33. Smith KS, Rudolph U (2012): Anxiety and depression: Mouse genetics and pharmacological approaches to the role of GABA(A) receptor subtypes. Neuropharmacology 62:54–62.
34. Leil TA, Chen ZW, Chang CS, Olsen RW (2004): GABAA receptor-associated protein traffics GABAA receptors to the plasma membrane in neurons. J Neurosci 24:11429–11438.
35. O'Sullivan GA, Kneussel M, Elazar Z, Betz H (2005): GABARAP is not essential for GABA receptor targeting to the synapse. Eur J Neurosci 22:2644–2648.
36. Hylin MJ, Zhao J, Tangavelou K, Rozas NS, Hood KN, MacGowan JS, *et al.* (2018): A role for autophagy in long-term spatial memory formation in male rodents. J Neurosci Res 96:416–426.

37. Rea K, Lang Y, Finn DP (2009): Alterations in extracellular levels of gamma-aminobutyric acid in the rat basolateral amygdala and periaqueductal gray during conditioned fear, persistent pain and fear-conditioned analgesia. J Pain 10:1088–1098.
38. Heldt SA, Ressler KJ (2007): Training-induced changes in the expression of GABAA-associated genes in the amygdala after the acquisition and extinction of Pavlovian fear. Eur J Neurosci 26:3631–3644.
39. Hernandez D, Torres CA, Setlik W, Cebrián C, Mosharov EV, Tang G, *et al.* (2012): Regulation of presynaptic neurotransmission by macroautophagy. Neuron 74:277–284.
40. Yin Y, Zhao Y, Han S, Zhang N, Chen H, Wang X (2017): Autophagy-ERK1/2-involved disinhibition of hippocampal neurons contributes to the pre-synaptic toxicity induced by Abeta42 exposure. J Alzheimers Dis 59:851–869.
41. Tretter V, Revilla-Sanchez R, Houston C, Terunuma M, Havekes R, Florian C, *et al.* (2009): Deficits in spatial memory correlate with modified {gamma}-aminobutyric acid type A receptor tyrosine phosphorylation in the hippocampus. Proc Natl Acad Sci U S A 106:20039–20044.
42. Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999): GABA(A)-receptor-associated protein links GABA(A) receptors and the cytoskeleton. Nature 397:69–72.
43. Kawaguchi SY, Hirano T (2007): Sustained structural change of GABA(A) receptor-associated protein underlies long-term potentiation at inhibitory synapses on a cerebellar Purkinje neuron. J Neurosci 27:6788–6799.
44. Fields RD, Araque A, Johansen-Berg H, Lim SS, Lynch G, Nave KA, *et al.* (2014): Glial biology in learning and cognition. Neuroscientist 20:426–431.
45. Yamamoto A, Yue Z (2014): Autophagy and its normal and pathogenic states in the brain. Annu Rev Neurosci 37:55–78.
46. Valet C, Levade M, Chicanne G, Bilanges B, Cabou C, Viaud J, *et al.* (2017): A dual role for the class III PI3K, Vps34, in platelet production and thrombus growth. Blood 130:2032–2042.