Activation of a novel α₂A-AR-spinophilin-cofilin axis determines the effect of α₂ adrenergic drugs on fear memory reconsolidation

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INTRODUCTION

Posttraumatic stress disorder (PTSD) after the pandemic has emerged as a major neuropsychiatric component of post-acute COVID-19 syndrome, yet the current pharmacotherapy for PTSD is limited. The use of adrenergic drugs to treat PTSD has been suggested; however, it is hindered by conflicting clinical results and a lack of mechanistic understanding of drug actions. Our studies, using both genetically modified mice and human induced pluripotent stem cell-derived neurons, reveal a novel α₂A adrenergic receptor (α₂AAR)-spinophilin-cofilin axis in the hippocampus that is critical for regulation of contextual fear memory reconsolidation. In addition, we have found that two α₂ ligands, clonidine and guanfacine, exhibit differential abilities in activating this signaling axis to disrupt fear memory reconsolidation. Stimulation of α₂AAR with clonidine, but not guanfacine, promotes the interaction of the actin binding protein cofilin with the receptor and with the dendritic spine scaffolding protein spinophilin to induce cofilin activation at the synapse. Spinophilin-dependent regulation of cofilin is required for clonidine-induced disruption of contextual fear memory reconsolidation. Our results inform the interpretation of differential clinical observations of these two drugs on PTSD and suggest that clonidine could provide immediate treatment for PTSD symptoms related to the current pandemic. Furthermore, our study indicates that modulation of dendritic spine morphology may represent an effective strategy for the development of new pharmacotherapies for PTSD.

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Mounting evidence supports that previously stored memories can be reactivated by retrieval, entering a labile or destabilized state, and restabilized in an updated form [22–24]. This process is referred to as memory reconsolidation. Disruption of reconsolidation using pharmacological agents has been suggested as a promising therapeutic strategy for the treatment of PTSD [25]. Currently, a complete understanding of molecular mechanisms underlying memory reconsolidation is still lacking. Here we show, for the first time, that reconsolidation of fear memory requires dynamic changes in the activity and synaptic localization of coflin.

Cofilin is an actin-severing protein and controls dendritic spine morphology and synaptic plasticity through regulating actin dynamics [26, 27]. Morphological changes of dendritic spines have been indicated as an essential cellular event that underlies learning and memory [28, 29] and disruption of these events is associated with many neuropsychiatric disorders including PTSD [30–32]. Activation of coflin by dephosphorylation at Ser3 causes dendritic spine remodeling in hippocampal neurons, leading to transformation of mature mushroom-shaped spines into immature long thin spines [33]. Consistently, coflin-deficient neurons show an increase in the number of mature neurons with large spine heads [34]. Changes in coflin activity are required for both long-term potentiation (LTP) [35, 36] and long-term depression (LTD) [37, 38]. Given the pivotal role of coflin in these processes, precise regulation of its synaptic activity is essential to support proper actin reorganization in learning and memory. To date, our knowledge regarding the spatial and temporal control of coflin activity during a physiological process, and how this can be manipulated by neurotransmitters and hormones as a means to regulate learning and memory, remains largely scarce. In the present study, we provide the first example that the spatial and temporal dynamics of coflin at the synapse can be regulated by an FDA-approved adrenergic drug to modulate fear memory.

We identified coflin as a novel downstream signaling effector that mediates α2AR-elicited regulation of fear memory reconsolidation. The two α2AR agonists, clonidine and guanfacine, show distinct ability in activating coflin. Clonidine, but not guanfacine, promotes the interaction of coflin with the receptor and with a synaptic scaffolding protein, spinophilin, which is essential in enhancing coflin activity at the synapse. This ligand-selective activation of the α2AR-spinophilin-cofilin signaling axis leads to distinct regulation of contextual fear memory reconsolidation by clonidine and guanfacine and informs interpretation of differential clinical observations of these drugs on PTSD. Our study further suggests that pharmacological manipulation of spine morphology modulators such as coflin represents an effective strategy for modification of fear memory reconsolidation, and thus has far-reaching implications for the development of active pharmacotherapies for PTSD.

MATERIALS AND METHODS

Animals

All experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees (IACUC) of University of Alabama at Birmingham and Augusta University. Mice were maintained on a 12-h light/dark cycle with food and water continuously available and used at 3–5 months of age. α2AR-deficient (Adra2a−/−) were originally obtained from The Jackson Laboratory (stock number 004367) and bred and maintained on site at the C57BL/6 background. The generation of spinophilin deficient (Ppp1r19b−/−) mice has been described previously [39], and the line has been backcrossed more than 10 generations to the C57BL/6 background. Both males and females were used, and we did not observe a significant difference between the sexes.

Reagents and antibodies

All peptides were synthesized and purified by GenScript, USA, Inc. Peptides containing a 16 aa sequence of the coflin Ser3 site (MASGVAVSDGVIKFVN, referred to as s3 peptides) or phosphor-Ser3 site [MASp]GVAVSDGVIKFVN, referred to as pS3 peptides] were used. These peptides were fused to a TAT-like polyarginine membrane permeability sequence (GRRRRRRRRRRRRRR) to facilitate its entrance into cells and to a biotin molecule to allow detection. The TAT-like peptide (GRRRRRRRRRRRRRRRRRRRRRRR) was used as a control. Antibodies to coflin (cat #51755, dilution 1:1000), phosphorylated coflin (p-cofilin) (cat# 33115, dilution 1:500) and Myc-Tag (9B11) (cat# #22765, dilution 1:1000) were purchased from Cell Signaling Technology. HA.11 antibody (cat#901515) was from Biolegend. Clonidine (cat# C7897), guanfacine (cat#G1043), BRL44408 (cat B4559) were from Sigma-Aldrich, and JP1302 (cat# 26-661-0) and imiloxan (cat# 09-861-0) were purchased from Thermo Fisher Scientific.

Cannulation and infusion of peptide

Stainless-steel bilateral guide cannulae (26 gauge, RWD Life Science, Inc) were implanted into the dorsal hippocampus (AP −1.8 mm, ML ±1.7, DV −2.0), under isoflurane anesthesia, using standard stereotaxic procedures. Coordinates were chosen based on a mouse brain atlas. The cannula was anchored to the skull using screws and acrylic cement. The mice were administered a recovery period of at least 1 week after surgery. The injection cannula was connected via PE Tubing (1.50*0.50 mm, RWD Life Science, Inc) to a 10 µl Hamilton micro syringe, driven by a microinjection pump (Dual Syringe, Model ‘11’, Harvard apparatus; MA-70-2209). Infusions were administered in a volume of 1 μl over 5 min, and an additional 1 min was allowed for diffusion before the infusion cannulas were removed. Tat-S3, Tat-pS3 and Tat-control (100 µM, 1 µl, per side), were administered into the hippocampus immediately after re-exposure to conditioned stimuli.

Fear conditioning and test

For the fear conditioning paradigm, mice were placed into a standard fear-conditioning chamber (Coulbourn instruments, Habtest System). Mice were habituated to experimental chamber (Context A) for five minutes. On the next day (training day, TR), mice were placed into the same chamber (Context A) for 2 min and then received 2 sessions of tone-shock pairings (a tone for 30 s and an electric foot shock for 2 s at 0.5 mA) with an interval of 60 s between sessions. Following the presentation of the final stimulus, mice remained in the context for 60 s. Then 24 h later on reactivation day (RE), mice were first re-exposed to Context A for 3 min to test contextual fear memory. Mice were then moved to a novel context (Context B) and received 2 pairs of tones for 30 s without the shock to test cued fear memory. Saline, clonidine or guanfacine (0.5 mg/kg for both drugs) was injected intraperitoneally (i.p) immediately after re-exposure to both contextual and cued stimuli. 48 h (test session 1, TS1) and 96 h (test session 2, TS2) later, freezing behavior was measured and the percentage of freezing score was calculated as the percentage of time for which the mice remained immobile [40–44]. Heavy breathing, minimal movement and other movements required for normal respiration and autonomic function were considered as freezing behavior. Schematic presentation of the procedure is shown in Fig. 1A.

Preparation of hippocampal total lysates and the crude synaptosomal fraction

Hippocampi were dissected out and homogenized in a Dounce glass homogenizer with sucrose buffer (0.32 M sucrose, 4.2 mM HEPES buffer, pH7.4 with 0.1 mM CaCl2, 1 mM MgCl2 with protease and phosphatase inhibitors) and spun down at 10,000 × g for 10 min at 4 °C. The supernatant was collected, and one-fifth was used as total lysate and the remaining supernatant was centrifuged at 100,000 × g for 20 min at 4 °C. The resulting pellet containing the crude synaptosomal fraction was re-suspended in 1.5x lysis buffer (75 mM Tris, pH6.8, 15% Glycerol and 3% SDS), and protein concentration was quantified using a Bradford assay (Biorad) and protein concentrations were measured using the BioTek (Synergy 2) plate reader.
Cell culture and transfection

Neuro2A cells (ATCC, Cat# CCL-131) were cultured in 1:1 DMEM/Opti-MEM mix (Thermo Fisher Scientific) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK293 cells (ATCC, Cat# CRL1573) were cultured in DMEM with 10% fetal bovine serum plus 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific). Cells were tested periodically for mycoplasma contamination.

Primary culture of hippocampal neurons was performed as described previously [45]. Briefly, newborn (postnatal day 0) mouse hippocampi were dissected out, minced, and digested with papain for 15 min at 37 °C. Neurons (2.5 × 10⁴-1 × 10⁵ cells/well) were plated in 24-well plates with Neurobasal-A medium supplemented with 5% FBS, 2% B27, 2% glutamax and 0.2% gentamycin. Feeding medium contained Neurobasal-A medium with 2% B27, 2% glutamax, 0.2% gentamycin. Neurons were treated after being cultured for 13–14 days in vitro (DIV).

Transfection of plasmids YFP-tagged cofillinWT and cofillinS3D, cofillinS3A (Addgene) and myc tagged-spinophilin were performed using PureFec-tion™ transfection reagent (System Biosciences) according to manufacturer instructions.
buffered saline with 0.1% Tween-20 (TBST). The membrane was then incubated with proper primary antibody diluted in blocking buffer overnight at 4 °C. Next day, the membrane was extensively washed with TBST and incubated with a fluorescence- or HRP-conjugated secondary antibody. The membrane was scanned, and images were obtained using the Li-COR Odyssey system. The signal intensity was quantified using the Li-COR Image Studio software.

**Fluorescence lifetime imaging microscopy (FLIM)-fluorescence resonance energy transfer (FRET) assay**

Fluorescence lifetime imaging microscopy was used to study α2AR-spinophilin interaction in live cells as previously described [45]. Briefly, HEK293 cells plated onto 8-well micro slides were co-transfected with CFP-α2AR and YFP-spinophilin constructs. 48 h post transfection, cells were analyzed with an one-photon FLIM imaging system attached to a Zeiss LSM710 confocal microscope. FLIM-FRET efficiency (E) was calculated as: 

\[ E = 1 - \left( \frac{\text{FRET} + \text{CFP}}{\text{FRET} - \text{CFP}} \right) \]

where FRET and CFP were the CFP lifetimes obtained for cells expressing CFP and YFP (unstimulated or stimulated prior to imaging) or CFP alone, respectively [45].

**cAMP assay**

The cAMP Hunter™ CHO-K1 ADRA2A-Gi cell line was purchased from Eurofins DiscoverX. Cells were seeded into white-walled 96-well tissue culture treated plates (5 × 10^3 cells/well). After 24 h, cells were stimulated with vehicle, clonidine or guanfacine through i.p. injection. 2 h post treatment, samples were compared for statistical significance by two-tailed unpaired Student’s t-test. One-way and two-way ANOVA with post hoc multiple comparisons were applied when comparing multiple groups with one and two variables, respectively. P < 0.05 denotes significance. Data are expressed as means ± SE. Sample sizes, statistical tests used, and statistical results are indicated in the figure legends.
RESULTS
Clonidine and guanfacine show differential effects on fear memory reconsolidation and coflin activation
To better understand the conflicting clinical effects of two α2AR agonists, clonidine and guanfacine, on PTSD, we evaluated their effects on fear memory reconsolidation, following a Pavlovian fear conditioning procedure (Fig. 1A). Mice treated with clonidine immediately after re-exposed to the conditioned stimuli showed significantly less freezing time when tested in the same contextual environment than saline-treated mice in both test sessions, TS1 and TS2 (Fig. 1B). By contrast, guanfacine treatment had no effect on the freezing behavior (Fig. 1D). Furthermore, cotreatment with clonidine and guanfacine attenuated clonidine-induced disruption of fear memory reconsolidation (Fig. 1F). Neither clonidine nor guanfacine affected the amount of freezing time induced by the conditioned cue in test sessions (Fig. 1C, E). These data clearly demonstrate the distinct effects of clonidine and guanfacine on contextual fear memory reconsolidation; while clonidine effectively disrupts contextual fear memory, guanfacine acts as a competitive antagonist for this response.

α2AR is the primary α2AR subtype expressed in the brain [53]. We next tested the role of this receptor subtype in clonidine-elicited impairment of contextual memory reconsolidation using mice lacking α2AR expression (Adra2A−/−) [54]. Contrasting with observations in WT mice shown in Fig. 1, clonidine treatment immediately after reactivation of fear memory had no effect on the freezing behavior in response to the conditioned context in the test sessions in α2AR deficient mice (Fig. 1G). Intriguingly, α2AR deficient mice showed a stronger level of freezing in response to conditioned contextual (Fig. 1H), but not cued (Fig. 1I), stimuli in test sessions when compared to WT mice. Consistently, treatment with a selective α2A blocker, BRL44408, immediately after re-exposure resulted in an increased amount of freezing time in response to conditioned context on both test days (Supplementary Fig. S1). Conversely, ligands blocking the α2B or α2C receptor subtypes showed no significant effect on freezing behavior as compared to saline (Supplementary Fig. S1). Collectively, these data suggest that α2AR is a crucial regulator of contextual fear memory reconsolidation and mediates clonidine-elicited impairment of this process.

Clonidine and guanfacine share similar binding affinities at the α2AR [55]. To understand potential molecular mechanisms that could account for their distinct effects on contextual fear memory reconsolidation, we examined the abilities of these ligands to induce downstream signaling that can regulate learning and memory. We first tested two known α2AR downstream signaling effector cascades, cAMP inhibition and ERK1/2 activation. Clonidine and guanfacine exhibited similar efficacies in inhibiting cAMP levels (Supplementary Fig. S2A), and both effectively induced activation of ERK1/2 in primary neurons (Supplementary Fig. S2A). The given importance of synaptic structures in learning and memory [28, 29] and the key role of coflin in regulating dendritic spine dynamics [26, 27], we next asked whether coflin can be activated downstream of α2AR and, if so, whether there is any difference between clonidine and guanfacine in inducing this signaling event. Coflin activity is controlled by the phosphorylation status at Ser3; coflin becomes inactive when it is phosphorylated at this residue [56]. Clonidine stimulation of Neuro2a cells led to a dose-dependent reduction of phospho-coflin (at Ser3) levels compared to vehicle treatment (Fig. 2A and Supplementary Fig. S3), indicating activation of this protein by clonidine treatment. However, guanfacine stimulation failed to induce significant changes in coflin phosphorylation (Fig. 2A, B). We further validated the differential effects of these two α2AR agonists on coflin activation in human neurons. As shown in Fig. 2C, D, in human iPSC-derived neurons, clonidine treatment, but not guanfacine treatment, led to a significant reduction in coflin phosphorylation (Fig. 2C, D). We have therefore identified a novel signaling effector downstream of α2AR, namely coflin, that can be activated by clonidine, but not by guanfacine.

Clonidine induces coflin activation to regulate dendritic spine morphology in a spinophilin-dependent manner
Heterotrimeric G proteins and β arrestins are well-known signaling transducers of GPCRs. There is no significant difference between clonidine and guanfacine in inducing Gai or Gao association with α2AR (Supplementary Fig. S4). Both ligands also dose-dependently induced β-arrestin2 recruitment to the receptor (Supplementary Fig. S5A), an event critical for receptor internalization [48]. Consistently, clonidine and guanfacine induced a similar level of α2AR internalization in primary hippocampal neurons (Supplementary Fig. S5B). Our previous studies have revealed another key regulator of α2AR signaling and function, spinophilin [46, 57–60]. We found that clonidine, but not guanfacine, was able to drive rapid recruitment of spinophilin to the receptor in live cells (Fig. 2E and Supplementary Fig. S6). To determine whether spinophilin plays a role in clonidine-induced coflin activation, we examined coflin phosphorylation in primary hippocampal neurons derived from WT and spinophilin deficient (Ppp1r19b−/−) mice. While clonidine treatment induced a time-dependent reduction of phospho-coflin in WT neurons, it failed to do so in neurons lacking spinophilin (Fig. 2, F, G). Furthermore, in vivo treatment with clonidine significantly increased coflin activity (indicated by the reduced phospho-coflin level) in the hippocampus of WT, but not Ppp1r19b−/−, mice (Fig. 2H, I). These data suggest that spinophilin is required for clonidine-induced coflin activation.

Given the critical role of coflin in regulating dendritic spine morphology [26, 27], we predicted that clonidine-induced coflin activation would lead to morphological changes in dendritic spines. Indeed, in WT hippocampal neurons, clonidine treatment induced elongation of dendritic spines compared to vehicle treatment (Fig. 3A); the overall spine length (Fig. 3B) and the percentage of thin, long (>1.5 μm) spines (Fig. 3C) were significantly increased by clonidine treatment while spine densities were not changed (Fig. 3D). These clonidine-induced changes are similar to spine remodeling induced by coflin activation [33]. Consistent with its requirement in clonidine-induced coflin activation, spinophilin is also essential for clonidine-induced spine remodeling. In neurons without spinophilin expression (Ppp1r19b−/−), clonidine failed to induce changes in spine morphology (Fig. 3A, B). In addition, there appeared to be a trend of increase in spine length in spinophilin-deficient neurons as compared to WT neurons under baseline conditions (Fig. 3B). Since guanfacine did not induce coflin activation (Fig. 2A, B), we predicted that it would not alter spine morphology either. Indeed, guanfacine treatment failed to induce spine remodeling in WT hippocampal neurons (Supplementary Fig. S7).

To further validate that clonidine-induced changes in spine morphology rely on coflin activation, we examined the effects of overexpression of wild type coflin vs an inactive form of coflin bearing the S3D mutation. While in hippocampal neurons with expression of wild type coflin, clonidine treatment induces dendritic spine elongation compared to vehicle treatment, clonidine failed to do so when the coflin-S3D mutant was overexpressed in neurons (Fig. 3E, F). Taken together, these data suggest that clonidine induces coflin activation to regulate dendritic spine morphology in a spinophilin-dependent manner.

Spinophilin preferentially interacts with active coflin and is required for maintaining coflin activity and synaptic localization
Spinophilin is a scaffolding protein that contains a PDZ domain and the C-terminal end of coflin possesses a PDZ-binding motif, although interaction between these two proteins has not been reported. Our co-immunoprecipitation (IP) assays revealed that the two proteins formed a complex in the mouse brain (Fig. 4A).
We then asked whether changes in cofilin activity could affect its interaction with spinophilin. We examined cofilin interaction with the constitutively active mutant form of cofilin, cofilin-S3A, and the inactive mutant of cofilin, cofilin-S3D, and detected a significantly higher level of cofilin-S3A than cofilin-S3D in the spinophilin-IP complex (Fig. 4B, C). The level of cofilin-S3A co-immunoprecipitated with spinophilin was nearly three-fold higher versus cofilin-S3D (Fig. 4C), suggesting that spinophilin preferentially interacts with active cofilin. We further determined whether spinophilin could regulate cofilin activity by examining the level of phospho-cofilin in the hippocampus of WT and Ppp1r9b−/− (spinophilin null) mice. In mice without spinophilin expression, the level of phospho-cofilin was significantly elevated as compared to that in WT mice (Fig. 4D, E), suggesting that spinophilin not only preferentially binds active cofilin but also is required for maintaining its activity in the hippocampus.
From four independent cultures. For mice was significantly increased at both 0.5 and 2 h time points compared to the baseline level prior to re-exposure (Fig. 5A, B), suggesting that the memory reconsolidation process is accompanied by hippocampal co-inhibition.

To understand the importance of hippocampal co-inhibition activity in memory reconsolidation, we activated or inhibited co-inclusion using the co-inclusion S3 and pS3 peptide [64], respectively. TAT or TAT-fused S3 (TAT-S3) or pS3 (TAT-pS3) peptide was infused via bilateral intra-hippocampal cannula immediately after mice were re-exposed to the conditioned stimuli (see Fig. 1A). Mice treated with the TAT-S3 peptide showed a reduced amount of freezing time in response to the conditioned context in test sessions when compared to mice treated with the TAT peptide or TAT-pS3 peptide (Fig. 5C). These data suggest that hippocampal co-inclusion inactivation is required for contextual fear memory reconsolidation; elevation of co-inclusion activity by the S3 peptide effectively disrupts this process. Consistent with the notion that the hippocampus is not involved in cued fear conditioning [65], hippocampal injection of the S3 peptide had no effect on freezing time in response to the conditioned cue in test sessions (Fig. 5D).

Concurrently with the change in co-inclusion activity during the reconsolidation process, we observed alterations in co-inclusion levels at the synapse. Reactivation of fear memory induced a significant reduction in co-inclusion levels in synaptosomes of the hippocampus at the 2-h time point (Fig. 5E, F). Furthermore, this re-exposure-induced decrease in synaptic co-inclusion levels was gradually reversed on test days (Fig. 5H, I), correlated with gradually reduced memory strength on these days. Since spinophilin is important for the synaptic localization of co-inclusion, as revealed in Fig. 4, we next tested whether spinophilin plays a role in the synaptic dynamics of co-inclusion during fear memory reconsolidation. In mice lacking spinophilin expression, synaptic levels of co-inclusion were not altered after re-exposure to conditioned stimuli on any days tested (Fig. 5E, G, H), suggesting that spinophilin is required for the dynamic changes of co-inclusion at the synapse during fear memory reconsolidation.

Given the importance of spinophilin in regulating hippocampal co-inclusion dynamics revealed above, we predicted that spinophilin would play a role in contextual fear memory reconsolidation. Indeed, in Ppp1r9b null (spinophilin null) mice, the amount of freezing time in response to the conditioned context in test sessions was significantly increased as compared to WT mice (Fig. 5J), suggesting that the absence of spinophilin expression enhanced contextual fear memory reconsolidation in mice. The freezing on the reactivation day was comparable between Ppp1r9b null and WT mice (Fig. 5J), suggesting normal memory consolidation and retrieval in Ppp1r9b null mice. These data suggest that spinophilin is particularly important for regulating contextual fear memory reconsolidation while being dispensable for memory consolidation and retrieval. In mice without spinophilin expression, the reconsolidation process is enhanced, and fear memory persists despite repeated re-exposure to conditioned stimuli.

Clonidine, but not guanfacine, promotes the co-inclusion interaction with α2AAR and spinophilin to disrupt contextual fear memory reconsolidation

Because of the essential role of synaptic co-inclusion dynamics in regulating fear memory reconsolidation as revealed above, to gain mechanistic insight into the differential regulation of contextual fear memory by clonidine and guanfacine, we tested the ability of these ligands to induce the interaction between α2AAR and co-inclusion. Minimal interaction was detected between the two proteins under baseline conditions. In cells treated with clonidine, α2AAR was readily co-immunoprecipitated with co-inclusion. However, guanfacine

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**Fig. 3** Clonidine induces spine remodeling in a spinophilin- and co-inclusion-dependent manner. A-D Hippocampal neurons derived from WT or Ppp1r9b−/− mice were treated with vehicle (Veh) or clonidine (Clon, 10 µM) for 30 min, and stained for microtubule (MT, green) and actin (red). Representative images of dendritic spines are shown in A. Quantification of spine length (B), the percentages of spines at different lengths (C) and spine density (D) are also shown. **p < 0.01; ****p < 0.0001 by two-way ANOVA Sidak’s multiple comparisons test. For WT, n = 12 and 17 neurons for vehicle and clonidine, respectively, from four independent cultures. For Ppp1r9b−/−, n = 15/group from four independent cultures. E, F Co-inclusion activation is required for clonidine-induced spine remodeling. Primary hippocampal neurons derived from WT mice were transfected with YFP-tagged co-inclusion WT or S3D mutant construct, treated with vehicle or clonidine (10 µM, 30 min) and stained for YFP-co-inclusion (WT or mutant, green) and actin (red). Representative images (E) and quantification (F) of spine length are shown. **p < 0.01 by two-way ANOVA Sidak’s multiple comparisons test. N = 10/group. All data are expressed as mean ± SEM.

Since spinophilin is enriched in dendritic spines [39, 61], we further examined whether interaction with spinophilin affects co-inclusion localization at the synapse using Ppp1r9b−/− (spinophilin null) mice. The level of co-inclusion in synaptosomes isolated from these mice was significantly reduced compared to that in synaptosomes from WT mice (Fig. 4G, H), whereas co-inclusion levels in the total hippocampal lysates were comparable between WT and spinophilin deficient mice (Fig. 4D, F). Taken together, our data suggest that spinophilin plays a crucial role in retaining active co-inclusion at the synapse.

The spinophilin-dependent co-inclusion dynamics at the synapse are required for fear memory reconsolidation

Although a role for co-inclusion in regulating learning and memory has been suggested [62], its activity and function in memory reconsolidation have not been addressed thoroughly. We first tested whether co-inclusion activity is changed during the fear memory reconsolidation process in the hippocampus, a brain region that is critical for encoding and maintaining contextual fear memory [43, 63]. We examined co-inclusion phosphorylation in the hippocampus at different time points after mice were re-exposed to conditioned stimuli. Phospho-co-inclusion (i.e., inactive co-inclusion) levels were significantly increased at both 0.5 and 2 h time points compared to the baseline level prior to re-exposure (Fig. 5A, B), suggesting that the memory reconsolidation process is accompanied by hippocampal co-inclusion inactivation.
treatment failed to induce coflin interaction with the receptor (Fig. 6A, B). Furthermore, the presence of guanfacine markedly reduced the level of α2AAR-cofilin interaction induced by clonidine (Fig. 6C, D), suggesting that an antagonistic action of guanfacine in this process.

Clonidine treatment also significantly enhanced the interaction between coflin and spinophilin in cells (Supplementary Fig. S8). Furthermore, in mice treated with clonidine in vivo, the level of spinophilin in complex with coflin in the mouse hippocampus was significantly increased compared to saline treatment. By contrast, guanfacine treatment in vivo had no effect on the spinophilin-cofilin interaction (Fig. 6E, F). Taking these data together with the finding that clonidine stimulation increased the α2AAR-spinophilin interaction (Fig. 2E), these data suggest that clonidine, but not guanfacine, promotes the complex formation among α2AAR, spinophilin, and coflin to facilitate coflin activation.

We next investigated whether clonidine disrupts contextual memory reconsolidation through regulating coflin dynamics during the process. As shown above in Fig. 5A, re-exposure to conditioned stimuli resulted in coflin inactivation in the hippocampus. However, in mice receiving clonidine injection immediately after re-exposure to conditioned stimuli, the level of phospho-cofilin at the 2-hr time point was reduced to a level comparable to the basal level prior to re-exposure/reactivation (Fig. 6G, H), suggesting that clonidine treatment can efficiently prevent coflin inactivation during fear memory reconsolidation. We then determined whether the change of coflin activity is required for clonidine-elicted regulation of contextual fear memory reconsolidation. We infused, through bilateral intrahippocampal cannula, the TAT-pS3 peptide to block coflin activation, or the TAT control peptide, immediately after mice were re-exposed to conditioned stimuli and injected with clonidine or saline. In mice receiving with the TAT peptide, clonidine treatment was able to reduce the freezing time in response to the conditioned context (Fig. 6I), consistent with our data shown in Fig. 1B. However, in mice infused with the TAT-pS3 peptide, clonidine failed to elicit changes in contextual fear memory reconsolidation (Fig. 6J). These data strongly suggest that clonidine-elicted disruption of contextual fear memory reconsolidation requires coflin activation in the hippocampus.

Since clonidine induces coflin activation in a spinophilin-dependent manner, we further determined the importance of spinophilin in clonidine-elicted regulation of contextual fear memory reconsolidation. As expected, contrasting with observations in WT mice (Fig. 1B), in spinophilin deficient mice, clonidine injection immediately after reactivation showed no effect on freezing behaviors in response to contextual stimuli in test sessions compared to saline injection (Fig. 6K). These data suggest that clonidine-induced effect on contextual fear memory reconsolidation requires the presence of spinophilin.

**DISCUSSION**

In the present study, we have identified a novel α2AAR-spinophilin-cofilin axis in the hippocampus that is critical in regulating synaptic coflin dynamics and contextual fear memory reconsolidation (Supplementary Fig. S9). Fear memory reconsolidation after
re-exposure to conditioned stimuli is accompanied by and requires a reduction in cofilin activity at the synapse. Stimulation of α2AAR by clonidine, but not guanfacine, promotes the interaction of cofilin with α2AAR and spinophilin to enhance the activity and synaptic localization of cofilin. When administered immediately after re-exposure to conditioned stimuli, clonidine, but not guanfacine, disrupts reconsolidation of contextual fear memory, and this effect relies on spinophilin-dependent cofilin activation (SupplementaryFig. S9). Our study thus uncovers a new molecular mechanism that regulates fear memory reconsolidation, which will facilitate future development of therapeutic strategies for emotional disorders such as PTSD.

Recent research has suggested that intervention of the reconsolidation process after retrieval/reactivation of previously re-consolidated memories can disrupt the consolidation of untrained memories. Such an intervention might be a potential therapeutic strategy for treating memory-related disorders such as PTSD. Understanding the molecular mechanisms underlying this process can provide new insights into the development of effective therapeutic strategies. Our study highlights the importance of cofilin and spinophilin in the regulation of memory reconsolidation and opens up new avenues for the development of targeted therapeutic interventions.
respective, in J protein, co-memory is accompanied by inactivation of an actin severing remains largely elusive. We now show that reconsolidation of fear spines are reshaped to facilitate the reconsolidation process effective reconsolidation of contextual fear memory. Furthermore, established memory can result in long-lasting modification of the memory [22–24]. Although changes in actin dynamics and dendritic spine morphology are well associated with learning and memory formation [66–69], evidence regarding whether and how dendritic spines are reshaped to facilitate the reconsolidation process remains largely elusive. We now show that reconsolidation of fear memory is accompanied by inactivation of an actin severing protein, coflin, in the hippocampus, and this change is required for effective reconsolidation of contextual fear memory. Furthermore, reactivation of fear memory induces a significant reduction in synaptic localization of coflin. Changes in coflin activity and synaptic localization during reconsolidation are predicted to cause dendritic spine remodeling in this process, which warrants further investigation. Nonetheless, our study provides clear evidence suggesting modulators of actin dynamics as a promising target for modification of fear memory reconsolidation.

Despite the essential role of coflin in regulating dendritic spine structure, information regarding mechanisms that regulate its

Fig. 6 Clonidine, but not guanfacine, promotes coflin interaction with α2A-AR and spinophilin, and clonidine-elicited effects on contextual fear memory reconsolidation requires both coflin and spinophilin. A, B Clonidine stimulation increases the complex formation between coflin and α2A-AR. Neuro2A cells were stimulated with vehicle, clonidine (10 µM) or guanfacine (10 µM) for 30 min, and cell lysates were subjected to co-IP assays using a coflin antibody. Representative blots (A) and quantification of the level of α2A-AR in the IP complex (B) are shown. ***p < 0.001 by one-way ANOVA Tukey’s multiple comparisons test. N = 4/group. C, D The presence of guanfacine blocks clonidine-induced α2A-AR-coflin interaction. Cells were stimulated with vehicle, clonidine or clonidine + guanfacine for 30 min, and cell lysates were subjected to co-IP assays using a coflin antibody. Representative blots (C) and quantification of the level of α2A-AR in the IP complex (D) are shown. *p < 0.05 by one-way ANOVA Tukey’s multiple comparisons test. N = 3/group. E, F Clonidine, but not guanfacine, treatment enhances the coflin-spinophilin interaction in the mouse brain. Mice were injected i.p. with vehicle, clonidine (0.5 mg/kg) or guanfacine (0.5 mg/kg). 2 h post injection, hippocampal lysates were prepared and subjected to co-IP assays using a coflin antibody. Representative blots (E) and quantification of the level of spinophilin in coflin complex (F) are shown. *p < 0.05 versus vehicle by one-way ANOVA Tukey’s multiple comparisons. N = 3/group. G, H Clonidine treatment prevents re-exposure-induced coflin inactivation during the reconsolidation process. Clonidine (0.5 mg/kg) or saline was injected (i.p) immediately after re-exposure to conditioned stimuli. Representative Western blots (G) show the phospho- and total coflin and actin at baseline (BL) prior to re-exposure and 2 h after re-exposure on reactivation day. Quantification of phospho-coflin/coflin levels over baseline is shown in H. *p < 0.05, **p < 0.01 by two-way ANOVA Tukey’s multiple comparisons test. For WT, n = 3, 3 and 4 for baseline (BL), vehicle and vehicle and guanfacine group, respectively; for Ppp1r9b−/−, n = 5, 4 and 4 for BL, vehicle and clonidine group. I, J Cofilin activation is required for clonidine-induced disruption of contextual fear memory reconsolidation. Immediately after re-exposure to contextual and cued stimuli, mice were infused bilaterally with TAT (I) or TAT-PS3 (J) peptide through intrahippocampal cannula and injected (i.p.) with saline or coflin (0.5 mg/kg). N = 22 for TAT (with saline) and n = 8 for TAT-Clon group in I. **p < 0.001, versus TAT group by two-way ANOVA; *p < 0.05, **p < 0.01, versus TAT by Sidak’s multiple comparisons test. N = 10 and 9 for TAT-PS3 and TAT-PS3 + clonidine, respectively, in J. K Clonidine fails to alter contextual fear memory reconsolidation in Ppp1r9b−/− mice. Clonidine (0.5 mg/kg, i.p.) is administered immediately after reactivation. Freezing time is quantified in response to the conditioned context. All values are presented as mean ± SEM.
The phenomenon that clonidine and guanfacine selectively activate α2AAR signaling pathways is referred to as ligand-selective agonism, a common feature observed for GPCRs and presumably caused by distinct structural conformations of the same receptor induced when in complex with different ligands [74–76]. Consistent with this notion, our in-silico docking studies suggest differences in residual-interaction patterns between clonidine and guanfacine binding to α2AAR. Despite both ligands binding to the same site and forming a salt-bridge/hydrogen bond with residue D113 of α2AAR, the basic head of guanfacine displays a different binding pattern than the imidazole head of clonidine and forms two additional hydrogen bonds with E189 (Supplementary Fig. S10). E189 is a key orthostatic residue and part of an interaction network regulating ligand binding [77], and an α2AAR antagonist, yohimbine, is predicted to form H-bond with E189 [78]. Our experimental evidence indeed suggests antagonistic features of guanfacine in blocking clonidine-induced α2AAR cofilin interaction and in attenuating clonidine-induced disruption of fear memory reconsolidation. Intriguingly, the predicted difference in receptor binding between clonidine and guanfacine does not cause distinction in G protein coupling or arrestin interaction with α2AAR. However, it sufficiently leads to differential complex formation of the receptor with spinophilin and cofilin, suggesting a higher sensitivity of spinophilin-mediated signaling to conformational changes in α2AAR. Thus, in addition to G proteins and arrestins, spinophilin can act as effective mediator of GPCR ligand-biased signaling.

Our current study has strong clinical implications. Following global pandemics, including COVID-19, PTSD can affect over 20% of all populations, with even stronger effects on infected patients and frontline health workers [4]. The dearth of effective FDA-approved treatments has driven off-label usage of other medications, including adrenergic ligands, for PTSD treatment. Effectively repurposing existing medications for the immediate treatment of PTSD requires a better understanding of molecular and cellular mechanisms underlying drug actions. Our current study reveals a novel α2AAR-spinophilin-cofilin signaling axis that regulates contextual fear memory reconsolidation and distinguishes the efficacies of α2AAR agonists in disrupting this process. Our observation of no effect of guanfacine on fear memory reconsolidation in mice could help interpret the failure of double-blinded, placebo-controlled clinical trials with this agonist [20, 21]. On the other hand, our data support the usefulness of clonidine in treating PTSD. Although no large placebo-controlled trials have been conducted for clonidine, its usage has been observed to be effective in multiple clinical practice and trials [13–19]. Large scale, placebo-controlled clinical trials for clonidine in PTSD are warranted. If successful, clonidine would provide immediate treatment to PTSD in general populations and veterans and help combat the mental health issues associated with the COVID-19 pandemic. Furthermore, cofilin activation could provide an effective screening tool for selecting other α2AAR ligands or pharmacotherapeutic agents for PTSD treatment. Our study thus has far-reaching implications for the development of active pharmacotherapies for PTSD.

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AUTHOR CONTRIBUTIONS
QW, KJ, NL, XL conceived, designed and/or planned experiments; SS, HR, YC, CC, HW, SL performed behavioral, biochemical, cell biological and/or pharmacological experiments; SZ and CA performed in-silico modeling study, QW, KJ, SS, CC, XL analyzed the data, QW, KJ, SS, CC, XL prepared manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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