β-Arrestin–biased signaling mediates memory reconsolidation

Xing Liu, Li Ma[1], Hao Hong Li[1], Bing Huang, You Xing Li, Ye Zheng Tao, and Lan Ma[2]

The State Key Laboratory of Medical Neurobiology, School of Basic Medical Sciences and the Institutes of Brain Science, and the Collaborative Innovation Center for Brain Science, Fudan University, Shanghai 200032, China

Edited by Robert J. Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC, and approved February 26, 2015 (received for review November 26, 2014)

A long-standing hypothesis posits that a G protein-coupled signaling pathway mediates β-adrenergic nervous system functions, including learning and memory. Here we report that memory retrieval (reactivation) induces the activation of β_1-adrenergic β-arrestin signaling in the brain, which stimulates ERK signaling and protein synthesis, leading to postreactivation memory restabilization. β-Arrestin2-deficient mice exhibit impaired memory reconsolidation in object recognition, Morris water maze, and cocaine-conditioned place preference paradigms. Postreactivation blockade of both brain β-adrenergic Gs protein- and β-arrestin–dependent pathways disrupts memory reconsolidation. Unexpectedly, selective blockade of the Gs/cAMP/PKA signaling but not the β-arrestin/ERK signaling by the biased β-adrenergic ligands does not inhibit reconsolidation. Moreover, the expression of β-arrestin2 in the entorhinal cortex of β-arrestin 2–deficient mice rescues β_1-adrenergic ERK signaling and reconsolidation in a G protein pathway-independent manner. We demonstrate that β-arrestin–biased signaling regulates memory reconsolidation and reveal the potential for β-arrestin–biased ligands in the treatment of memory-related disorders.

β-arrestin2 | β-adrenergic receptor | memory reconsolidation | biased receptor signaling

Alongside classical G protein pathways, activation of G protein-coupled receptors (GPCRs) stimulates β-arrestin–dependent signaling, leading to ERK phosphorylation and other downstream events (1, 2). Biased agonists, which induce functionally selective or biased receptor states and, thus, selectively activate one of the signaling pathways, have recently been identified for several GPCRs (3). Biased receptor agonism offers theoretical guidance for the discovery of a new generation of GPCR-targeted drugs with greater efficacy but fewer adverse effects. However, the lack of knowledge about the signaling pathways specifically eliciting a beneficial effect is a major obstacle in the understanding of disease mechanisms and the development of biased drugs targeting most GPCRs, especially those expressed in the central nervous system (CNS) with psychiatric importance.

Besides their important roles in the cardiovascular and pulmonary systems, β-adrenergic receptors (β-ARs) are critically involved in CNS functions such as arousal, cognition, and stress-related behaviors (4, 5). β-Adrenergic neuronal signaling is important for neuroplasticity, including long-term potentiation (6) and memory formation (7). Accumulating cell biological evidence suggests that β-ARs also signal via G protein-independent, β-arrestin–dependent pathways (8–10). However, functions of β-AR in the CNS have been primarily ascribed to their classical role of stimulating Gs protein. The differential neurophysiological consequences for the G protein- and β-arrestin–dependent pathways, if any, have not been delineated.

A longstanding hypothesis posits that a β-AR/Gs/protein kinase A (PKA) signaling pathway mediates memory reconsolidation (11–13), a process that strengthens, updates, or erases a previously acquired memory after recall (memory reactivation). This hypothesis is largely based on observations that β-ARs and molecules in the classical GPCR signaling pathway—such as cAMP (cAMP), PKA, and cAMP response element-binding protein (CREB)—are required for reconsolidation, which was determined by using receptor antagonists, kinase inhibitors, or gene knockout mice (11, 14, 15). Most of these molecules are also required for basal neural activity or plasticity, and there has been no direct evidence demonstrating that the function of β-ARs in reconsolidation is mediated by G protein/PKA or other signaling pathway (12). In the current study, we tested the potential involvement of G protein/cAMP/PKA-dependent pathway versus β-arrestin–dependent signaling in memory reconsolidation by using object recognition paradigm.

Results

Reconsolidation of Object Recognition Memory Is Mediated by a Gs Protein-Independent β_1-AR Signaling Pathway. Mice tend to explore a novel object more than the familiar one, and this preference reflects the use of recognition memory (16). In the reconsolidation of object recognition memory (ORM) test, mice were first trained to recognize object A and object B (Fig. S1*A*), and 24 h after reexposure to the two objects to retrieve/reactivate ORM acquired in the training session, they were subjected to memory retention (reconsolidation) test. During the 5-min memory test, mice were allowed to explore a novel object (object C) and a familiar object (object A). The time spent exploring each object was recorded (Fig. S1 *A*–*G*) and the animal's preference for

Significance

β-Adrenergic receptors (β-ARs) are hormone and neurotransmitter receptors. The data we present in this paper challenge the assumption that memory reconsolidation is governed by the traditional β-AR/G protein signaling pathway. We found that memory reconsolidation is mediated by a β-arrestin–dependent β-adrenergic signaling pathway. Our experiments demonstrate that upon memory retrieval, a β1-AR/β-arrestin2/ERK pathway is activated in distinct brain areas, stimulating de novo protein synthesis and inducing postretrieval memory restabilization. Moreover, memory reconsolidation can be disrupted by propranolol, but not biased β-blockers such as carvedilol and alprenolol. Our study thus demonstrates that memory reconsolidation is mediated by a β-arrestin–biased β-adrenergic signaling pathway and reveals the therapeutic potential for β-arrestin–biased ligands in the treatment of memory-related disorders.

Author contributions: X.L. and Lan Ma designed research; X.L., Li Ma, H.H.L., B.H., and Y.X.L. performed research; X.L., Li Ma, H.H.L., B.H., and Y.Z.T. analyzed data; and X.L., Li Ma, Y.Z.T., and Lan Ma wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Li Ma and H.H.L. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: lanma@fudan.edu.cn.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1421758112/-/DCSupplemental.

object C over object A was designated as preference index and compared with those for object B over object A determined during memory reactivation process. We first examined the effect of antagonist treatment given immediately (within 2 min) after memory reactivation on ORM reconsolidation (Fig. 1*A*). Two-way repeated measures (RM) ANOVA indicates a drug treatment by session test interaction [$F_{\text{treatment}}$ (4, 93) = 15.082, $P < 0.001$, F_{session} (1, 93) = 80.298, $P < 0.001$, $F_{\text{treatment} \times \text{session}}$ (4, 93) = 13.051, $P < 0.001$, two-way RM ANOVA]. During the memory retention test, C57BL/6 mice treated with vehicle immediately after reexposed to objects A and B (memory reactivation) exhibited a preferential exploration for object C versus object A, indicating a normal object recognition memory, whereas mice i.p. administrated propranolol (a nonselective blocker of β-AR) or betaxolol (a selective β1-AR antagonist) after memory reactivation did not (Bonferroni's post hoc comparison, Fig. 1*A* and Fig. S1 *B*, *H*, and *I*). Moreover, ORM reconsolidation could not be blocked by i.p. administration of nadolol, a blood–brain barrier-impermeable β-blocker (Fig. S1*M*) or β_2-AR–selective antagonist ICI 118, 551 (Fig. 1*A* and Fig. S1*J*). These data suggest the critical involvement of brain β_1 adrenergic signaling in ORM reconsolidation.

Unexpectedly, in contrast to the strong inhibitory effect of propranolol and betaxolol, postreactivation i.p. administration of the biased β-AR ligand carvedilol, an antagonist of the G protein pathway and a weak agonist of β-arrestin–dependent ERK signaling (8, 9), failed to block ORM reconsolidation (Fig. 1*A* and Fig. S1*K*). Intracerebroventricular (i.c.v.) injection of carvedilol immediately after memory reactivation also failed to inhibit ORM reconsolidation, whereas the combined pretreatment of betaxolol and carvedilol impaired ORM reconsolidation [Fig. 1*B*, $F_{\text{treatment}}$ (1, 29) = 3.691, $P = 0.065$, F_{session} (1, 29) = 32.048, $P < 0.001$, $F_{\text{treatment} \times \text{session}}$ (1, 29) = 13.220, $P = 0.001$, two-way RM ANOVA]. The analysis of total time spent exploring each object confirmed the above results (Fig. S1 *B* and *C*). These data argue that the β-AR/β-arrestin signaling, but not the β-AR/Gs-protein signaling, is required for ORM reconsolidation.

To confirm that the Gs/cAMP/PKA pathway in the brain was selectively blocked by carvedilol administered via i.p. and i.c.v. injection during ORM reconsolidation, the level of cAMP and the activation of PKA and ERKs in the entorhinal cortex (Enc), a brain region critically involved in ORM, were determined. Upon memory reactivation, cAMP level in the Enc of C57BL/6 mice was increased and reached peak value at ∼5 min after memory reactivation (Fig. S2*A*). Administration of carvedilol via i.p. or i.c.v. abolished memory reactivation-induced cAMP accumulation and PKA activation (Fig. 1 *C* and *D* and Fig. S2 *A* and *B*), but stimulated β_1-AR–mediated ERK activation (Fig. S2 *C* and *D*). Moreover, postmemory reactivation (i.p. or i.c.v.) administration of another biased β-AR ligand alprenolol, which also selectively antagonizes Gs signaling and stimulates the β-arrestin signaling, did not inhibit memory reconsolidation either [Fig. 1*E*, $F_{\text{treatment} \times \text{session}}$ (1, 19) = 0.225, $P = 0.650$; Fig. S1*D*; Fig. 1*F*, $F_{\text{treatment}}$ (1, 21) = 0.416, $P = 0.526$, F_{session} (1, 21) = 14.413, $P = 0.001$, $F_{\text{treatment} \times \text{session}}$ (1, 21) = 4.767, $P = 0.041$; Fig. S1 *E* and *L*, two-way RM ANOVA]. The preference index for the novel object

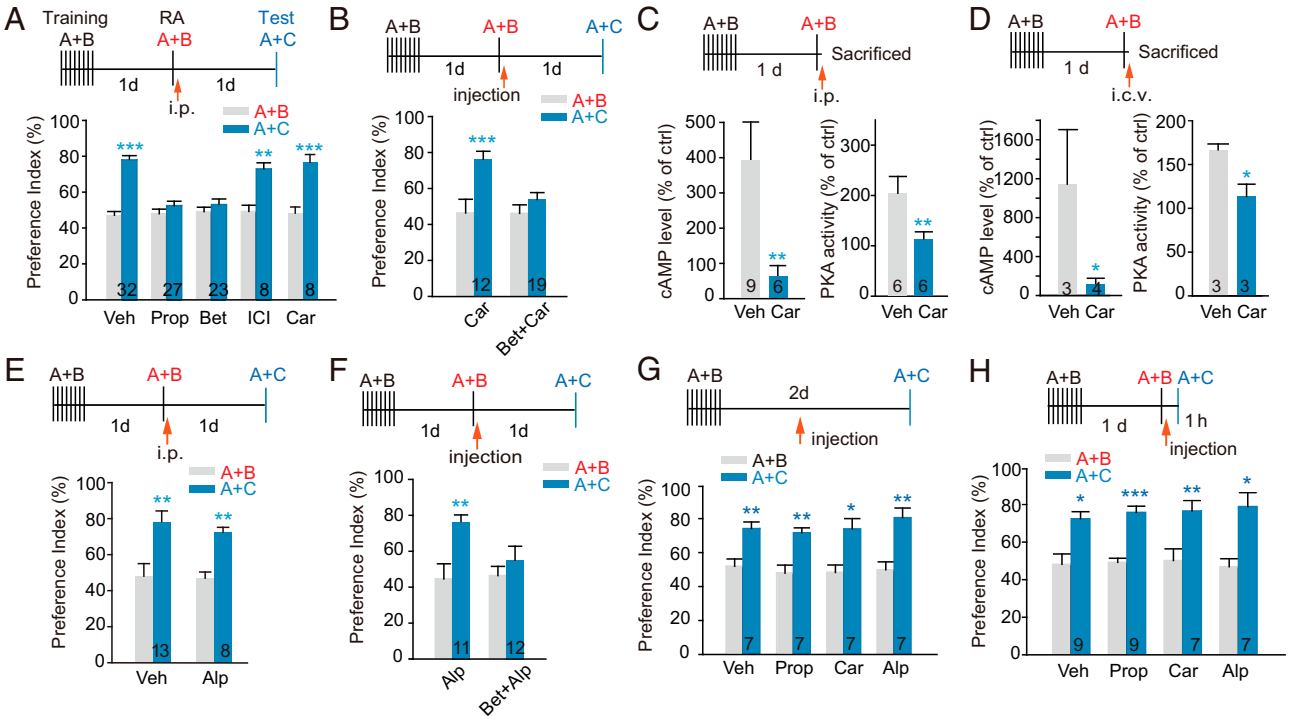

Fig. 1. ORM reconsolidation is mediated by a Gs protein-independent β_1-AR signaling pathway. Mice trained with object A and object B were reexposed to both objects (A+B) for memory reactivation (RA) 24 h later. Drug injection was given within 2 min after memory reactivation. Memory retention was tested by exposure to object A and object C (A+C). Values in the bar indicate number of mice per group. (*A* and *E*) Postreactivation i.p. injection of propranolol (Prop, 10 mg/kg) or betaxolol (Bet; 1.0 mg/kg) inhibited reconsolidation, whereas ICI 118,551 (ICI; 10 mg/kg), carvedilol (Car; 3.0 mg/kg), alprenolol (Alp; 10 mg/kg), or vehicle (Veh; 4.0 mL/kg) did not. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RA (A+B) with the same drug treatment. (*B* and *F*) Administration of Bet (1.0 mg/kg, i.p.) before Car (10 μg i.c.v.) or Alp (10 μg, i.c.v.) decreased preference index, whereas injection of Car or Alp alone did not. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. (A+B) with same drug treatment. (*C* and *D*) Injection of Car (3.0 mg/kg, i.p. or 10.0 μg, i.c.v., within 2 min after memory reactivation) decreased cAMP level and PKA activity in the Enc as determined 5 min after RA. Data are expressed as percentage of basal level determined before reactivation. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Veh, *t* test. (*G*) ORM retention was tested 2 d after training. β-Blockers were given 1 d after training without memory reactivation. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Training (A+B) with same drug treatment. (*H*) ORM retention was tested 1 h after memory reactivation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RA (A+B) within same treatment.

was not altered when propranolol, carvedilol, or alprenolol was administered at the corresponding time point but without memory reactivation [Fig. 1*G*, $F_{\text{treatment} \times \text{session}}$ (3, 24) = 0.537, $P = 0.663$, two-way RM ANOVA; Fig. S1*F*] or determined 1 h after memory reactivation [Fig. 1*H*, $F_{\text{treatment} \times \text{session}}$ (3, 28) = 0.424, $P = 0.738$; two-way RM ANOVA; Fig. S1*G*]. These data suggest that Gs/cAMP/PKA-independent β_1-AR signaling mediates ORM reconsolidation.

β-Arrestin2 Is Required for Postreactivation Memory Restabilization. We next explored the possible involvement of β-arrestin/ERK-dependent signaling. In the long-term memory test after memory reactivation, the performance of wild-type C57BL/6 ($Arrb2^{+/+}$) and β-arrestin2 knockout ($Arrb2^{-/-}$) mice was compared. $Arrb2^{+/+}$ showed significant preference for the novel object in ORM test, whereas $Arrb2^{-/-}$ mice exhibited no preference. ANOVA showed a genotype-by-memory session interaction [Fig. 2*A*, F_{genotype} (1, 28) = 9.209, $P = 0.005$; F_{session} (1, 28) = 12.076, $P = 0.002$; $F_{\text{genotype} \times \text{session}}$ (1, 28) = 18.328, $P < 0.001$ two-way RM ANOVA]. The attenuation of memory retention by β-arrestin2 ablation was memory reactivation-dependent [Fig. 2*B*, F_{genotype} (1, 27) = 0.244, $P = 0.625$; F_{session} (1, 27) = 29.791, $P < 0.001$; $F_{\text{genotype} \times \text{session}}$ (1, 27) = 0.398, $P = 0.534$, two-way RM ANOVA] and long-lasting (Fig. S3 *A* and *B*), but it was not detected within 3 h of reactivation [Fig. 2*C*, F_{genotype} (1, 16) = 0.509, $P = 0.486$; F_{session} (1, 16) = 125.929, $P < 0.001$; $F_{\text{genotype} \times \text{session}}$ (1, 16) = 0.800, $P = 0.384$, two-way RM ANOVA; Fig. S3*C*]. Both $Arrb2^{+/+}$ and $Arrb2^{-/-}$ mice could form consolidated memory 24 h after training (Fig. S3*D*). These data suggest that β-arrestin2, like β_1-AR, functions in ORM reconsolidation via restabilization of the postreactivation long-term memory. Moreover, the postreactivation treatment of carvedilol did not restore the impaired ORM reconsolidation in $Arrb2^{-/-}$ mice [Fig. 2*D*, $F_{\text{genotype} \times \text{session}}$ (1, 12) = 0.153, $P = 0.702$, two-way RM ANOVA]. The analysis of total time spent exploring each object confirmed the above results (Fig. S3 *H–O*). $Arrb2^{-/-}$ mice showed no change in locomotor activity in the open field task, whereas the treatment of propranolol did not inhibit locomotion of $Arrb2^{+/+}$ or $Arrb2^{-/-}$ mice (Table S1). No impairment of ORM reconsolidation was found in β-arrestin1 knockout ($Arrb1^{-/-}$) mice, suggesting β-arrestin1 is not critically involved in ORM reconsolidation (Fig. S3 *E–G*).

The role of β-arrestin2 in reconsolidation of spatial memory was tested in the Morris water maze task. $Arrb2^{-/-}$ mice performed comparably to $Arrb2^{+/+}$ mice in cued or spatial training (Fig. S4 *A* and *B*). Two probe trials were sequentially carried out after mice learned to find the hidden platform (Fig. 2 *E* and *F*). To avoid possible memory extinction, a short probe trial of 60 s was used as memory reactivation session, which has been shown by other groups to cause no extinction (17–19). The first probe test was carried out 1 d after spatial training, and both $Arrb2^{-/-}$ mice and wild-type littermates demonstrated a similar preference for the target quadrant. The results of the second probe test (memory retention test) revealed that $Arrb2^{-/-}$, but not $Arrb2^{+/+}$ mice, forgot the location of the platform 1 d after the first probe test [Fig. 2*E* and Fig. S4*C*, $F_{\text{target} \times \text{genotype}}$ (3, 104) = 5.038, $P = 0.003$], although both genotypes retained this information 1 h after the first probe trial [Fig. 2*F*, $F_{\text{target} \times \text{genotype}}$ (3, 88) = 2.189, $P = 0.095$]. No extinction in Probe Test 2 was detected in the wild-type littermates; however, significant decrease of preference for the target quadrant in $Arrb2^{-/-}$ mice was found in the second probe test, indicating that β-arrestin2 is required for the reconsolidation of hippocampus-dependent spatial memory.

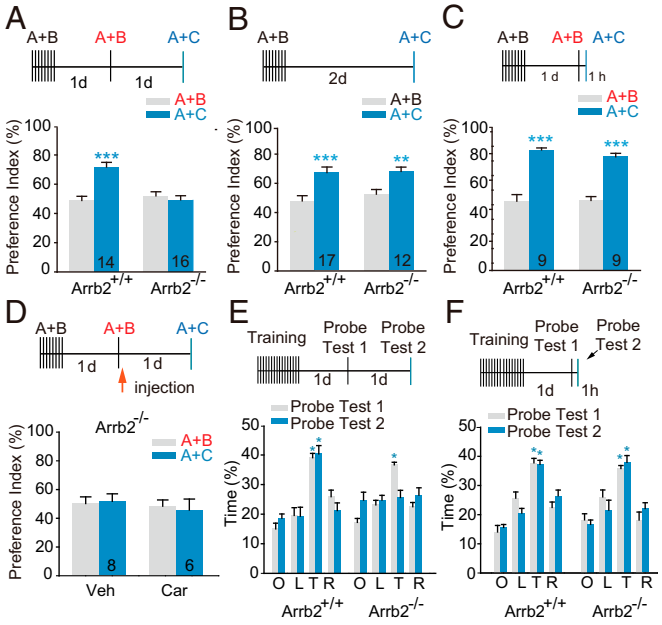

Fig. 2. β-Arrestin2 is required for postreactivation restabilization of ORM and spatial memory. $Arrb2^{+/+}$ and $Arrb2^{-/-}$ mice were tested for ORM retention 48 h after training with (*A*) or without memory reactivation (*B*), or tested 1 h after memory reactivation (*C*). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. (A+B) within the same genotype. (*D*) Reconsolidation of ORM after postreactivation carvedilol treatment in $Arrb2^{-/-}$ mice (3.0 mg/kg, i.p.). (*E* and *F*) Reconsolidation of spatial memory. Mice were trained to find the hidden platform in the Morris water maze; two probe trials were performed sequentially with the platform removed, and the percentage of time spent in each quadrant was calculated. Probe Test 1 (memory reactivation) was carried out 24 h after the last training trial. Probe Test 2 was performed 24 h (*E*) or 1 h (*F*) after Probe Test 1. Quadrants: L, left; O, opposite; R, right; T, target. $n = 6$–10 per group; $^{*}P < 0.05$ vs. O, L, and R in each probe test, three-way ANOVA.

Memory Reactivation Triggers β-Arrestin2–Mediated β-AR-ERK Translational Signaling. The activation of ERK cascade, a major target of β-arrestin–dependent signaling, was tested. Reactivation of ORM induced a time-dependent increase of ERK phosphorylation in the Enc of $Arrb2^{+/+}$ mice (Fig. S5*A*), which could be further enhanced by carvedilol treatment [Fig. 3*A* and Fig. S5*B*, $F_{\text{treatment} \times \text{session}}$ (5, 86) = 7.935, $P < 0.001$, two-way ANOVA]. The peak of ERK activation was detected 15 min after memory reactivation in the Enc, but not the ventral hippocampus or cerebellum in $Arrb2^{+/+}$ mice (Fig. S5 *A* and *C*). Postreactivation inhibition of ERK activation in the brain by U0126 blocked ORM reconsolidation (Fig. S5*D*). The increase in phosphorylation of ERK in Enc neurons induced by memory reactivation could be abolished by postreactivation administration of propranolol or betaxolol, or ablation of β-arrestin2 [Fig. 3*B* and Fig. S5*E*, $F_{\text{genotype} \times \text{treatment} \times \text{session}}$ (1, 55) = 5.726, $P = 0.020$; Fig. 3 *C* and *D*, $F_{\text{genotype} \times \text{treatment} \times \text{session}}$ (1, 36) = 5.230, $P = 0.028$, three-way ANOVA; Fig. S5*F*]. The i.c.v. injection of isoproterenol increased pERK level in the Enc of $Arrb2^{+/+}$, but not $Arrb2^{-/-}$ mice, and this increase could be suppressed by pretreatment of betaxolol (Fig. S5*G*).

Previous studies have shown that reconsolidation of spatial and fear memories requires postreactivation neurotransmission and de novo protein synthesis (20, 21), and that the binding of β-arrestin–biased ligand with AT1R stimulates ERK-dependent protein translation in HEK293 cells (22, 23). As shown in Fig. 3 *C*, *E*, and *F*, increased phosphorylation of 90-kDa ribosomal S6 kinase (p90-RSK) and eukaryotic translation initiation factor 4B (eIF4B), downstream targets of ERKs and key regulators of protein synthesis, was observed in the Enc of $Arrb2^{+/+}$ mice after ORM reactivation [Fig. 3*E*, $F_{\text{genotype} \times \text{treatment} \times \text{session}}$ (1, 36) = 11.109, $P = 0.003$; Fig. 3*F*, $F_{\text{genotype} \times \text{treatment} \times \text{session}}$ (1, 36) = 12.55, $P = 0.001$, three-way ANOVA]. In contrast, no increase in phosphorylation of ERK1/2, p90-RSK, or eIF4B was observed in

NEUROSCIENCE

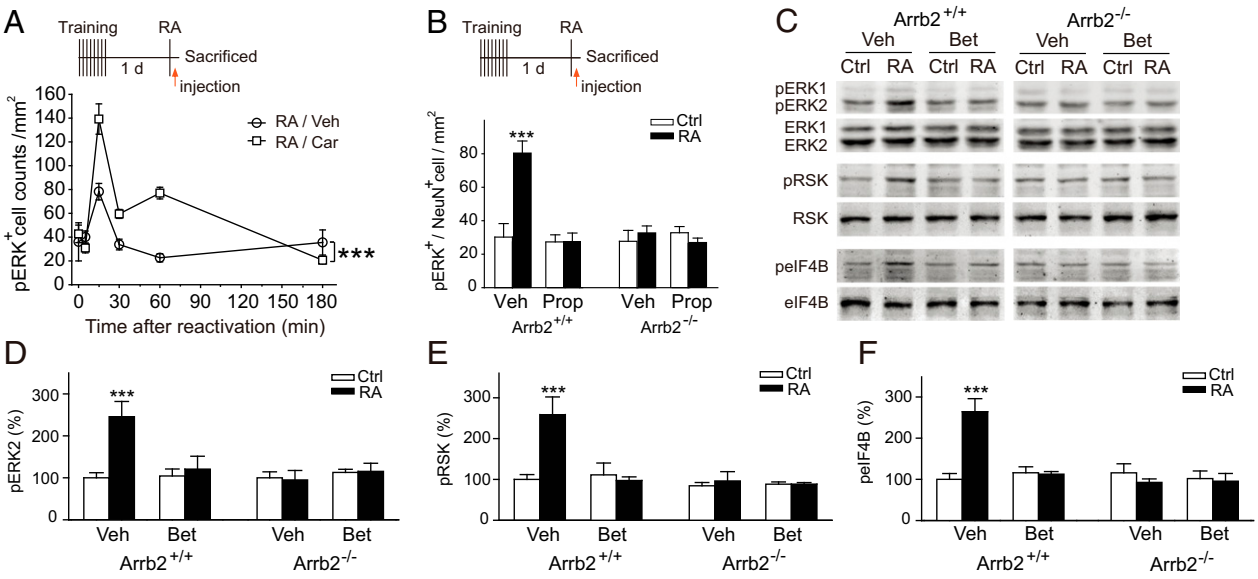

Fig. 3. Memory reactivation induces β-arrestin2–mediated β-AR-ERK translational signaling in the Enc. (*A*) Postreactivation immediate injection of Car (3.0 mg/kg, i.p.) enhanced reactivation-stimulated ERK activation in the Enc. $n = 4$–11. ***$P < 0.001$. (*B–F*) Arrb2$^{+/+}$ and Arrb2$^{-/-}$ mice were treated with β-blocker or vehicle immediately after ORM reactivation, and brain samples were collected 15 min later for analysis of phosphorylation status. (*B*) Postreactivation injection of Prop (10 mg/kg, i.p.) and ablation of β-arrestin2 inhibited reactivation-induced increase in phosphorylated ERK (pERK)/NeuN double-positive cell counts in the Enc ($n = 5$–7 mice per group). ***$P < 0.001$ vs. no RA control (Ctrl). (*C–F*) Postreactivation injection of Bet (1.0 mg/kg, i.p) and ablation of β-arrestin2 inhibited reactivation-induced increase of phosphorylation of ERK (pERK, $n = 5$–6 per group), phosphorylation of ribosomal S6 kinase (pRSK, $n = 5$–6 per group), and phosphorylation of eukaryotic translation initiation factor 4B (peIF4B, $n = 5$–6 per group) in the Enc. ***$P < 0.001$ vs. Ctrl.

Arrb2$^{-/-}$ mice, or mice treated with betaxolol after reactivation (Fig. 3 *C–F*). These data suggest that memory recall stimulates β_1-AR/β-arrestin/ERK–mediated de novo protein synthesis, which could positively regulate memory reconsolidation.

β_1-AR/β-Arrestin2/ERK Signaling Mediates Memory Reconsolidation. The physiological consequence of β-arrestin–biased β_1-AR signaling was investigated. Local viral expression of β-arrestin2/GFP, but not the control protein β-galactosidase/GFP, in the Enc of Arrb2$^{-/-}$ mice rescued the ORM reconsolidation phenotype [Fig. 4*A*, F_{genotype} (1, 64) = 5.186, $P = 0.026$; $F_{\text{viral construct}}$ (1, 64) = 2.126, $P = 0.150$; F_{session} (1, 64) = 55.898, $P < 0.001$; $F_{\text{genotype} \times \text{viral construct} \times \text{session}}$ (1, 64) = 12.642, $P < 0.001$, three-way ANOVA]. The number of β-arrestin2/GFP-expressing neurons in the Enc of these mice was positively correlated with ORM reconsolidation (Fig. 4*B* and Fig. S6*A*). Expressing β-arrestin2 in the Enc of Arrb2$^{-/-}$ mice restored β-AR–mediated ERK activation in infected neurons [Fig. 4*C* and Fig. S6*B*, $F_{\text{viral expression} \times \text{treatment}}$ (1, 20) = 5.106, $P = 0.035$ for pERK; $F_{\text{viral expression} \times \text{treatment}}$ (1, 20) = 12.494, $P = 0.002$ for pERK/GFP, two-way ANOVA]. The restored ORM reconsolidation by infection of AAV-Arr2 in the Enc of Arrb2$^{-/-}$ mice could be interrupted by postmemory reactivation treatment of betaxolol [Fig. 4*D*, $F_{\text{viral construct}}$ (1, 66) = 5.125, $P = 0.027$; $F_{\text{treatment}}$ (1, 66) = 6.300, $P = 0.014$; $F_{\text{viral construct} \times \text{session} \times \text{treatment}}$ (1, 66) = 7.993, $P = 0.006$, three-way ANOVA], just as what was observed with the AAV-Gal injected wild-type control mice (Fig. S6*C*). Similar to the results obtained with the wild-type C57BL/6 mice (Fig. 1 *B* and *F*), betaxolol plus carvedilol, but not carvedilol treatment alone showed a significant inhibition on memory reconsolidation in Arrb2$^{-/-}$ mice infected with AAV-Arr2 (Fig. S6*D*). These results indicate that the β-arrestin/ERK-biased β_1-AR signaling, but not the conventional β-adrenergic Gs/cAMP/PKA signaling in the Enc mediates reconsolidation of ORM.

Combining memory reactivation with the pharmacological disruption of reconsolidation was recently proposed as a strategy to treat drug addiction and fear-related disorders, including posttraumatic stress disorder; however, the distinct receptor-mediated signaling pathway responsible for reconsolidation has not been identified. We hypothesized that β-arrestin–biased, and not Gs protein-mediated signaling, also underlies reconsolidation of drug-conditioned place preference (CPP) and conditioned fear memories, and possibly other types of memories. Consistent with the results of ORM, Arrb2$^{-/-}$ mice showed reduced preference for the drug-paired chamber 1 d after reactivation of a cocaine-associated memory [Fig. 4*E*, *Left*, $F_{\text{genotype} \times \text{session}}$ (1, 33) = 6.028, $P = 0.020$, two-way RM ANOVA]. The reconsolidation of cocaine-related memory was disrupted by postreactivation injection of propranolol, but not carvedilol [Fig. 4*E*, *Right*, $F_{\text{treatment} \times \text{session}}$ (2, 70) = 6.820, $P = 0.003$, two-way RM ANOVA]. The involvement of β-arrestin–biased signaling was also shown in conditioned fear memory model. During training phase, the freezing levels before and right after footshock were not different between Arrb2$^{+/+}$ and Arrb2$^{-/-}$ mice (Fig. S6*E*). In the memory retention test, propranolol and betaxolol inhibited, whereas carvedilol and alprenolol enhanced, freezing behavior when memory retention was tested 1 d after reexposure to the context, in which a one-trial fear training was given [Fig. 4*F*, $F_{\text{treatment} \times \text{session}}$ (4, 68) = 17.249, $P < 0.001$, two-way RM ANOVA].

Discussion

Consolidated memories are transiently destabilized upon reactivation, and subsequently restabilized through reconsolidation, an active, de novo protein synthesis-dependent process (21, 24, 25). Studies have shown that blocking β-adrenergic transmission by propranolol impairs memory reconsolidation in auditory fear conditioning, spatial radial maze, and cocaine-induced CPP (12, 26–30); inhibition of basal and stimulated activities of certain components of G protein-coupled pathways associated with neuroplasticity, such as PKA, ERK, and CREB, disrupts reconsolidation of fear and object recognition memories (31–34). In cocaine-associated reward memory task, bilateral intra-amygdalar infusions of the PKA inhibitor Rp-cAMPS following light/tone stimulus reactivation decreases subsequent cue-induced

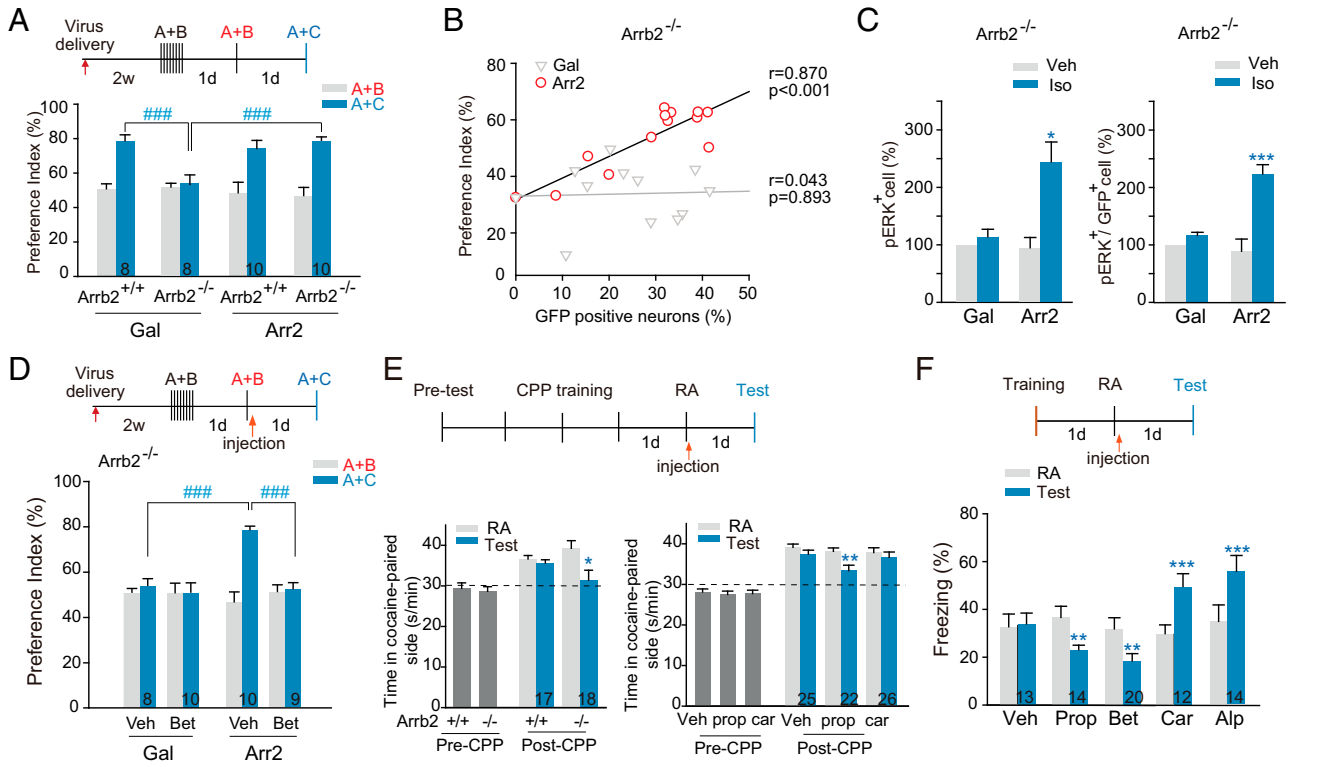

Fig. 4. β_1-AR/β-arrestin2/ERK signaling mediates memory reconsolidation. (*A*) Reconsolidation of ORM in Arrb2$^{+/+}$ and Arrb2$^{-/-}$ mice infected with AAV-encoding β-galactosidase (Gal) or β-arrestin2 (Arr2) in the Enc. $^{###}P < 0.001$ vs. indicated groups. (*B*) Plot of preference index for object C against percentage of GFP-positive neurons in the Enc of Arrb2$^{-/-}$ mice infected with Gal or Arr2. Each symbol represents data obtained from a single animal. (*C*) Levels of pERK- and pERK/GFP-positive cells in the Enc of Arrb2$^{-/-}$ mice locally infected with virus 15 min after i.c.v. injection of vehicle or isoproterenol (Iso, 10 μg). $n = 4$–9. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. Veh. (*D*) Reconsolidation of ORM. Arrb2$^{-/-}$ mice expressing Gal or Arr2 in the Enc were given Bet (1.0 mg/kg, i.p.) or Veh injection after memory reactivation. $^{###}P < 0.001$ vs. indicated groups. (*E*) Reconsolidation of cocaine CPP memory. Time (s/min) spent in drug-paired side before and after 3 d of place preference conditioning of cocaine (10 mg/kg) was presented. (*Left*) Arrb2$^{+/+}$ and Arrb2$^{-/-}$ mice were tested. (*Right*) Wild-type mice were injected with Prop (10 mg/kg, i.p.) or Car (10 μg, i.c.v.) after memory reactivation. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RA. (*F*) Reconsolidation of contextual fear memory. Postreactivation treatment with Prop (10 mg/kg, i.p.) or Bet (1.0 mg/kg, i.p.) inhibited freezing, whereas Car (10 μg, i.c.v.) or Alp (10 μg, i.c.v.) increased cue-induced freezing as determined 24 h after. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. RA.

reward memory reconsolidation (35). Activation of amygdalar PKA is sufficient to enhance memory only when it is retrieved; in contrast, PKA inhibition impaired reconsolidation (11). Although it has also been reported that memory reconsolidation requires protein synthesis but not PKA activation 24 h after training (36), the classical G protein/cAMP/PKA signaling pathway has been proposed to mediate the function of β-ARs in memory (6), and the signaling pathways leading to protein synthesis and memory restabilization are not clear. In this study, we showed that upon reactivation of a particular memory trace, β_1-AR/β-arrestin2/ERK signaling and downstream effectors involved in protein translation, such as p90RSK and eIF4B (9, 37), are activated in a distinct brain area. We found that this pathway, but not the conventional Gs protein-coupled PKA pathway, mediates memory reconsolidation, revealing an unexpected role of β-arrestin–biased signaling in brain physiology. Our data suggest that memory reactivation-triggered β_1-AR/β-arrestin2/ERK signaling positively regulates postreactivation protein synthesis and memory restabilization. Our data showed that in parallel to β-arrestin-dependent ERK activation, reactivation of ORM induced an increase of cAMP production and PKA activation in the Enc; however, selective blockade of β-AR–mediated Gs/PKA signaling by G protein-biased β-AR antagonist failed to inhibit ORM reconsolidation. The physiological consequence of memory reactivation-stimulated neuronal β-AR/G protein signaling remains to be investigated.

The signaling pathways that mediate or regulate memory reconsolidation are therefore potential pharmacological targets for memory enhancement or erasure. It has been reported earlier that in HEK293 cells stably expressing β-AR, carvedilol and alprenolol can stimulate ERK1/2, but have inverse efficacy for Gs-dependent adenylyl cyclase activation (8, 9). Consistently, our results showed that carvedilol enhanced memory reactivation-induced ERK activation and inhibited cAMP accumulation and PKA activation in the brain. Our results suggest that β-arrestin–dependent adrenergic signaling regulates postreactivation memory retention, which implicates that agents up-regulating this pathway may improve memory.

In therapeutic contexts, disrupting the process of reconsolidation could change or erase pathophysiological memories (28, 30, 38). Blockade of β-AR signaling disrupts reconsolidation of memory for learned behaviors. In human and clinical studies, administration of propranolol before memory reactivation suppresses the behavioral expression of the fear memory (39), and postreactivation treatment reduces symptoms of posttraumatic stress disorder (40, 41). In animal studies, propranolol has been shown to inhibit reconsolidation of cocaine- and morphine-conditioned place preference (27, 42). Postreactivation propranolol administration also attenuates reconsolidation of memories for craving and cue reactivity in cocaine addicts and abstinent heroin addicts (31, 43). Previous studies have shown that Arrb2$^{-/-}$ mice respond well to cocaine in CPP (44), but not to amphetamine-induced locomotor activity, compared with wild-type mice (45). In this study, we show that Arrb2$^{-/-}$ mice could acquire cocaine CPP, but exhibit impaired reconsolidation of CPP. Moreover,

propranolol, but not carvedilol inhibits reconsolidation of CPP and conditioned fear memory. The proposal that the function of β-AR in reconsolidation is regulated by β-arrestin/ERK signaling, not the conventional Gs protein/PKA pathway, suggests that β-AR/β-arrestin pathway may be an effective target of β-blockers in pharmacological intervention of memory reconsolidation. To our knowledge, β-arrestin–biased β-AR antagonist has not been reported. Our results highlight a need to develop novel β-blockers with specific β-arrestin–biased antagonism, which may produce fewer side effects than antagonists against both the G protein- and β-arrestin–dependent pathway for the treatment of posttraumatic stress disorder and drug addiction.

In addition, β-blockers are routinely used to treat hypertension and heart failure. Consistent with the report that β-blockers can aggravate memory loss in cognitively impaired elderly patients and cause clinically significant side effects (46), our data suggest that chronic use of β-blockers that antagonize both G protein and β-arrestin signaling and are capable of penetrating the blood–brain barrier may have secondary effects on cognitive processes.

Materials and Methods

Object Recognition Memory Task. Mice were submitted to a 30-min familiarization session daily in the empty arena for 3 d. In the extensive training session, mice were exposed to Object A and Object B for 4 blocks of two 5-min trials, with 60-min interval between blocks and 15-min interval between trials. In the memory reactivation session, mice were reexposed to Object A and Object B for 5 min 24 h after training to reactivate the memory trace. To test reconsolidation of memory for objects A and B, a 5-min memory retention test was carried out 1 h, 3 h, 24 h, or 6 d after reactivation by presenting mice with a duplicate of Object A and a novel object (Object C) in the same location of Object B.

Additional Methods. The memory tasks, cannula implantation and drug delivery, Western blotting, cAMP assay, PKA activity assay, immunohistochemistry, viral constructs, and microinjection methods are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. R. J. Lefkowitz (Duke University Medical Center) for Arrb1$^{-/-}$ and Arrb2$^{-/-}$ mice. This research was supported by Ministry of Science and Technology Grants 2014CB942801 (to Lan Ma) and 2013CB835102 (to X.L.), and National Natural Science Foundation of China Grants 31371136 (to X.L.) and 91232307, 31430033, and 31421091 (to Lan Ma).

1. Rajagopal S, Rajagopal K, Lefkowitz RJ (2010) Teaching old receptors new tricks: Biasing seven-transmembrane receptors. *Nat Rev Drug Discov* 9(5):373–386.
2. Shukla AK, Xiao K, Lefkowitz RJ (2011) Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem Sci* 36(9):457–469.
3. Whalen EJ, Rajagopal S, Lefkowitz RJ (2011) Therapeutic potential of β-arrestin- and G protein-biased agonists. *Trends Mol Med* 17(3):126–139.
4. Kopin IJ (1984) Avenues of investigation for the role of catecholamines in anxiety. *Psychopathology* 17(Suppl 1):83–97.
5. McCormick DA, Pape HC, Williamson A (1991) Actions of norepinephrine in the cerebral cortex and thalamus: implications for function of the central noradrenergic system. *Prog Brain Res* 88:293–305.
6. O'Dell TJ, Connor SA, Gelinas JN, Nguyen PV (2010) Viagra for your synapses: Enhancement of hippocampal long-term potentiation by activation of beta-adrenergic receptors. *Cell Signal* 22(5):728–736.
7. Cahill L, Prins B, Weber M, McGaugh JL (1994) Beta-adrenergic activation and memory for emotional events. *Nature* 371(6499):702–704.
8. Wisler JW, et al. (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. *Proc Natl Acad Sci USA* 104(42):16657–16662.
9. Kim IM, et al. (2008) Beta-blockers alprenolol and carvedilol stimulate beta-arrestin-mediated EGFR transactivation. *Proc Natl Acad Sci USA* 105(38):14555–14560.
10. Patel PA, Tilley DG, Rockman HA (2008) Beta-arrestin-mediated signaling in the heart. *Circ J* 72(11):1725–1729.
11. Tronson NC, Wiseman SL, Olausson P, Taylor JR (2006) Bidirectional behavioral plasticity of memory reconsolidation depends on amygdalar protein kinase A. *Nat Neurosci* 9(2):167–169.
12. Johansen JP, Cain CK, Ostroff LE, LeDoux JE (2011) Molecular mechanisms of fear learning and memory. *Cell* 147(3):509–524.
13. Stern SA, Alberini CM (2013) Mechanisms of memory enhancement. *Wiley Interdiscip Rev Syst Biol Med* 5(1):37–53.
14. Besnard A, Caboche J, Laroche S (2013) Recall and reconsolidation of contextual fear memory: differential control by ERK and Zif268 expression dosage. *PLoS ONE* 8(8): e72006.
15. Tronson NC, et al. (2012) Distinctive roles for amygdalar CREB in reconsolidation and extinction of fear memory. *Learn Mem* 19(5):178–181.
16. Antunes M, Biala G (2012) The novel object recognition memory: Neurobiology, test procedure, and its modifications. *Cogn Process* 13(2):93–110.
17. Suzuki A, et al. (2004) Memory reconsolidation and extinction have distinct temporal and biochemical signatures. *J Neurosci* 24(20):4787–4795.
18. Artinian J, De Jaeger X, Fellini L, de Saint Blanquat P, Roullet P (2007) Reactivation with a simple exposure to the experimental environment is sufficient to induce reconsolidation requiring protein synthesis in the hippocampal CA3 region in mice. *Hippocampus* 17(3):181–191.
19. Artinian J, et al. (2008) Protein degradation, as with protein synthesis, is required during not only long-term spatial memory consolidation but also reconsolidation. *Eur J Neurosci* 27(11):3009–3019.
20. Morris RG, et al. (2006) Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron* 50(3):479–489.
21. Nader K, Schafe GE, Le Doux JE (2000) Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature* 406(6797):722–726.
22. Hansen JL, et al. (2009) Lack of evidence for AT1R/B2R heterodimerization in COS-7, HEK293, and NIH3T3 cells: How common is the AT1R/B2R heterodimer? *J Biol Chem* 284(3):1831–1839.
23. Doyère V, Debiec J, Monfils MH, Schafe GE, LeDoux JE (2007) Synapse-specific reconsolidation of distinct fear memories in the lateral amygdala. *Nat Neurosci* 10(4): 414–416.
24. Tronson NC, Taylor JR (2007) Molecular mechanisms of memory reconsolidation. *Nat Rev Neurosci* 8(4):262–275.
25. Chen X, et al. (2005) PI3 kinase signaling is required for retrieval and extinction of contextual memory. *Nat Neurosci* 8(7):925–931.
26. Bernardi RE, Lattal KM, Berger SP (2006) Postretrieval propranolol disrupts a cocaine conditioned place preference. *Neuroreport* 17(13):1443–1447.
27. Diergaarde L, Schoffelmeer AN, De Vries TJ (2006) Beta-adrenoceptor mediated inhibition of long-term reward-related memory reconsolidation. *Behav Brain Res* 170(2):333–336.
28. Przybyslawski J, Roullet P, Sara SJ (1999) Attenuation of emotional and nonemotional memories after their reactivation: role of beta adrenergic receptors. *J Neurosci* 19(15): 6623–6628.
29. Debiec J, Ledoux JE (2004) Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience* 129(2):267–272.
30. Gazarini L, Stern CA, Carobrez AP, Bertoglio LJ (2013) Enhanced noradrenergic activity potentiates fear memory consolidation and reconsolidation by differentially recruiting α1- and β-adrenergic receptors. *Learn Mem* 20(4):210–219.
31. Zhao LY, et al. (2011) Effects of β-adrenergic receptor blockade on drug-related memory reconsolidation in abstinent heroin addicts. *Drug Alcohol Depend* 118(2-3): 224–229.
32. Duvarci S, Nader K, LeDoux JE (2005) Activation of extracellular signal-regulated kinase- mitogen-activated protein kinase cascade in the amygdala is required for memory reconsolidation of auditory fear conditioning. *Eur J Neurosci* 21(1):283–289.
33. Kelly A, Laroche S, Davis S (2003) Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase in hippocampal circuitry is required for consolidation and reconsolidation of recognition memory. *J Neurosci* 23(12):5354–5360.
34. Miller CA, Marshall JF (2005) Molecular substrates for retrieval and reconsolidation of cocaine-associated contextual memory. *Neuron* 47(6):873–884.
35. Sanchez H, Quinn JJ, Torregrossa MM, Taylor JR (2010) Reconsolidation of a cocaine-associated stimulus requires amygdalar protein kinase A. *J Neurosci* 30(12):4401–4407.
36. Kemenes G, Kemenes I, Michel M, Papp A, Müller U (2006) Phase-dependent molecular requirements for memory reconsolidation: Differential roles for protein synthesis and protein kinase A activity. *J Neurosci* 26(23):6298–6302.
37. Tilley DG, Kim IM, Patel PA, Violin JD, Rockman HA (2009) beta-Arrestin mediates beta1-adrenergic receptor-epidermal growth factor receptor interaction and downstream signaling. *J Biol Chem* 284(30):20375–20386.
38. Sara SJ (2000) Retrieval and reconsolidation: Toward a neurobiology of remembering. *Learn Mem* 7(2):73–84.
39. Kindt M, Soeter M, Vervliet B (2009) Beyond extinction: Erasing human fear responses and preventing the return of fear. *Nat Neurosci* 12(3):256–258.
40. Pitman RK, et al. (2002) Pilot study of secondary prevention of posttraumatic stress disorder with propranolol. *Biol Psychiatry* 51(2):189–192.
41. Donovan E (2010) Propranolol use in the prevention and treatment of posttraumatic stress disorder in military veterans: Forgetting therapy revisited. *Perspect Biol Med* 53(1):61–74.
42. Robinson MJ, Franklin KB (2007) Central but not peripheral beta-adrenergic antagonism blocks reconsolidation for a morphine place preference. *Behav Brain Res* 182(1):129–134.
43. Saladin ME, et al. (2013) A double blind, placebo-controlled study of the effects of post-retrieval propranolol on reconsolidation of memory for craving and cue reactivity in cocaine dependent humans. *Psychopharmacology (Berl)* 226(4):721–737.
44. Bohn LM, et al. (2003) Enhanced rewarding properties of morphine, but not cocaine, in beta(arrestin)-2 knock-out mice. *J Neurosci* 23(32):10265–10273.
45. Beaulieu JM, et al. (2005) An Akt/beta-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* 122(2):261–273.
46. Gliebus G, Lippa CF (2007) The influence of beta-blockers on delayed memory function in people with cognitive impairment. *Am J Alzheimers Dis Other Demen* 22(1): 57–61.