Regulation of amygdalar PKA by β-arrestin-2/phosphodiesterase-4 complex is critical for fear conditioning

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β-arrestins, key regulators of receptor signaling, are highly expressed in the central nervous system, but their roles in brain physiology are largely unknown. Here we show that β-arrestin-2 is critically involved in the formation of associative fear memory and amygdalar synaptic plasticity. In response to fear conditioning, β-arrestin-2 translocates to amygdalar membrane where it interacts with PDE-4, a cAMP-degrading enzyme, to inhibit PKA activation. *Arrb2*$^{-/-}$ mice exhibit impaired conditioned fear memory and long-term potentiation at the lateral amygdalar synapses. Moreover, expression of the β-arrestin-2 in the lateral amygdala of *Arrb2*$^{-/-}$ mice, but not its mutant form that is incapable of binding PDE-4, restores basal PKA activity and rescues conditioned fear memory. Taken together, our data demonstrate that the feedback regulation of amygdalar PKA activation by β-arrestin-2 and PDE-4 complex is critical for the formation of conditioned fear memory.

β-arrestins interact with most seven-membrane-spanning receptors following their activation by neurotransmitters, cytokines, or hormones to promote receptor endocytosis and inhibit receptor signal transduction. They also function as scaffolding/signaling molecules to recruit Src, MAP kinases, MDM2, IκBα, and Akt, and are involved in mediating cross-talk to other signal pathways. β-arrestins play a critical role in a wide variety of physiological and pathophysiological cellular processes (1)

Although ubiquitously expressed, β-arrestins are found in high abundance in the immune and central nervous systems (2, 3). The critical roles for β-arrestins in innate immune responses and autoimmunity have been demonstrated in many studies. β-arrestin-2 negatively regulates CXCR2-mediated neutrophil chemotaxis (4) and plays a role in allergic-asthmatic inflammation (5). The interaction of β-arrestin-2 with TRAF6 negatively modulates Toll-like receptor signaling in innate immunity (6). β-arrestin-1 functions as a positive regulator for CD4$^+$ T cell survival and autoimmunity (7). *Arrb2*$^{-/-}$ natural killer cells display enhanced killing activity and *Arrb2*$^{-/-}$ mice are less susceptible to MCMV challenge (8). Whereas, the functions of β-arrestins in brain physiology are largely unknown. It has been shown that morphine-induced analgesia is enhanced in *Arrb2*$^{-/-}$ mice (9). Our earlier work demonstrated that in response to opiate stimulation, β-arrestin-1 recruits P300 and activates gene transcription (10). Up-regulation of β-arrestin-2 was observed in the striatum of patients of Parkinson's disease with dementia (11). Recent study has revealed that dopaminergic signaling induces the formation of the β-arrestin-2-Akt-PP2A complex and thus modulates the effect of lithium in psychiatric disorders (12, 13). The emerging evidence implicates that β-arrestin-2 may play an important role in regulating basic brain functions.

The lateral nucleus of the amygdala (LA) is a core locus involved in fear memories. Fear conditioning is a classic experimental model for emotional associative memory. The structural integrity of the LA is essential for the establishment of fear conditioning (14). Evidence has shown that fear conditioning induces synaptic plasticity in LA neurons of both thalamo- and cortico-amygdala pathways (15, 16), and disruption of these two pathways or plasticity in the LA neurons blocks fear conditioning (17).

Accumulating evidence reveals that the formation of associative fear memory involves multiple signal cascades including cAMP/PKA and ERK/MAPK. Perfusion of PKA or ERK inhibitor into the LA before fear conditioning results in the impairment of the fear memory (18, 19). The cAMP response element binding protein (CREB), a key target of PKA, is also activated in the amygdala after fear conditioning (20). Furthermore, protein kinases, such as PKC, CaMKII, PI-3 kinase, and Akt, which are known to converge on the MAPK signaling pathway, have been shown to be critically involved in associative fear memory (21).

cAMP-specific phosphodiesterase 4 (PDE-4) degrades cAMP and thus underpins compartmentalized cAMP/PKA signaling. Houslay and colleges demonstrated that inhibition of PDE-4 affects CNS responses including depression, schizophrenia and memory (22–26). β-arrestin-2 can form stable complex with PDE-4s (27, 28), and PDE-4D5 is the preferred binding partner of β-arrestin-2 (29). In this study, we reveal a role of β-arrestin-2/PDE-4D complex in feedback regulation of amygdalar PKA activity in response to fear conditioning and demonstrate β-arrestin-2 is critical for the formation of associative fear memory.

Results

Conditioned Fear Memory is Impaired in Arrb2$^{-/-}$ Mice. The performance of *Arrb2*$^{-/-}$ mice in fear conditioning test was examined first. Mice were exposed to a single pairing of a tone (conditional stimulus, CS) with a foot shock (unconditional stimulus, US) and were then re-exposed to the conditioning chamber in the absence of CS 1 h or 1 day after training to evaluate the contextual fear memory. As shown in Fig. 1*A*, when exposed to the same context where they received the foot shock, the wild-type (WT) mice spent significantly longer time freezing than they did 30-s post-pairing (ANOVA, 1 h, $F_{1, 22} = 32.74$; 1 day, $F_{1, 22} = 230.72$; $P < 0.001$). In contrast, *Arrb2*$^{-/-}$ (KO) mice exhibited no significant increase in freezing when tested 1 h after training, as compared to the result obtained when tested 30 s after pairing, and significantly less freezing response 1 h and 1 day after training than their wild-type counterpart (Fig. 1*A*, two-way ANOVA, interaction of genotype × times, $F_{3, 88} = 25.31$, $P < 0.001$; post-hoc analysis of genotype effect, 30 s, $F_{1, 22} = 0.00033$,

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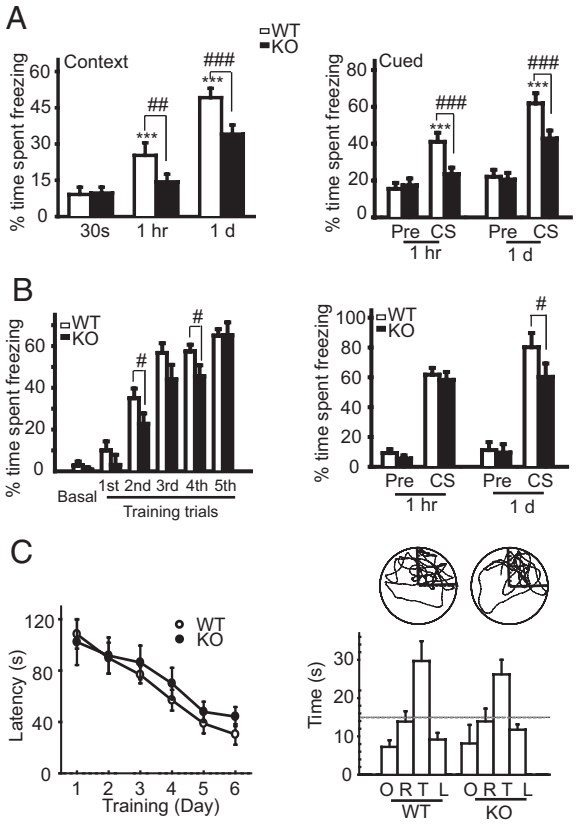

Fig. 1. *Arrb2*$^{-/-}$ mice are deficient in fear memory. (*A*) Fear conditioning using a 1× CS-US pairing protocol; $n = 12$ per group; ***$P < 0.001$ versus training group, ##$P < 0.01$, ###$P < 0.001$ versus WT mice; Context or Cued: contextual or cued fear conditioning; Pre: before presentation of tone; CS: conditioned stimulus (i.e., tone). (*B*) Fear conditioning using a 5× CS-US pairings protocol. $n = 13$ per group. ***$P < 0.001$ versus each relative Pre group; #$P < 0.05$ versus WT mice. (*C*) Escape latencies during training (left), spatial preferences and representative paths (right) in probe trial after training in Morris water maze. L, O, and R represent left, opposite, and right of the target quadrant (T); WT, $n = 14$; KO, $n = 12$.

$P = 0.986$; 1 h, $F_{1, 22} = 10.76$, $P = 0.003$; 1 day, $F_{1, 22} = 85.01$, $P < 0.001$). Mice were next placed in a conditioning chamber with novel context and presented with the same tone as in the training to test amygdala-dependent cued fear memory. Compared with WT mice, *Arrb2*$^{-/-}$ mice displayed decreased freezing at the onset of the tone given 1 h and 1 day after training (Fig. 1*A*, two-way ANOVA, interaction of genotype × times in CS, $F_{2, 66} = 1.55$, $P = 0.220$; post-hoc analysis of genotype effect in CS groups, 1 h, $F_{1, 22} = 103.21$; 1 day, $F_{1, 22} = 42.85$; $P < 0.001$).

Possible involvement of β-arrestin-2 in conditioned fear memory was further examined using an intense training paradigm, including five tone-foot shock pairing trials. As shown in Fig. 1*B*, WT mice displayed an increase in freezing from the first to the third trials and then their responses remained at the same level until the last trial. The freezing response of *Arrb2*$^{-/-}$ mice reached the level of WT at the fifth training trial (ANOVA, fifth trial, $P = 0.386$), but these mice apparently learned at a slower rate, as less freezing than WT mice was shown across the tests of the second to fourth trials (ANOVA, second trial, $P = 0.025$; third trial, $P = 0.145$, fourth trial, $P = 0.044$). *Arrb2*$^{-/-}$ mice showed similar freezing in response to the tone when tested 1 h after the intense training, but significantly reduced freezing 1 day after the training (ANOVA, genotype effects in CS groups, $F_{1, 24} = 6.02$, $P = 0.022$). The thresholds of electric shock required to elicit movement, vocalization, or jump responding were determined and the results indicated that the decreased freezing observed in the *Arrb2*$^{-/-}$ mice was not due to the reduced sensitivity to the electric shock (Fig. S1*A*). Taken together, these results suggest that β-arrestin-2 is important for the acquisition and consolidation of fear memories.

The performance of *Arrb2*$^{-/-}$ mice in the spatial memory task of the Morris water maze was also tested. Mice were trained to learn the position of the hidden escape platform in a circular pool and tested for their ability to navigate with the assistance of the cues surrounding the pool. Both the wild-type and *Arrb2*$^{-/-}$ mice showed a similar level of decrease in escape latency for reaching the hidden platform during 6 days of training and a similar level of the preference for the target quadrant during the probe trial on the last day of the experiment (Fig. 1*C*). Furthermore, the normal performance of *Arrb2*$^{-/-}$ mice in the open field and elevated plus maze (Fig. S1 *B* and *C*), two models of anxiety-considered as fear without clearly associated stimulus revealed unaltered anxiety level in these mice.

Amygdalar Synaptic Plasticity Is Impaired in Arrb2$^{-/-}$ Mice. Amygdalar LTP has been implicated in the formation of conditioned fear memory (30). We therefore examined whether β-arrestin-2 plays a role in amygdalar synaptic plasticity. The levels of the synaptic-related proteins AMPARs-GluR1 and GluR2 were not significantly different in hippocampus and amygdala of WT and Arrb2$^{-/-}$ mice (Fig. S2 *A* and *C*). The mRNA levels of amygdalar and hippocampal AMPARs and NMDARs were also unaltered in Arrb2$^{-/-}$ mice (Fig. S2*B*). Furthermore, the brain size or gross morphology of Arrb2$^{-/-}$ mice showed no detectable abnormalities. Data of ultrastructure of hippocampal and lateral amygdalar synapses also show normal appearing synapses and postsynaptic densities (Fig. S2 *D* and *E*). LTP from two independent inputs, cortical and thalamic, which carry auditory signals essential for fear conditioning to individual neurons in the LA, was recorded in slices in the presence of picrotoxin (100 μM). As shown in Fig. 2 *A* and *B* when measured 45–50 min after LTP induction, the thalamo-amygdala EPSC (Fig. 2*A*) was potentiated to 154 ± 7% of its initial value in the WT mice. In *Arrb2*$^{-/-}$ mice, however, LTP was depressed to 117 ± 4% (*t*-test, $P < 0.001$). Similarly, impaired LTP in the cortico-amygdala pathway was also observed in *Arrb2*$^{-/-}$ mice (Fig. 2*B*, *t*-test, $P < 0.001$). The effect of β-arrestin-2 deficiency on basal synaptic properties was also examined. As shown in Fig. 2*D*, Arrb2$^{-/-}$ mice displayed decreased paired pulse ratio (PPR), an index of presynaptic release probability, at cortico-amygdala synapses (1.66 ± 0.23 for WT; 1.16 ± 0.06 for KO; *t*-test, $P < 0.05$), but unaltered PPR at the thalamo-amygdala synapses (Fig. 2*C*) (1.17 ± 0.35 for WT and 1.11 ± 0.15 for KO; *t*-test, $P = 0.651$). Furthermore, the current-voltage curves of NMDAR and AMPAR were unaltered in Arrb2$^{-/-}$ mice. The ratios of synaptic NMDAR to AMPAR currents at thalamo- and cortico-amygdala synapses in the wild-type mice were not significantly different from those of Arrb2$^{-/-}$ animals, indicating that synaptic conductance is not affected by deletion of β-arrestin-2 (Fig. 2 *E–H*).

Fear Conditioning Promotes Interaction of β-arrestin-2 with PDE-4D and Regulates PKA Activity in Amygdala. To further explore how β-arrestin-2 is involved in fear conditioning, levels of β-arrestin-2 were measured during fear conditioning. Immunoblot analysis of total cell lysates prepared from amygdala of WT mice revealed no change in β-arrestin-2 levels during fear conditioning (Fig. 3*C*). We next examined the subcellular distribution of β-arrestin-2. As shown in Fig. 3*A*, notably, β-arrestin-2 associated with amygdala membrane increased rapidly to a peak value in 5–30 min in response to the pairing of tone with shock (Fig. 3*A*, *t*-test, $P < 0.01$) and gradually declined to the basal level within 3 h. However, no change was observed when presentation of tone

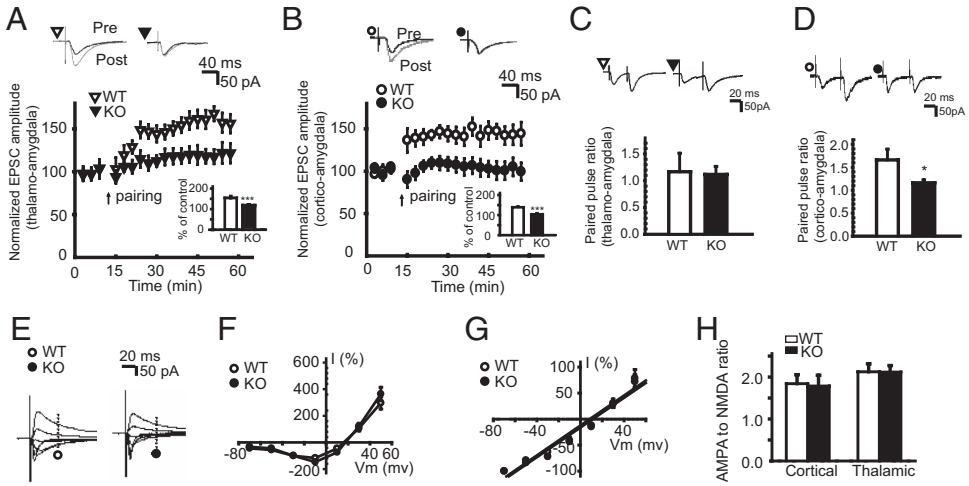

Fig. 2. LTP is impaired in thalamo-amygdala and cortico-amygdala synapses in *Arrb2*$^{-/-}$ mice. (*A* and *B*) Pairing-induced LTP of thalamo-amygdala (*A*) and cortico-amygdala (*B*) EPSCs in LA neurons in the presence of picrotoxin. Representative EPSC traces recorded before (Pre: black line) and 45 min after the pairing procedure (Post: gray line) (top), LTP time courses (middle), and quantitative summaries of LTP experiments (bottom) are shown; $n = 10$ cells from five mice of each genotype for each pathway, $***P < 0.001$ versus the WT mice. (*C* and *D*) Traces and PPF ratios obtained from thalamic (*C*) or cortical (*D*) input to LA. Top: traces of 10 responses represent PPF of EPSC recorded following a 40-ms interstimulus interval. Bottom: PPFs were calculated as the ratio of the second EPSC amplitude to the first EPSC amplitude, $*P < 0.05$ versus the WT mice. (*E*) Representative amygdalar EPSC traces evoked by the stimulation at holding potentials ranging from −70 to + 50 mV in slices from WT and KO mice. Traces are averages of 10 EPSCs recorded at each holding potential. (*F*) Current-voltage plot of the NMDAR-mediated cortico-amygdala EPSCs measured 50 ms after the peak AMPAR-mediated EPSC. Values for the graphs were obtained by normalizing the mean NMDAR current at each holding potential to the mean NMDAR-mediated EPSC recorded at a holding potential of −30 mV. (*G*) Current-voltage plot of the peak current mediated by the AMPARs in slices. Values for the graph were obtained by normalizing the mean AMPAR-mediated peak current at each holding potential to the mean amplitude of the AMPAR-mediated EPSC recorded at a holding potential of −70 mV. (*H*) AMPA/NMDA receptor ratio for the cortico-amygdala and thalamo-amygdala EPSCs recorded in slices. The ratio was calculated by dividing the amplitude of the AMPAR component measured at −70 mV by the amplitude of the NMDAR component measured 50 ms after the peak at + 50 mV. $n = 10$ cells obtained from five mice of each genotype.

and shock was unpaired. Perry et al. has demonstrated that β-arrestin-2 can directly bind to PDE-4D3 and PDE-4D5 (31). It is also reported that PDE-4D knockout mice exhibit the impaired fear conditioning (32) and PDE-4 selective inhibitor rolipram reverses MK-801-induced impairments in fear memory (33). Therefore, the levels of PDE-4D during fear conditioning

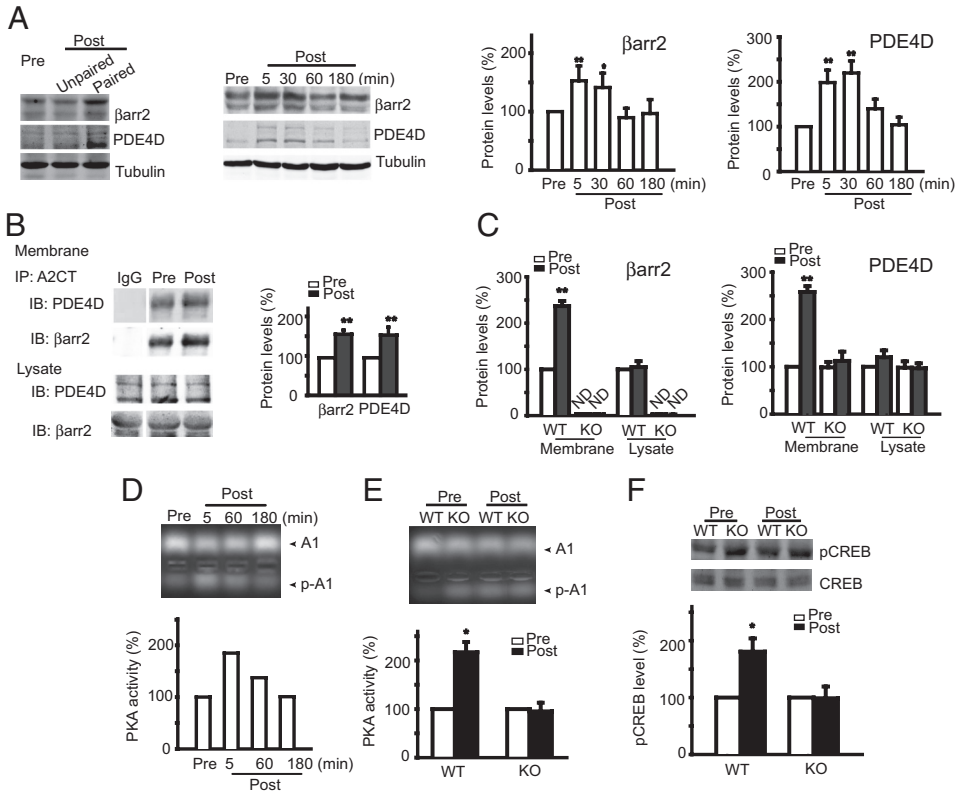

Fig. 3. β-arrestin-2 interacts with PDE-4D to regulate PKA activity in fear conditioning. (*A*) Immunoblots of PDE-4D (PDE-4D3, 95 kDa; PDE-4D5, 105 kDa) and β-arrestin-2 (48 kDa) in amygdala membrane fractions in the control, Unpaired and Paired groups (Left). A pair of mice per group (left); Immunoblots of PDE-4D and β-arrestin-2 in amygdala membrane fractions before and 5 min, 30 min, 1 h, and 3 h after fear conditioning. $n = 4$ per group; $*P < 0.05$, $**P < 0.01$ versus preshock group of each protein. (*B*) Coimmunoprecipitation of PDE-4D and β-arrestin-2 in amygdala membrane fractions in WT mice before and 5 min after fear conditioning. $**P < 0.01$ versus the relative protein levels in the total lysates. (*C*) Protein levels of PDE-4D and β-arrestin-2 in the amygdala membrane fractions and total lysates obtained from WT and KO mice before and 5 min after fear conditioning; $n = 3$ per group; $**P < 0.01$ versus WT in the preshock group. (*D*) PKA activity in amygdala membrane fractions before (Pre) and 5 min, 1 and 3 h after (Post) fear conditioning. (*E*) Representative gel graph and statistic summary of amygdala membrane PKA activity before and 5 min after fear conditioning. $n = 8$ per group. $*P < 0.05$ versus WT in the preshock group. (*F*) Representative immunoblots and quantitative summary of phosphorylated CREB (CREB, 43 kDa; *p*-CREB, 43 kDa) in nuclear fractions from amygdala before and 5 min after conditioning; $n = 3$ per group; $*P < 0.05$ versus WT in the preshock group.

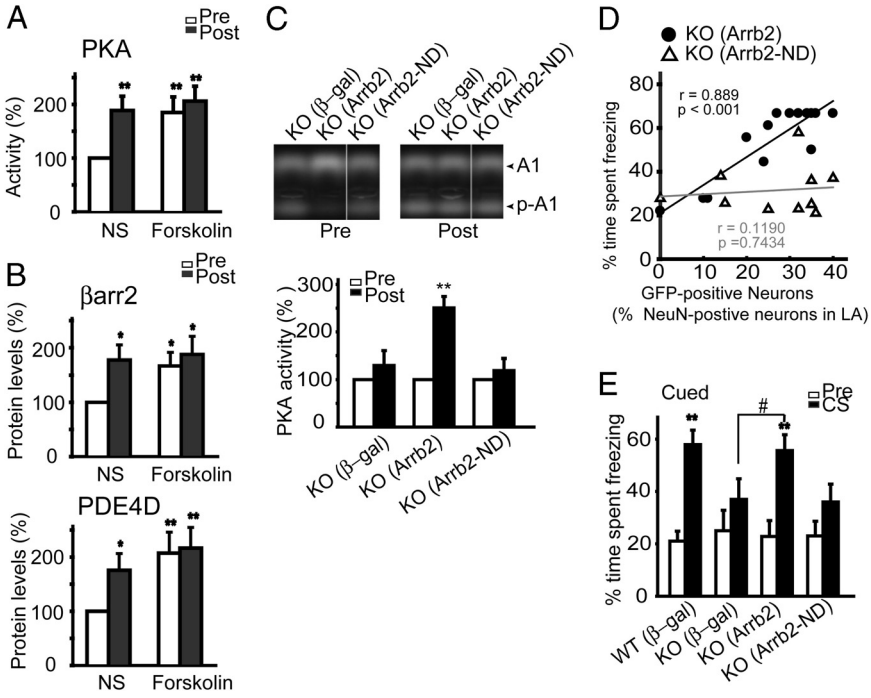

Fig. 4. Feedback regulation of amygdalar PKA activity by β-arrestin-2/PDE-4D complex during fear conditioning. (*A* and *B*) Levels of PKA activity (*A*) and β-arrestin-2 and PDE-4D (*B*) in amygdala membrane fractions of WT mice before and after fear conditioning, 1 h following administration of forskolin (0.05 mg/kg, i.p.). $*P < 0.05$, $**P < 0.01$ versus preshock group of each protein. (*C*) Representative blots and statistical summary of membrane PKA activity in amygdala of mice infected with β-gal-GFP, Arrb2-GFP, or Arrb2-ND in LA. $n = 3$ mice per group; $**P < 0.01$ versus preshock group. (*D*) Correlation between freezing behavior and β-arrestin-2 or β-arrestin-2-ND expression in LA neurons in *Arrb2*$^{-/-}$ mice infected with Arrb2-GFP virus ($n = 13$) and Arrb2-ND-GFP ($n = 10$). Each symbol represents data obtained from a single animal. (*E*) Cued fear memory was tested 1 day after fear training in mice receiving microinjection of adenovirus in LA. $n = 8$–18 mice per group. $**P < 0.01$ versus preshock group; $\#P < 0.05$ versus KO (β-gal) in the postshock group.

were examined. In parallel with the increase of β-arrestin-2, the level of amygdalar membrane-associated PDE-4 was also increased following fear conditioning (Fig. 3*A*) (*t*-test, $P < 0.01$). Data of co-immunoprecipitation showed that β-arrestin-2 and PDE-4D were present as a complex on the amygdalar membrane of WT mice and the amount of the complex increased in response to fear conditioning (Fig. 3*B*). As expected, this activity-stimulated PDE-4 membrane translocation was not detected in β-arrestin-2-deficient mice (Fig. 3*C*) (*t*-test, $P = 0.296$). Moreover, a parallel increase of amygdala PDE-4 activity in response to fear conditioning stimuli was observed in WT, but not *Arrb2*$^{-/-}$ mice (Fig. S3*A*).

PDE-4 can down-regulate PKA/CREB signaling pathway through degrading cAMP, an activator of PKA. It has also been shown that inhibition of PKA activity in the LA impairs fear conditioning (18). Therefore, the potential involvement of β-arrestin-2 and PDE-4 in regulation of PKA activity was investigated during fear conditioning process. Fear conditioning induced no change in PKA activity and membrane-associated β-arrestin-2 and PDE-4D in hippocampus of WT and KO mice (Fig. S4). However, as shown in Fig. 3*D*, the PKA activity in amygdalar membrane fractions from the WT mice increased rapidly to a peak value in 5 min after fear conditioning and gradually declined to the basal level within 3 h. Furthermore, fear conditioning induced a 90–100% increase in PKA activity and phosphorylation of CREB in the LA of WT mice (*t*-test, $P < 0.05$), however, in *Arrb2*$^{-/-}$ mice, the fear conditioning-induced increase of PKA activity and CREB phosphorylation was not observed (Fig. 3 *E* and *F*). Inhibiting PDE-4 activation with rolipram, a specific inhibitor of PDE-4 (25), resulted in a dose-dependent increase in amygdalar PKA activity in WT mice, but caused no change in amygdalar PKA activity in *Arrb2*$^{-/-}$ mice (Fig. S3 *B* and *C*). Furthermore, administration of forskolin, an adenylyl cyclase activator, results in an increase of PKA activity and paralleled increases of β-arrestin-2 and PDE4D in amygdalar membrane fractions in WT mice (Fig. 4 *A* and *B*). PDE4D can be phosphorylated and activated by PKA, thus limiting PKA activation (34). It is likely that β-arrestin-2 recruits PDE-4 to the membrane compartment where PKA is localized and allows PDE-4D to be phosphorylated and activated by PKA, thereby facilitating the degradation of cAMP to feedback regulate PKA activation in response to fear conditioning.

Feedback Regulation of Amygdalar PKA Activity by β-arrestin-2/PDE-4 Complex in Response to Fear Conditioning. To further confirm the role of β-arrestin-2/PDE-4 complex in fear memory, adenovirus expressing β-arrestin-2 or β-arrestin-2-ND was delivered into the bilateral amygdala of *Arrb2*$^{-/-}$ mice by microinjection and histological analysis was performed after fear memory test. As shown in Fig. S5, β-arrestin-2 and its mutation β-arrestin-2-ND were expressed at moderate-to-high levels in 25.6 ± 11.7% and 26.4 ± 12.8% (mean ± SD) NeuN-positive LA neurons. The expression of β-arrestin-2 in the LA of *Arrb2*$^{-/-}$ mice restored the fear conditioning-induced amygdalar PKA activation (Figs. S6, S7, and Fig. 4*C*, ANOVA, $F_{1,\,4} = 57.15$, $P = 0.006$ versus KO-Arrb2 in preshock group). However, expression of β-arrestin-2-ND, which is incapable to interact with PDE-4 (35), failed to rescue PKA activation in response to fear conditioning (Fig. 4*C*) (ANOVA, $F_{1,\,4} = 0.094$, $P = 0.774$ versus the KO-Arrb2-ND in preshock group). As shown in Fig. 4 *D* and *E*, the expressing β-arrestin-2 in LA of virus-infected *Arrb2*$^{-/-}$ mice correlated positively to their memory scores tested post fear conditioning (Fig. 4*D*) ($r = 0.889$, $P < 0.001$) and caused an average of 35% increase in freezing responding 1 day after fear training (ANOVA, different adenovirus effects in the CS group, $F_{1,\,32} = 7.24$, $P = 0.011$ compared with KO-β-gal group), and rescued fear memory in *Arrb2*$^{-/-}$ mice (ANOVA, different adenovirus effects in the CS group, $F_{1,\,25} = 0.41$, $P = 0.530$ compared with WT-β-gal group). Furthermore, in contrast to the rescue effect observed with the wild-type β-arrestin-2, infection of the adenovirus expressing β-arrestin-2-ND did not improve fear memory scores (Fig. 4 *D* and *E*) (ANOVA, $F_{1,\,23} = 0.80$, $P = 0.379$). All these results suggest that β-arrestin-2 regulates associative fear memory, likely through recruiting PDE-4 for feedback regulation of amygdalar PKA activation.

Discussion

In the present study, we found that the acquisition and consolidation of associative fear memory induced by noxious foot shock were impaired in *Arrb2*$^{-/-}$ mice. In addition, we showed that the

Arrb2$^{-/-}$ mice performed well in the Morris water maze, a task for spatial learning and memory. Consistent with the previous electrophysiological and behavioral observations that LTP in thalamo- and cortico-amygdala pathways is important for fear-related memory (15, 17), impaired associative fear memory with inhibition of LTP in both of those pathways was observed in *Arrb2*$^{-/-}$ mice. Furthermore, we showed that local expression of β-arrestin-2 in the LA of *Arrb2*$^{-/-}$ animals rescued associative fear memory. These results suggest that β-arrestin-2 in LA is critical for regulation of amygdalar neuroplasticity that may underlie associative fear memories.

During fear conditioning-induced CS-US pairing, protein kinase second messenger pathways are directly or indirectly activated. As one of these, PKA has been extensively studied in relation to LTP and fear conditioning. It has been shown that both activation and inhibition of PKA hinder amygdalar early-LTP and late-LTP in both thalamo- and cortico-amygdala pathways (36, 37). PKA inhibitor infused into the LA impairs long-term fear memory (18); however, inhibition of the elevated PKA activity rescues short- and long-term fear memory deficits in Gαs* mice (38). These studies implicate that both activity-dependent activation of PKA and the stringent regulation of PKA activation are critical for the amygdalar synaptic plasticity and associative fear memory. In the current study, we found that amygdalar PKA was activated immediately after fear conditioning in the wild-type animals, and this was correlated with a parallel increase of β-arrestin-2/PDE-4 complex in amygdalar membrane fractions and PDE-4 activity. These data support the notion that β-arrestin-2 serves as a critical mediator by recruiting PDE-4 to regulate PKA activity during fear conditioning. Consistent with this proposal, expression of β-arrestin-2 in the amygdala of *Arrb2*$^{-/-}$ mice not only restored the deregulation of amygdalar PKA activity but also rescued the impaired fear memory in *Arrb2*$^{-/-}$ mice.

PDE-4, a degrading enzyme of cAMP, plays a critical role in regulation of PKA activation and PKA-mediated physiological functions. Park et al. reported that both overexpression and knock-down of apPDE-4 impair 5-HT-stimulated PKA activation and short- and long-term facilitation (39). Kandel and colleagues also demonstrated that a lower dose of PDE-4 inhibitor rolipram improves fear memory in normal mice trained with a mild shock but a higher dose of rolipram does not. Consistently, it has also been shown that a low concentration of rolipram enhances LTP while a high concentration decreases it (40). The converging evidence suggests that optimum PDE-4 activity is required for normal synaptic plasticity and conditioned fear memory. Evidence has demonstrated that β-arrestins can form stable complexes with PDE-4s in the desensitization system independent of agonist treatment (31). Behavior experiment also implicated the role of PDE-4, especially PDE-4D, in amygdala-dependent fear conditioning responses (32, 33, 38). Although β-arrestin-2 is ubiquitously expressed in CNS, *Arrb2*$^{-/-}$ mice exhibit impaired fear conditioning and normal Morris water maze. The possible mechanisms underlying the differential contributions of β-arrestin-2 to hippocampus and amygdala-dependent memories may be due to the presence of distinct upstream signaling pathways, such as preferentially amygdala expressed 5-HT4 and 5-HT6 (41, 42). In our study, we demonstrated that during fear conditioning, as the modulator of PKA activity, β-arrestin-2/PDE-4 complex was activated to limit PKA activation. Conversely, knockout of β-arrestin-2, a binding partner of PDE-4, led to deregulation of amygdalar PKA activation and impaired LTP at amygdalar synapses and fear memory. All these effects could be rescued by expression of the wide-type β-arrestin-2, but not its mutant form incapable of binding PDE-4, in LA of these β-arrestin-2 KO mice. Consistent with this, we further observed that administration of rolipram, a specific inhibitor of PDE-4, induced a dose-dependent increase of PKA activity in WT mice but not in β-arrestin-2 KO mice. Furthermore, rapidly responding to the changes of PKA activity caused by treatments of forskolin, the level of amygdalar membrane-associated β-arrestin-2/PDE-4D was changed correspondingly. Our results suggest that interaction of β-arresti-2 with PDE-4D in amygdala plays a critical role in limiting PKA activation and the formation of fear memory.

In addition to their classical role in mediation of receptor endocytosis, accumulating evidence has demonstrated that β-arrestins also function as signaling scaffold proteins to mediate crosstalk to many signal pathways. Studies have shown that β-arrestin-2 can bind Raf, MEK, and ERK, and activates MAPK signal pathways (1). It is reported that upon β_2AR stimulation, β-arrestin-2/PDE-4D complex is vitally involved in ERK1/2 activation and as PDE-4D recruitment and activity is completely inhibited by PDE-4D mutant PDE-4D-D556A, ERK1/2 activation is potentiated (43). Conversely, the PDE-4Ds activities can be also modified by its phosphorylation through ERK2 (44). MAPK/ERK signaling plays an inhibitory role in regulating PDE-4 activity in the hippocampus to mediate long-term memory (45). This indicates that PDE-4D is likely to be an important link between the cAMP/PKA and MEK/ERK signaling pathways in the mediation of memory. Activation of ERK/MAPK cascades is important for the LTP induction and the consolidation of fear memory (19). It has been shown that PI3K/Akt, serving as a critical intermediate in the PKA-mediated MAPK signaling pathway, is also required for amygdalar LTP and fear memory (46). Furthermore, Beaulieu et al. have demonstrated recently that in response to dopamine receptor activation, β-arrestin-2 forms a complex with Akt and PP2A and knockout of β-arrestin-2 results in disruption of dopamine-stimulated interaction of Akt with its negative regulator PP2A (12). This raises a possibility that direct β-arrestin-2-Akt interaction may also contribute to fear memory formation.

Methods

Mouse Strains and Conditions. *Arrb2*$^{-/-}$ mice were generated by laboratory of R. J. Lefkowitz (Duke University Medical Center, Durham, NC) and backcrossed onto a C57BL/6J background. The animals used in the present study were wild-type or *Arrb2*$^{-/-}$ littermates resulting from heterozygous breeding and descendents of mice that were separately bred as homozygous wild-type or *Arrb2*$^{-/-}$ mice after the initial backcrossing. Comparing data between *Arrb2*$^{-/-}$ animals in the two breeding colonies did not reveal any significant differences. *Arrb2*$^{+/-}$ mice were backcross onto a C57BL/6J background every six generations. All mice were maintained on a 12-h light/dark cycle with food and water available ad libitum. All animals' treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For all behavioral tasks, mutant and control littermates (males, 2–3 months old) were used and the experimenter was blind to the genotype in all studies.

Fear Conditioning Test (47). On the training day, mice were placed in the conditioning chamber for 2 min before conditioning. Two training protocols were used. For one CS-US pairing protocol training, mice were presented with one tone (CS, 2800 Hz/85 dB for 30 s) that coterminated with an electric foot shock (US, 0.7 mA, 2 s). Alternatively, in the five CS-US pairings paradigm, mice were presented with five tone-foot shock pairing trials (1 s at 0.7 mA, 1 s for each US) with intertrial interval of 2 min and freezing behavior was measured during each tone presentation. Mice were returned to home cages after conditioning and tested at 1 h and 1 day after training by scoring freezing behavior. For contextual fear memory, mice were tested in the training chamber. For cued fear memory, mice were placed in a novel chamber for 3 min (pre-CS) and then given 3 min tone (CS) that had been presented during training. As a control experiment, the intensity of electric foot shock required to elicit running, vocalization, and jumping was determined for each mouse by delivering a 1 s shock every 30 s starting at 0.08 mA and increasing the shock 0.02 mA each time. Testing was stopped after all behaviors had been noted. For biochemical analysis, mice were placed in the contextual chamber and received foot-shock pairing. Unpaired controls received the same intensity of CS and US presentation, but in an unpaired pseudorandom fashion.

Electrophysiology. Amygdala slices (300 μm) were prepared from 3- to 5-week-old wild-type and *Arrb2*$^{-/-}$ mice (littermates) with a vibratome. The recording electrode was placed in the LA and a stimulation electrode was placed at thalamo- or cortico-amygdala synapses. All recordings were performed at 25 °C. Details can be seen in *SI Text*.

Immunoprecipitation and Western Blotting. For Western blotting, the antibodies to β-arrestins (rabbit, polyclonals A1CT and A2CT) were the gift from R. J. Lefkowitz (Duke University Medical Center, NC). Mouse antibody to β-arrestin-2 was from BD Bioscience. Other antibodies were also used: synaptophysin (rabbit monoclonal, Sigma), PSD95 (rabbit monoclonal, Chemico), CREB and phosphorylated CREB (rabbit polyclonals, Upstate Biotechnology), GluR1 and GluR2 (rabbit polyclonals, Chemicon International, Inc.). Endogenous PDE-4D or β-arrestins was immunoprecipitated with A2CT, followed by treatment with protein G-Sepharose (Amersham Biosciences). The complex sample was separated by SDS/PAGE and blotted using A2CT and antibodies to β-arrestin-2 and PDE-4D (rabbit polyclonals, Cat PDE4–400p, FabGennix Inc.) antibodies.

Microinjection and Expression of Recombinant Adenovirus in LA. Replication-deficient adenoviruses encoding GFP-tagged full-length of β-arrestin-2 (Arrb2-GFP adenovirus), GFP-tagged β-galactosidase (gal-GFP adenovirus), and HA-tagged β-arrestin-2 mutant with deletion of its N-terminal (Arrb2-ND-GFP adenovirus) were prepared as before (6, 48). The titers of these three adenoviruses were about 1.0×10^{11} PFU/mL. For surgery and microinjection, chloral hydrate-anesthetized mice were positioned in a stereotaxic apparatus (NARISHIGE Scientific Instruments); a glass electrode aimed at the bilateral amygdala was injected. The intended stereotaxic coordinates were: AP −1.75 mm; ML ± 2 mm (with an angle of 17° from the middle to the lateral); DV 4.5 mm. Adenovirus for one side was microinjected into the LA at a rate of 0.1 μL/min. The glass electrode was left in place for additional 2 min. Fear conditioning test was conducted 10–14 days after virus microinjection. We then detected the expression of GFP-tagged aim protein and analyzed the infection levels inside and outside of LA. Infected animals with 30% or more of the total infection levels outside LA were excluded in the behavior test.

Statistical Analysis. The data are represented as mean ± SEM, and analyzed using Sigma Stat 3.5. The statistical significant was determined by one-way analysis of variance (ANOVA) for multiple comparisons followed by Bonferroni post-hoc test or the two-tailed Student's *t*-test. The two-way ANOVA was used for behavior tests.

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