M₁-Muscarinic Receptors Promote Fear Memory Consolidation via Phospholipase C and the M-Current

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Neuromodulators released during and after a fearful experience promote the consolidation of long-term memory for that experience. Because overconsolidation may contribute to the recurrent and intrusive memories of post-traumatic stress disorder, neuromodulatory receptors provide a potential pharmacological target for prevention. Stimulation of muscarinic receptors promotes memory consolidation in several conditioning paradigms, an effect primarily associated with the M₁ receptor (M₁R). However, neither inhibiting nor genetically disrupting M₁R impairs the consolidation of cued fear memory. Using the M₁R agonist cevimeline and antagonist telenzepine, as well as M₁R knock-out mice, we show here that M₁R, along with β_2 -adrenergic (β_2 AR) and D₅-dopaminergic (D₅R) receptors, regulates the consolidation of cued fear memory by redundantly activating phospholipase C (PLC) in the basolateral amygdala (BLA). We also demonstrate that fear memory consolidation in the BLA is mediated in part by neuromodulatory inhibition of the M-current, which is conducted by KCNQ channels and is known to be inhibited by muscarinic receptors. Manipulating the M-current by administering the KCNQ channel blocker XE991 or the KCNQ channel opener retigabine reverses the effects on consolidation caused by manipulating β_2 AR, D₅R, M₁R, and PLC. Finally, we show that cAMP and protein kinase A (cAMP/PKA) signaling relevant to this stage of consolidation is upstream of these neuromodulators and PLC, suggesting an important presynaptic role for cAMP/PKA in consolidation. These results support the idea that neuromodulatory regulation of ion channel activity and neuronal excitability is a critical mechanism for promoting consolidation well after acquisition has occurred.

Introduction

Long-term consolidation of fear memory in the basolateral amygdala (BLA) depends on the activity-dependent induction of intracellular signaling pathways that promote gene expression and protein synthesis (Johansen et al., 2011). Regulation of these signaling pathways by G-protein-coupled neuromodulatory receptors can affect the strength of fear memory consolidation, a process that may underlie the recurrent and intrusive memories of post-traumatic stress disorder (PTSD). Traditionally, these effects on consolidation have been attributed to signaling activated by G_s proteins (Sara, 2009; Tully and Bolshakov, 2010; Johansen et al., 2011). However, although consolidation of fear memory can be enhanced by pharmacological activation of neuromodulatory receptors in the BLA, it is not reliably blocked by antagonism of individual receptors.

Recently it has been proposed that, rather than a single neuromodulatory system, a combination of neuromodulatory sys-

tems coupled to phospholipase C (PLC) redundantly mediates consolidation in the BLA (Ouyang et al., 2012). Activation of either the G_{q/10}-coupled β_2 -adrenergic receptor (β_2 AR) or the G_{q/11}-coupled D₅-dopaminergic receptor (D₅R) enhances consolidation; however, both receptors must be blocked to impair consolidation. Further, β_2 AR and D₅R in the BLA redundantly activate PLC as an initial signaling mechanism that is necessary for consolidation.

Based on the observation that PLC, and neuromodulatory receptors that control its activity, can regulate fear memory consolidation, we asked whether other G_{q/11}-coupled receptors contribute to the redundant relationship of β_2 AR and D₅R. In this study, we investigated a potential role for G_{q/11}-protein-coupled muscarinic receptors. Release of acetylcholine (ACh) increases following the presentation of fearful stimuli (Acquas et al., 1996), and cholinergic neurons in the basal forebrain project to the BLA (Rao et al., 1987; Kordower et al., 1989), where muscarinic agonists initiate PLC-dependent intracellular calcium release (Power and Sah, 2007), increase the excitability of neurons (Womble and Moises, 1992, 1993), and enhance fear memory consolidation (Vazdarjanova and McGaugh, 1999; Barros et al., 2002).

Both M₁- and M₃-muscarinic receptors couple to G_{q/11} and are expressed in the BLA; however, *in situ* hybridization results suggest that the M₁ receptor (M₁R) is most abundant (Buckley et al., 1988). Activation of M₁R in the BLA enhances consolidation of inhibitory avoidance and contextual fear memory (Vazdarjanova and McGaugh, 1999; Power et al., 2003). However, studies using M₁R-selective antagonists or M₁R-deficient mice report no

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impairments in cued fear memory (Fornari et al., 2000; Anagnostaras et al., 2003). Given that stimulation of M₁R in the BLA enhances consolidation without being required (Robinson et al., 2011), we hypothesized that M₁R redundantly contributes to the consolidation of fear memory.

Here, we use pharmacological, genetic, and biochemical approaches in mice to demonstrate that M₁R redundantly regulates the consolidation of fear memory with β_2 AR and D₅R by activating PLC. Further, we examine whether the muscarine-sensitive M-current in the BLA is a downstream target of PLC activation that is important for fear memory consolidation. Finally, we examine the relationship between the role of cAMP and protein kinase A (cAMP/PKA) signaling in consolidation and that for the neuromodulatory signaling coupled to PLC.

Materials and Methods

Animals. Stock *Chrm1*^{−/−} (M₁R knock-out) and wild-type (WT) mice on a C57BL/6 background were from The Jackson Laboratory and bred at the University of Pennsylvania (Gerber et al., 2001). All other mice were on a hybrid 129/Sv × C57BL/6 background and bred locally. M₁R knock-out (KO) mice were generated by mating heterozygotes or homozygotes, and genotype was determined by PCR. Animals were maintained on *ad libitum* food and water and a 12 h light/dark cycle, with lights on beginning at 7:00 A.M. Mice were 3- to 6-months-old when tested and of either sex. No significant differences were found by sex or parental genotype, so data were combined. Studies were performed during the light phase, with most experiments taking place between 9:00 A.M. and 5:00 P.M. Studies were in accordance with NIH guidelines and had the approval of the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Classical fear conditioning. Animals were habituated to handling and drug administration for two (systemic injection) or four (BLA infusion) days before behavioral experimentation. On habituation days, animals were handled for 4 min, and either injected with vehicle (systemic experiments) or given a simulated infusion (infusion experiments). Animals were then placed in individual plastic holding buckets (12 cm diameter) with bedding and lids for 30–60 min. Before behavioral experimentation, animals were held in the buckets for 30–60 min. For conditioning, animals were placed in the training apparatus (ENV-010MC with ENV-414S, Med Associates) for 2 min, after which an 84 dB, 4.5 kHz tone was activated for 30 s that coterminated with a 2 s footshock (moderate = 0.4 mA or strong = 1 mA). Animals were removed from the apparatus and injected or infused 30 s after shock, and then returned to the home cage. The apparatus was cleaned with Versa-Clean (Fisher Scientific) between subjects. Individual subjects were tested for either contextual or cued fear memory, but not both, the day after training. Contextual fear was tested for 5 min in the conditioning apparatus in the absence of the tone. Cued fear was tested in a Plexiglas cylinder (21 cm diameter, 24 cm tall) with green wire grid floor and vertical green and white wall stripes 240° around that was cleaned with lemon-scented Ajax between subjects. After 2 min, the training tone was turned on for 3 min. Percentage freezing was estimated by scoring the presence or absence of nonrespiratory movement every 5 s.

BLA infusions. Two guide cannulae mounted on a base plate (C315GS system, Plastics One) were implanted under pentobarbital anesthesia (72.5 mg/kg) using a stereotax (SAS75/EM40M, Cartesian Research). The guides were placed 1.25 mm posterior to bregma and 3.5 mm bilateral. The guide and dummy cannulae projected 3 mm below the base plate. Habituation of the animals to the investigator and the infusion procedure began a couple of days later with a 4 min handling session followed by 3 min of immobilization (gently holding the nape of the neck and body) that mimicked infusion. Handling sessions were conducted on each of the 4 d preceding training and once more 1 h before training. Immediately after training, mice were infused bilaterally using injection cannulae that extended 2.8 mm below the tip of the guide cannulae. All BLA infusions were 0.2 μ l/BLA at 0.08 μ l/min, and infusion cannulae were left in place for 30 s after infusion.

Drugs. Cevimeline HCl, telenzepine 2HCl, SCH 23390 HCl, ICI 118,551 HCl, XE991 2HCl, SKF 83959 HBr, procaterol HCl (all Tocris Bioscience), retigabine 2HCl (Ryan Scientific), $\pm$ 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP; Abcam), Sp-8-Br-cAMPS (Sp8; Santa Cruz Biotechnology), and myr-PKI[14–22]amide (PKI; Invitrogen) were administered intraperitoneally or infused into the BLA immediately after training. Drugs were dissolved in 0.9% saline (SKF 83959 contained 0.1 mg/ml ascorbic acid and <1% DMSO, pH 7.4; Sigma-Aldrich). Vehicle was saline with or without 0.1 mg/ml ascorbic acid and <1% DMSO. Systemic injection volumes were 10 μ l/g body weight.

IP₃ levels. Mice were anesthetized with CO₂, killed by cervical dislocation, and brains were rapidly removed, frozen in 2-methylbutane on dry ice, and stored at −80°C. Two frozen coronal sections (400 μ m) that contained the BLA were cut by cryostat (HM505E, Microm) from each mouse, and a 0.5 mm diameter punch of BLA tissue was collected bilaterally from each slice. The four punches per mouse were pooled and homogenized on ice with three 2 s pulses (5 s interval) in 125 μ l of 4% perchloric acid using a Sonic Dismembrator 100 set on level 3 (Fisher Scientific). After 15 min on ice, samples were stored overnight at −80°C. The next day samples were centrifuged at 4°C and 2000 × g for 15 min, and the pellet was stored at −80°C for subsequent Bradford assay to determine total protein. Supernatants were neutralized on ice with 10 M KOH (to precipitate the perchloric acid) and centrifuged at 4°C and 2000 × g for 15 min. Supernatant (100 μ l) was then used in the [³H]-IP₃ radioreceptor assay (PerkinElmer) according to instructions.

Statistics. Data were analyzed with Statistica 9.1 (StatSoft) using one- or two-way ANOVA with $\alpha = 0.05$. The Bartlett χ^2 test was used to analyze homogeneity of variances. *Post hoc* comparisons were made using the Newman–Keuls test. Data are presented as mean $\pm$ SE. For all figures: * $p < 0.05$; ^ $p < 0.01$, and # $p < 0.001$. Comparisons marked as significant are to the reference group except where indicated.

Results

Activating M₁R in the BLA enhances fear memory consolidation

To determine whether signaling by M₁R influences the consolidation of classical auditory fear conditioning, we explored whether immediate post-training administration of cevimeline (Cev), an M₁R-selective agonist, affected freezing in response to the training tone the following day. Mice were trained with a moderate shock intensity (0.4 mA) that elicits relatively low levels of freezing in response to the training tone. Systemic injection of Cev caused a dose-dependent increase in freezing in response to the tone during testing (Fig. 1A; $F_{(3,16)} = 5.09$, $p = 0.012$, $n = 5$ /group). These data suggest that signaling by M₁R can enhance cued fear memory consolidation.

Given that the BLA is a crucial site for fear memory consolidation (Pape and Pare, 2010; Ouyang et al., 2012), we next tested whether the effects of systemically injected Cev could be replicated by infusing it into the BLA immediately after moderate conditioning. Compared with vehicle-treated controls, mice given BLA infusions of Cev exhibited dose-dependent increases in freezing to the training tone during testing (Fig. 1B; $F_{(3,16)} = 8.49$, $p = 0.001$, $n = 5$ /group). These data suggest that the consolidation enhancement observed after systemic injection of Cev is mediated by the BLA.

Cev potentially activates M₁R and, to a lesser extent, M₃- and M₅-muscarinic receptors (Heinrich et al., 2009). To determine whether the enhancing effects of Cev on consolidation require M₁R, we tested the effect of Cev on mice congenitally lacking M₁R. M₁R KO mice injected with Cev (1 mg/kg) immediately after moderate fear conditioning did not exhibit a significant increase in freezing in response to the training tone during testing, whereas WT littermates exhibited enhancements similar to those described earlier (Fig. 1C; $F_{(1,16)} = 17.86$, $p = 0.0006$ for the

main effect of treatment; $F_{(1,16)} = 10.31$, $p = 0.0054$ for the main effect of genotype; and $F_{(1,16)} = 4.83$, $p = 0.043$ for the interaction of treatment and genotype, $n = 5/\text{group}$). These data indicate that the consolidation enhancements observed in response to post-training administration of Cev are mediated specifically by M₁R.

M₁R redundantly modulates consolidation with D₅R and β_2 AR

The G_{q/11}-protein coupled to M₁R activates PLC (Caulfield, 1993), whose activation by β_2 AR and D₅R in the BLA promotes consolidation (Ouyang et al., 2012). To determine whether M₁R redundantly regulates fear memory consolidation with β_2 AR and D₅R, we systemically administered the M₁R antagonist telanzepine (Tzp) alone or in combination with an antagonist of β_2 AR or D₅R (Schudt et al., 1988).

When combined with the D₅R antagonist SCH 23390 (SCH; 30 $\mu\text{g}/\text{kg}$) after conditioning with strong (1 mA) footshock, Tzp dose-dependently decreased the amount of freezing mice exhibited in response to the training tone during testing the next day (Fig. 2A; $F_{(5,24)} = 12.37$, $p < 0.0001$, $n = 5/\text{group}$). The smallest maximally effective dose of Tzp, when combined with SCH, was 1 mg/kg. Neither Tzp nor SCH significantly affected freezing when administered alone. These data suggest that M₁R and D₅R redundantly contribute to signaling mechanisms required for fear memory consolidation.

Given that the β_2 AR antagonist ICI 118,511 (ICI) also inhibits consolidation only when coadministered with SCH immediately after training (Ouyang et al., 2012), we next examined whether Tzp inhibits consolidation when combined with ICI. Mice injected with a combination of Tzp (1 mg/kg) and ICI (30 $\mu\text{g}/\text{kg}$) immediately after strong fear conditioning exhibited significantly less freezing in response to the training tone the following day compared with saline- or ICI-treated controls (Fig. 2B; $F_{(3,20)} = 30.82$, $p < 0.0001$, $n = 5\text{--}8/\text{group}$). Further, coadministering all three antagonists (Tzp, ICI, and SCH) inhibited consolidation more effectively than Tzp + ICI treatment. Given these observations, it is plausible that M₁R contributes to consolidation with β_2 AR and D₅R by converging on a common signaling mechanism in the BLA.

To determine the effect of Tzp in the BLA, we infused Tzp either alone or in combination with SCH or ICI (both at 50 ng/BLA), the latter of which affect consolidation when delivered together but not alone (Ouyang et al., 2012). Mice infused with Tzp into the BLA immediately after strong fear conditioning exhibited dose-dependent decreases in freezing in response to the training tone the next day, but only when Tzp was combined with either SCH or ICI (Fig. 2C; $F_{(5,22)} = 5.78$, $p = 0.0012$, $n = 5/\text{group}$). Infusion of Tzp alone at the lowest maximally effective dose (1 $\mu\text{g}/\text{BLA}$) from combination treatment did not affect consolidation (Fig. 2C). These data suggest that M₁R signaling is important for consolidation of cued fear memory occurs in the BLA.

To confirm that M₁R mediates the impairing effects of Tzp described above, we examined whether M₁R KO mice exhibit impairments in response to post-training administration of β_2 AR or D₅R antagonists. As previously reported (Anagnostaras et al., 2003), vehicle-treated M₁R KO and WT mice exhibited comparable freezing to the training tone (Fig. 2D; $F_{(2,32)} = 3.96$, $p = 0.0292$ for the main effect of treatment, $F_{(1,32)} = 15.19$, $p =$

Figure 1. Muscarinic stimulation promotes consolidation via M₁R. All treatments were given immediately after conditioning with moderate shock (0.4 mA). Animals were tested for retention in a novel context the following day. **A**, The M₁R agonist cevimeline dose-dependently increases freezing in response to the training tone. **B**, Directly infusing cevimeline into the BLA dose-dependently enhances freezing in response to the training tone. **C**, Either vehicle or Cev (1 mg/kg) was administered to M₁R KO and WT littermate control mice. Only WT mice exhibit increases in freezing in response to drug treatment (* $p < 0.05$; ^ $p < 0.01$).

0.005 for the main effect of genotype, and $F_{(2,32)} = 3.42$, $p = 0.045$ for the interaction of treatment and genotype; $n = 6\text{--}7/\text{group}$). However, mice lacking M₁R exhibited significantly impaired consolidation in response to either ICI or SCH treatment that was not observed in WT littermates. These data further support the idea that M₁R signals redundantly with β_2 AR and D₅R to mediate cued fear memory consolidation.

M₁R redundantly activates PLC with β_2 AR and D₅R

Coadministration of β_2 AR and D₅R antagonists immediately after fear conditioning impairs consolidation, and it also blocks PLC activation in the BLA that occurs 30 min after conditioning (Ouyang et al., 2012). Given that M₁R activates PLC (Caulfield, 1993) and also increases IP₃-dependent calcium release in the BLA (Power and Sah, 2007), we asked whether the behaviorally relevant Tzp and Cev treatments described above (Figs. 1, 2) influence PLC activity in the BLA. PLC hydrolyzes the membrane phospholipid phosphatidylinositol-bisphosphate (PIP₂), generating the second messengers inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). Thus we measured levels of IP₃ as a means of assessing PLC activity.

IP₃ levels in the BLA increase 30 min after systemic injection of a β_2 AR or D₅R agonist (Ouyang et al., 2012). Here, we observed that systemically injecting a dose of Cev that enhances consolidation also increases IP₃ levels in the BLA (Fig. 3A; $t_{(9)} = 2.27$, $p = 0.0496$, $n = 10/\text{group}$). This observation is consistent with the hypothesis that M₁R contributes to consolidation by activating PLC in the BLA. To determine whether PLC activation by M₁R might be important for its role in consolidation, we examined how the M₁R antagonist Tzp affects IP₃ levels in the BLA 30 min after strong fear conditioning (Ouyang et al., 2012). Immediate post-training injection of Tzp had no effect on the increase in IP₃ levels at 30 min compared with vehicle-treated controls (Fig. 3B; $F_{(3,36)} = 4.04$, $p = 0.0142$, $n = 10/\text{group}$). However, IP₃ levels were significantly decreased when Tzp treatment was combined with either SCH or ICI, the latter of which have no effect on IP₃ levels when administered alone (Ouyang et al., 2012). These data suggest that M₁R contributes to fear memory consolidation by redundantly activating PLC with β_2 AR and D₅R.

KCNQ potassium channels modulate fear memory consolidation in the BLA

Agonists of muscarinic receptors increase the excitability of neurons in the BLA by inhibiting the M-current (Womble and Moises, 1992, 1993), which is observed in several brain structures important for learning (Halliwell and Adams, 1982; Shen et al.,

Figure 2. M₁R signals redundantly with β_2 AR and D₅R to promote fear memory consolidation. All treatments were administered immediately after conditioning with strong shock (1 mA). Retention testing occurred the following day in a novel context. **A**, Tzp dose-dependently inhibits freezing to the training tone during testing when combined with SCH (30 μg/kg). Neither SCH nor Tzp affect consolidation on their own. **B**, ICI (30 μg/kg) impairs consolidation only when combined with Tzp. In addition, significantly lower freezing is observed when all three receptor antagonists are combined. **C**, Combining Tzp treatment with either SCH or ICI (both 50 ng) impairs fear memory consolidation when infused directly into the BLA. **D**, Vehicle, ICI, or SCH (both 30 μg/kg) was administered to M₁R KO and WT littermate mice. ICI and SCH impair consolidation only in M₁R KO mice (* $p < 0.05$; ^ $p < 0.01$; # $p < 0.001$).

Figure 3. M₁R redundantly activates BLA PLC induced by fear conditioning. **A**, BLA samples were taken 30 min after vehicle or Cev (1 mg/kg) injection. Cev elevates IP₃ in the BLA. **B**, BLA samples were taken 30 min after fear conditioning using 1 mA shock. Tzp (1 mg/kg) blocks conditioning-induced IP₃ levels only when combined with SCH or ICI (both 30 μg/kg; * $p < 0.05$).

2005; Santini and Porter, 2010). The M-current is a voltage-dependent potassium current conducted through noninactivating KCNQ channels that become active between resting membrane potential and action potential threshold (Brown and Adams, 1980). When active, these channels suppress depolarization by EPSPs and promote spike accommodation by enhancing the afterhyperpolarization (Brown and Yu, 2000). Importantly, M-current conductance by KCNQ channels depends on sufficient levels of PIP₂, which is hydrolyzed by PLC (Suh and Hille, 2002; Suh et al., 2006; Telezhkin et al., 2012).

Given that activating M₁R and other neuromodulatory receptors coupled to PLC enhances fear memory consolidation (Ouyang et al., 2012), we asked whether directly inhibiting the M-current with the KCNQ channel blocker XE991 would have a similar effect. Systemically injecting mice with XE991 immedi-

ately after moderate fear conditioning caused a dose-dependent enhancement of consolidation (Fig. 4A; $F_{(3,30)} = 4.81$, $p = 0.0075$, $n = 8$ –10/group). As with Cev, directly infusing XE991 into the BLA enhanced consolidation (Fig. 4B; $F_{(3,19)} = 10.37$, $p = 0.0003$, $n = 5$ –6/group). These data suggest that inhibition of the M-current in the BLA increases the strength of fear memory consolidation.

Having observed that pharmacologically blocking the M-current in the BLA enhances consolidation, we next explored whether enhancing the M-current would inhibit consolidation. To do this, we examined whether retigabine (Ret), a drug that maintains the open-state of KCNQ channels (Tatullian et al., 2001; Wuttke et al., 2005), would inhibit consolidation. Administering Ret immediately after strong fear conditioning, either by systemic injection or BLA infusion, dose-dependently inhibited freezing in response to the training tone the following day (Fig. 4C,D; $F_{(4,25)} = 3.82$, $p = 0.0147$, $n = 6$ /group for systemic injection; $F_{(3,16)} = 8.28$, $p = 0.0015$, $n = 5$ /group for BLA infusion).

So far our experiments have addressed the roles of PLC-coupled neuromodulators and the M-current in cued fear memory consolidation. However, we hypothesized that our findings in the BLA would apply to classically conditioned fear memory in general. To test this, we repeated two of our pharmacological experiments, but instead examined contextual fear memory 1 d after training. Post-training infusion of either Tzp+ICI or Ret into the BLA impaired the consolidation of contextual fear memory (Fig. 4E; $F_{(2,12)} = 9.83$, $p = 0.003$, $n = 5$ /group), demonstrating that consolidation of hippocampus-dependent fear memory is also sensitive to the impairing effects of dual neuromodulator blockade or augmentation of the M-current in the BLA.

Regulation of consolidation by PLC and PLC-coupled neuromodulators requires normal KCNQ channel activity

To test whether the effects of pharmacological manipulation of β_2 AR, D₅R, and M₁R are mediated by downstream effects on the M-current, we examined whether the impairing effects of coantagonist treatment could be reversed by coadministration of the M-current blocker XE991. XE991 alone had no effect on consolidation when administered immediately after strong fear conditioning (Fig. 5A; $F_{(2,24)} = 11.19$, $p = 0.0004$ for main effect of antagonist treatment, $F_{(1,24)} = 27.00$, $p < 0.0001$ for main effect of XE991 treatment; $F_{(2,24)} = 11.03$, $p = 0.0004$ for interaction of antagonist and XE991 treatments, $n = 5$ /group). However, XE991 rescued consolidation from the impairing effects of Tzp+SCH and SCH+ICI. These data suggest that inhibition of the M-current is sufficient for the enhancement of consolidation mediated by β_2 AR, D₅R, and M₁R.

Next, we asked whether potentiating the M-current with Ret could block the enhancing effects of β_2 AR, D₅R, and M₁R agonists on consolidation. Ret alone had no effect on consolidation when administered immediately after moderate fear conditioning (Fig. 5B; $F_{(3,42)} = 2.89$, $p = 0.0468$ for the main effect of

Figure 4. The M-current restrains fear memory consolidation in the BLA. All treatments were administered immediately after conditioning, with testing performed the following day. **A, B**, Mice were conditioned with 0.4 mA shock. **A**, The KCNQ channel blocker XE991 dose-dependently increases freezing in response to the training tone. **B**, Bilateral infusion of XE991 into BLA dose-dependently enhances cued fear memory consolidation. **C, D**, Animals were conditioned with 1 mA shock. **C**, The KCNQ channel opener Ret dose-dependently inhibits freezing in response to the training tone. **D**, Bilateral infusion of Ret into the BLA dose-dependently inhibits fear memory consolidation. **E**, Infusing Tzp + ICI (1 μ g + 50 ng) or Ret (1 μ g) into the BLA immediately after conditioning impairs contextual fear memory the following day (* p < 0.05; ^ p < 0.01; # p < 0.001).

agonist; $F_{(1,42)} = 20.54$, $p < 0.0001$ for the main effect of Ret; and $F_{(3,42)} = 3.85$, $p = 0.0161$ for the interaction between agonist and Ret, $n = 6$ –7/group). However, Ret completely blocked the enhancements of consolidation elicited by agonists of M₁R, β_2 AR, and D₅R. These data suggest that the ability of PLC activation by M₁R, β_2 AR, and D₅R to promote consolidation requires inhibition of the M-current.

To determine whether the effects of PLC activation on consolidation in the BLA are indeed mediated through inhibition of the M-current, we challenged the impairing effects of direct PLC inhibition with M-current blockade by XE991. As previously reported (Ouyang et al., 2012), infusing the PLC inhibitor edelfosine into the BLA immediately after strong fear conditioning impaired consolidation (Fig. 5C; $F_{(3,16)} = 12.33$, $p = 0.002$, $n = 5$ /group). However, coinjection with XE991 prevented this impairment, suggesting that the lack of consolidation observed in response to PLC inhibition is due to a failure to close KCNQ channels. Conversely, enhancement of consolidation by infusing the PLC activator m-3M3FBS (3M3) was inhibited by coadministration of the KCNQ channel opener Ret (Fig. 5D; $F_{(3,16)} = 14.23$, $p < 0.0001$, $n = 5$ /group). These data further support the notion that PLC promotes consolidation in large part by closing KCNQ channels and inhibiting the M-current.

Finally, we examined whether pharmacologically manipulating KCNQ channels would influence a distinct mechanism relevant to consolidation: that mediated by glutamatergic NMDA (GluN) receptor ion channels. Pretraining administration of antagonists or blockers of GluN impairs acquisition/consolidation of cued fear memory (Rodrigues et al., 2001; Bauer et al., 2002; Goosens and Maren, 2004). We first confirmed that injection of the competitive GluN antagonist ± 3 -(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP; 10 mg/kg) 1 h before strong fear conditioning decreases freezing in response to the training tone the following day (Fig. 5E; $F_{(4,20)} = 6.76$, $p = 0.0013$, $n = 5$ /group). Consistent with a critical role for activation of GluN during acquisition, the effect of CPP was greatly reduced when

Figure 5. Manipulating the M-current blocks the effects of neuromodulatory and GluN receptor manipulations on consolidation. All treatments were administered immediately after conditioning, with cued fear testing performed the next day. **A**, XE991 (1 mg/kg) has no effect on freezing in response to the training tone when conditioned with strong shock (1 mA). As expected, immediate post-training injection of Tzp + SCH or Tzp + ICI (both 1 mg/kg + 30 μ g/kg) inhibits freezing in response to the training tone, whereas XE991 (1 mg/kg) blocks these inhibitory effects. **B**, Ret (4 mg/kg) has no effect on freezing in response to the training tone when mice are conditioned with moderate shock (0.4 mA). Agonists of M₁R (Cev, 1 mg/kg), β_2 AR (procaterol, Proc; 50 μ g/kg), and D₅R (SKF, 2 mg/kg) enhance freezing in response to the training tone the next day, whereas Ret blocks these enhancing effects. **C, D**, All treatments were infused into the BLA immediately after conditioning. **C**, XE991 (XE, 1 μ g) has no effect on freezing in response to the training tone during testing when mice are conditioned with 1 mA shock. The PLC inhibitor edelfosine (Edel, 2 ng) significantly inhibits freezing in response to the training tone the next day, but coadministration with XE reverses that inhibition. **D**, Ret (1 μ g) has no effect on freezing in response to the training tone during testing when mice are conditioned with 0.4 mA shock. The PLC agonist 3M3 (0.2 ng) significantly enhances freezing in response to the training tone the next day, but coadministration with Ret reverses that enhancement. **E**, All subjects were injected both 60 min before and immediately after (+1 min) conditioning with 1 mA shock. The GluN antagonist CPP (C, 10 mg/kg) significantly inhibits consolidation when given before but not after training. XE991 (1 mg/kg) rescues CPP-induced impairment when injected with CPP before training and when injected immediately after training (* p < 0.05; ^ p < 0.01; # p < 0.001).

administered immediately after conditioning. Interestingly, immediate post-training injection of XE991 partially rescued the impairing effect of pretraining CPP injection, whereas concurrent pretraining administration of CPP + XE991 resulted in even greater but incomplete rescue. These results suggest that KCNQ channels may also contribute to some of the earliest postacquisition events that promote consolidation of fear memory.

Receptors that regulate PLC and KCNQ channels influence consolidation downstream of cAMP/PKA

Results from prior studies indicate a critical role for cAMP/PKA-dependent signaling in the BLA during fear memory consolidation (Schafe and LeDoux, 2000). Given the prominent role for neuromodulator-regulated PLC activity in consolidation, we explored the relationship between such signaling and that for

Figure 6. A critical aspect of cAMP/PKA signaling in fear memory consolidation is upstream of signaling by neuromodulators, PLC, and KCNQ channels. All compounds were infused directly into the BLA immediately after fear conditioning, and cued fear testing was performed one day later. **A, D.** Mice were conditioned with 0.4 mA shock. **A.** The cAMP analog Sp8 (0.5 μ g) increases freezing in response to the training tone. The impairing effect of Sp8 is mitigated by coinfusion with either combined M₁R + β_2 antagonists (Tzp + ICI, 1 μ g + 50 ng), a PLC antagonist (Edel, 2 ng), or a KCNQ channel opener (Ret, 1 μ g). **B, C.** Mice were conditioned with 1 mA shock. **B.** The impairing effect of Tzp + ICI (1 μ g + 50 ng) on consolidation is not reversed by Sp8 (2 μ g). **C.** The impairing effect of the protein kinase A inhibitor PKI (0.5 μ g) is blocked by coinfusion with either an M₁R agonist (Cev, 1 μ g), a PLC agonist (3M3, 0.2 ng), or a KCNQ channel blocker (XE, 1 μ g). **D.** The M₁R agonist Cev (1 μ g) enhances consolidation, and this effect is not significantly altered by coinfusion with PKI (0.5 μ g); * p < 0.05; ^ p < 0.01; # p < 0.001.

Figure 7. A model for an important aspect of neuromodulatory control over fear memory consolidation. At least three neuromodulatory receptors (M₁, β_2 , D₅) redundantly couple to PLC in the BLA to promote consolidation. A critical downstream effector of PLC activity is the KCNQ channel that underlies the M-current. Reduction in the M-current is hypothesized to enhance neuronal excitability and consolidation during a period beginning ~30 min after acquisition. Although the exact mechanism for KCNQ channel closure following acquisition has not been determined, it is likely to be a combination of PLC-mediated signaling events that include a reduction in PIP₂ levels, an increase in intracellular calcium, and activation of PKC. CaM, Calmodulin; DA, dopamine; ER, endoplasmic reticulum; NE, norepinephrine; PLC- β , phospholipase C- β .

cAMP/PKA. We first examined the enhancement of consolidation mediated by administration of the membrane-permeable cAMP analog Sp8. Sp8 was infused into the BLA immediately after moderate fear conditioning. As expected, Sp8 enhanced cued fear memory consolidation (Fig. 6A; $F_{(4,20)} = 6.25$, $p = 0.002$, $n = 5$ /group). Interestingly, coadministration of Tzp + ICI prevented Sp8-induced consolidation enhancement, suggesting that cAMP signaling is upstream of M₁R/ β_2 AR activation. Given this observation, we asked whether augmenting cAMP signaling would rescue the consolidation impairment elicited by these receptor antagonists. Sp8 and Tzp + ICI were coadministered after strong fear conditioning. The consolidation impairment by Tzp + ICI was not rescued by Sp8 (Fig. 6B; $F_{(2,12)} = 6.26$, $p = 0.0138$, $n = 5$ /group), further supporting the idea that an important effect of cAMP on consolidation is upstream of M₁R/ β_2 AR signaling.

Complementary to the above, we confirmed the requirement for cAMP/PKA signaling in consolidation by infusing the PKA inhibitor PKI immediately after strong fear conditioning (Fig. 6C; $F_{(4,20)} = 13.82$, $p < 0.0001$, $n = 5$ /group). Interestingly, coadministration of either the M₁R agonist Cev, the PLC agonist 3M3, or the KCNQ channel blocker XE991 rescued the impair-

ment of consolidation induced by PKI. Additionally, PKI did not block the enhancement of consolidation induced by Cev (Fig. 6D; $F_{(2,12)} = 7.02$, $p = 0.0096$, $n = 5$ /group). Together, these data indicate that an important role for cAMP/PKA signaling in fear memory consolidation is upstream of neuromodulatory receptor activation that is coupled to PLC and the M-current.

Discussion

Most research into the role of M₁R in fear memory consolidation suggests that stimulation of M₁R enhances but is not required for consolidation (Robinson et al., 2011). To address this seeming inconsistency, the present study explored whether

M₁R contributes to the redundant activation of PLC by β_2 AR and D₅R in the BLA that is necessary for consolidation (Ouyang et al., 2012). We show here that an M₁R-selective antagonist does not inhibit fear memory consolidation in the BLA unless coadministered with an antagonist of either β_2 AR or D₅R. We also demonstrate that antagonists of β_2 AR and D₅R impair consolidation in M₁R KO but not WT mice. These data support the existence of redundant signaling by M₁R, β_2 AR, and D₅R in fear memory consolidation. Further, we show that consolidation enhancement induced by an M₁R agonist is lost in M₁R KO mice, indicating that other G_{q/11}-coupled muscarinic receptors, such as M₃R, are not sufficient for the role of muscarinic receptors in promoting consolidation (Caulfield, 1993).

β_2 AR and D₅R stimulation elevates BLA IP₃ and contributes to the increase in IP₃ observed 30 min after conditioning (Ouyang et al., 2012). Here, we show that activation of M₁R also elevates BLA IP₃, and that M₁R antagonism, when combined with that for either β_2 AR or D₅R, inhibits conditioning-induced IP₃ increases. These observations suggest an important role for activation of BLA PLC by M₁R during consolidation. Anticipated mechanisms through which PLC regulates consolidation include those mediated by IP₃ and DAG generated via hydrolysis of PIP₂. IP₃ enhances intracellular calcium release that, together with DAG, could drive a number of relevant signaling pathways, including protein kinase C (PKC) and calcium- and calmodulin-dependent protein kinase II (Miller et al., 2002; Bonini et al., 2005). Indeed, activation of muscarinic receptors induces intracellular calcium release in BLA neurons (Power and Sah, 2007). However, broad electrophysiological effects of IP₃R antagonists (Ozaki et al., 2002) preclude pharmacological characterization of behavioral roles for IP₃-dependent mechanisms. Alternatively, conditional genetic approaches related to IP₃ signaling should be insightful (Chen et al., 2012).

In addition to the generation of IP₃ and DAG, breakdown of PIP₂ by PLC can affect the activity of a variety of ion channels (Suh and Hille, 2008; Logothetis et al., 2010). We examined KCNQ potassium channels here because they are inhibited by muscarinic agonists in the BLA (Womble and Moises, 1992, 1993), their inhibition by M₁R is well characterized (Suh and Hille, 2002; Kosenko et al., 2012), and selective KCNQ channel modulators have been validated in subunit KO mice (Tzingounis and Nicoll, 2008). Our experiments demonstrate that pharmacologically modulating the M-current in the BLA strongly affects consolidation of fear memory.

Inhibiting the M-current increases neuronal excitability by diminishing the afterhyperpolarization and reducing spike accommodation (Aiken et al., 1995; Peters et al., 2005). Our data and recent reports that XE991 enhances learning and memory (Santini and Porter, 2010; Fontan-Lozano et al., 2011) support the hypothesis that increased neuronal excitability following acquisition promotes consolidation (Giese et al., 2001). Of special note, increased firing rates have been observed in cat BLA neurons 30–50 min after inhibitory avoidance conditioning (Pelletier et al., 2005), a time interval similar to the period over which conditioning-induced increases in BLA IP₃ occur (Ouyang et al., 2012).

The M-current limits membrane depolarization by EPSPs, and thus reduction in the M-current could play a role in shaping EPSPs induced by fear conditioning (George et al., 2009; Shah et al., 2011). Increased excitability induced by KCNQ blockade may also enhance activation of L-type voltage-gated calcium channels that promote consolidation in the BLA (Bauer et al., 2002; Shinnick-Gallagher et al., 2003; McKinney and Murphy, 2006). Interestingly, enhanced excitability during the consolidation period that is sensitive to M-current manipulation also may result from internalization of GABA_A receptor subunits, suggesting that enhanced excitability may have multiple mechanisms and be of general importance (Chhatwal et al., 2005; Mou et al., 2011).

The observation that modulating BLA KCNQ channel activity can completely reverse the effects on consolidation of PLC manipulation suggests that consolidation may not require effects of IP₃ and DAG on targets other than the M-current, at least ~30 min after conditioning. PKC, which can be activated by elevated calcium secondary to IP₃ and/or by DAG, may also be required for fear memory consolidation. When infused into the BLA, an inhibitor of the PKC α and PKC β isozymes impairs consolidation of inhibitory avoidance memory (Bonini et al., 2005), and a role for BLA PKC in the maintenance of fear memory is likely (Serrano et al., 2008). Among PKC isozymes, PKC β but not PKC γ or PKC δ appears to be critical because only PKC β KO mice exhibit deficits in cued fear memory (Abeliovich et al., 1993; Weeber et al., 2000; Selcher et al., 2002). Although specific roles and mechanisms for PKC β in fear memory have not been delineated, PKC could contribute to consolidation in parallel with PIP₂ depletion by facilitating suppression of the M-current (Hoshi et al., 2003).

Notably, other ion channels that regulate excitability can be modulated by PLC activity. Decreases in PIP₂ inhibit voltage-dependent calcium channels, transient receptor potential channels, and inwardly rectifying potassium channels (Keselman et al., 2007; Suh and Hille, 2008). In addition, PKC can regulate the activity of multiple ion channels (Dai et al., 2009). Our observation that agonists of β_2 AR, D₅R, and M₁R lose their enhancing effects on consolidation when coadministered with the KCNQ channel opener retigabine suggests that modulation of other ion channels is insufficient for neuromodulator-enhanced consolidation. Conversely, the observation that XE991 overcomes the impairment of consolidation by receptor antagonist treatment suggests that blockade of KCNQ channels is sufficient to promote neuromodulator-mediated consolidation. Based on our findings, we predict that future studies will demonstrate suppression of the M-current in the BLA by agonists of β_2 AR and D₅R.

Our finding an interaction between the role of GluN and KCNQ channels in fear memory consolidation was unexpected. GluN channels are thought to contribute to acquisition and/or the beginning of memory consolidation by promoting some of the earliest signaling events that result from the convergence of conditioning and reinforcing sensory input to the BLA (Ro-

drigues et al., 2001; Bauer et al., 2002; Goosens and Maren, 2004). Our data are consistent with this idea. Interestingly, pretraining, and to some extent, even immediate post-training blockade of KCNQ channels considerably reduces the impairing effect of pretraining GluN antagonism on consolidation. As a potential intermediate mechanism that could explain these findings, it is possible that the calcium influx mediated by GluN channels activates PLC, which in turn suppresses KCNQ channel activity that is relevant to consolidation. Isozymes PLC δ and PLC η are activated by calcium rather than G-protein subunits (Delmas et al., 2004; Cockcroft, 2006), and specific roles for the coupling of GluN and PLC activity have been identified (Codazzi et al., 2006; Horne and Dell'Acqua, 2007). Mechanisms for the role of GluN in consolidation that are in addition to the suppression of the M-current are expected, and are supported by the observation that XE991 incompletely reverses the impairing effects of GluN receptor antagonism.

With respect to cAMP/PKA signaling in fear memory consolidation, our findings suggest that cAMP/PKA has an important role upstream of the neuromodulatory receptors that activate PLC. It may be that a prominent role for cAMP/PKA signaling in consolidation is the presynaptic regulation of neurotransmitter release within the BLA ~30 min after conditioning, when IP₃ levels rise. Indeed, cAMP/PKA signaling plays a critical role in presynaptically expressed LTP (Castillo et al., 2002; Lonart et al., 2003; Bayazitov et al., 2007; Fourcaudot et al., 2008). Such a role may be at least partly responsible for the enhanced neurotransmitter release observed *in vivo* following conditioning in some paradigms (Tronel et al., 2004; Guzmán-Ramos et al., 2010, 2012). Thus, we propose that cAMP/PKA regulates fear memory consolidation in part by augmenting the release of neuromodulators that activate and M₁R, β_2 AR, and D₅R. Our results do not exclude a postsynaptic role for cAMP/PKA signaling in consolidation, but do indicate that such signaling is not downstream of neuromodulator-driven PLC activity during this phase of consolidation.

In summary, we report a role for M₁R in the consolidation of fear memory as a redundant contributor to requisite PLC activity in the BLA (Fig. 7). We propose that PLC increases neuronal excitability in the BLA by suppressing KCNQ channel activity. Our experiments with retigabine offer the first report of an effect of this drug on learning and memory. Developed as an anticonvulsant, retigabine was recently approved by the FDA as an anti-epileptic (Orhan et al., 2012). Our observation that retigabine can inhibit the consolidation of fear memory suggests that these drugs might have an additional application in preventing PTSD when given shortly after a traumatic experience.

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