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A Specific Role for Ca^{2+}-Dependent Adenylyl Cyclases in Recovery from Adaptive Presynaptic Silencing

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Glutamate generates fast postsynaptic depolarization throughout the CNS. The positive-feedback nature of glutamate signaling likely necessitates flexible adaptive mechanisms that help prevent runaway excitation. We have previously explored presynaptic adaptive silencing, a form of synaptic plasticity produced by ongoing neuronal activity and by strong depolarization. Unsilencing mechanisms that maintain active synapses and restore normal function after adaptation are also important, but mechanisms underlying such presynaptic reactivation remain unexplored. Here we investigate the involvement of the cAMP pathway in the basal balance between silenced and active synapses, as well as the recovery of baseline function after depolarization-induced presynaptic silencing. Activation of the cAMP pathway activates synapses that are silent at rest, and pharmacological inhibition of cAMP signaling silences basally active synapses. Adenylyl cyclase (AC) 1 and AC8, the major Ca^{2+}-sensitive AC isoforms, are not crucial for the baseline balance between silent and active synapses. In cells from mice doubly deficient in AC1 and AC8, the baseline percentage of active synapses was only modestly reduced compared with wild-type synapses, and forskolin unsilencing was similar in the two genotypes. Nevertheless, after strong presynaptic silencing, recovery of normal function was strongly inhibited in AC1/AC8-deficient synapses. The entire recovery phenotype of the double null was reproduced in AC8-deficient but not AC1-deficient cells. We conclude that, under normal conditions, redundant cyclase activity maintains the balance between presynaptically silent and active synapses, but AC8 plays a particularly important role in rapidly resetting the balance of active to silent synapses after adaptation to strong activity.

Key words: **exocytosis; homeostasis; synaptic strength; epilepsy; cAMP; glutamate**

Introduction

Homeostatic mechanisms are activated by disturbances in the balance between excitation and inhibition and have been the subject of intense scrutiny recently (Burrone and Murthy, 2003; Turrigiano and Nelson, 2004; Rich and Wenner, 2007). We have described a form of presynaptic adaptation by which glutamatergic presynaptic terminals respond to changes in activity with a binary adjustment in the release competence of their synaptic vesicles (Moulder et al., 2004, 2006). Presynaptic silencing operates at normal levels of activity. In hippocampal cultures, ~25% of terminals are silent basally, and block of electrical activity decreases this percentage (Moulder et al., 2006). Furthermore, modest depolarization and associated spiking decrease the percentage of active synapses (Moulder et al., 2006). The mechanism of this adaptation to a wide range of activity levels appears to involve primarily a change in vesicle priming (Moulder et al., 2006). Such adaptive mechanisms may exist in part to limit the likelihood that glutamate signaling will run away unchecked, thus avoiding possible excitotoxicity.

Because appropriate alterations in activity can bidirectionally determine the percentage of active synapses, a balance must exist between mechanisms that activate and that silence synapses. Likewise, after presynaptic silencing, removal of the depolarizing stimulus should result in recovery of silenced synapses to an active state. Indeed, we have shown that recovery from strong presynaptic silencing requires ~4 h (Moulder et al., 2004). Although such recovery is presumably important for resumption of normal signaling after a depolarizing challenge, the mechanisms of synaptic activation and reactivation are primarily unexplored. Here we investigate the signaling pathways involving maintenance and recovery of an active status at glutamatergic presynaptic terminals.

Previous evidence has implicated the cAMP pathway in various forms of increased presynaptic efficacy, specifically the activation status of synaptic vesicles (Huang et al., 1994; Weisskopf et al., 1994; Tong et al., 1996; Ma et al., 1999; Kohara et al., 2001). We therefore explored the hypothesis that cAMP signaling participates in setting the baseline percentage of presynaptically active synapses and in recovery from adaptation to strong depolarization. We found that modulation of cAMP signaling indeed influenced the percentage of presynaptically active synapses, con-

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sistent with a cAMP "tone" maintaining basal activation status of glutamate terminals. To test whether Ca^{2+}-dependent isoforms of adenylyl cyclase (AC) are involved in maintaining activation status of terminals, we examined synapses from mice deficient in AC1 and AC8, which have been implicated previously in synaptic plasticity (Villacres et al., 1998; Wong et al., 1999; Wang et al., 2003; Gong et al., 2007). We found that AC1 and AC8 were not necessary for maintaining a strong majority of active synapses. However, when the system was challenged by depolarization-induced silencing, a strong role for AC8 was revealed during recovery from adaptive silencing. We conclude that redundant and/or compensatory activity of multiple cyclase isoforms other than AC1 and AC8 is capable of maintaining active synapses under normal conditions. However, when the system is perturbed, Ca^{2+}-dependent isoforms are particularly important for restoring normal ratios of active to inactive presynaptic terminals.

Materials and Methods

Cell culture. Hippocampal cultures were prepared as described previously (Mennerick et al., 1995). In brief, dissected postnatal (postnatal days 0–3) rat or mouse hippocampi were incubated with papain and then mechanically dissociated and plated at either ~650 cells/mm^2 as "mass" cultures or 100 cells/mm^2 on microdots of collagen as "microisland" cultures. Plating medium consisted of Eagle's medium (Invitrogen, Gaithersburg, MD) supplemented with heat-inactivated horse serum (5%), fetal bovine serum (5%), 17 mM glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultures were maintained at 37°C in a humidified incubator with 5%CO_2/95% air. Cytosine arabinoside at 6.7 μM was added 3–4 d after plating to inhibit cell division. At 4–5 d after plating, half the culture medium was replaced with Neurobasal medium (Invitrogen) plus B27 supplement.

Neurons were challenged with cAMP activators or inhibitors or with elevated $[K^+]_o$ at between 10 and 15 days *in vitro* (DIV), when synapses were well developed. $[K^+]_o$ was elevated from 5 to 35 mM for 4 or 16 h as indicated, with equimolar NaCl added to control cultures to match osmotic changes. D-2-Amino-5-phosphonovalerate (D-APV) at 25 μM and 1 μM 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline (NBQX) were added to control and experimental dishes during the depolarizing challenge. For experiments in which recovery from a depolarization challenge was assessed, agents present during the recovery period, e.g., Rp-cAMPS and KT5720 [(9*S*,10*R*,12*R*)-2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylicacid hexyl ester] (see Fig. 8), were also added for the last 30 min of the depolarization challenge. Recovery medium consisted of Neurobasal medium plus B27 supplement, which had been previously equilibrated to 37°C. For experiments in which electrical activity was blocked, 500 nM tetrodotoxin (TTX) plus 25 μM D-APV and 1 μM NBQX were added immediately at 4–5 DIV. These experiments were then conducted at 11–14 DIV (6–10 d of treatment), as described previously (Moulder et al., 2006). For agents dissolved in DMSO, final DMSO concentration was ≤0.1%.

FM1-43FX/vesicular glutamate transporter-1 assay for active synapses and other staining. Mass cultures plated on coverslips were used for all imaging experiments. Active synapses were labeled with a 2 min application of 10 μM FM1-43FX [fixable version of *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide] (Invitrogen, Eugene, OR) and 45 mM K^+ (equimolar substitution for Na^+) in extracellular recording saline containing the following (in mM): 138 NaCl, 4 KCl, 2$CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, and 0.025 D-APV, and 0.001 NBQX, pH 7.25. Cultures were washed for 10 s with extracellular recording saline containing 500 μM Advasep-7 (CyDex, Overland Park, KS) to remove nonspecific dye (Kay et al., 1999) and washed in saline alone for 10 min. Cultures were fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBS for 10 min.

For subsequent antibody staining, cells were washed with PBS and incubated in blocking solution (4% normal goat serum/0.04% Triton X-100 in PBS) for 15 min, followed by vesicular glutamate transporter 1 (vGluT-1) primary antibody (Millipore Bioscience Research Reagents, Temecula, CA) in blocking solution (1:2000 dilution for 3 h). Cells were washed with PBS and then incubated with cyanine 3 (Cy3)-conjugated anti-guinea pig antibody (1:500 in blocking solution; Millipore Bioscience Research Reagents) for 30 min. Coverslips were then washed with PBS and mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL).

All stains were examined by confocal microscopy performed using a 60× objective (1.4 numerical aperture), a C1 scanning confocal laser attached to an inverted Eclipse TE300 or TE2000 microscope (Nikon, Melville, NY), and Z-C1 software (Nikon). An observer naive to experimental conditions acquired images of representative fields in *z*-stack using alternating excitation by the 488 and 543 nm laser lines. Gain settings, dwell time, field of view size, and *z*-stack parameters were kept constant for all images within an experiment. Monochrome images were converted into projected images and analyzed using MetaMorph software (Universal Imaging, Downingtown, PA). Ten puncta per field and 5–10 fields per condition were analyzed for each experiment. Staining was thresholded independently in the vGluT-1 (Cy3) and FM1-43FX channels. Consistent thresholding algorithms were applied to all dishes from a single experiment (which were always stained simultaneously). vGluT-1 (Cy3)-positive puncta were defined first, without reference to the corresponding FM1-43FX stain. Regions identified in the vGluT-1 image were then transferred to the FM1-43FX channel. Regions with FM1-43FX staining exceeding a criterion of >10 thresholded pixels within the identified region were considered active presynaptic terminals. We verified in 15 images (150 terminals) that this criterion resulted in identification of terminals at or below background FM1-43FX fluorescence (assessed by moving the region of interest to a cell region just adjacent to the synaptic punctum). Experimental conditions were kept coded until analysis was complete.

For synapse density estimates, wild-type (WT) and matched AC1/AC8-deficient mouse cultures stained with the vGluT-1 antibody were evaluated. A rater naive to experimental conditions counted labeled puncta per 100 μm length of neurite. Segments of dendrite were pseudorandomly chosen without regard to branching order of the dendrite.

Electrophysiology. Whole-cell recordings were performed using an Axopatch 1D amplifier (Molecular Devices, Sunnyvale, CA) and a Digidata 1322 acquisition board (Molecular Devices). Electrodes had resistances of 3–5 MΩ, and access resistance was compensated 80–100%. In all instances, cells were excluded from analysis if a leak current >300 pA was observed.

For recording, the culture medium was exchanged for recording solution containing the following (in mM): 138 NaCl, 4 KCl, 2$CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, and 0.025 D-APV. The whole-cell pipette solution contained the following (in mM): 140 K-gluconate, 0.5 $CaCl_2$, 5 EGTA, and 10 HEPES, pH 7.25. For synaptic recordings, cells were stimulated with 1.5 ms pulses to 0 mV from −70 mV to evoke transmitter release (Mennerick et al., 1995).

Solutions were exchanged via a local multibarrel perfusion pipette with a common perfusion port placed within 0.5 mm of the cell under study. Liquid junction potential measurements exhibit exchange times between barrels of ~50 ms. For hypertonic solution (0.5 M sucrose), application time was 3 s. Sucrose responses were integrated to include responses beneath the transient peak of the response to 10% of the steady-state response. For vesicular release probability calculations, spike-evoked EPSCs were integrated over 50 ms and compared with integrated sucrose-evoked EPSCs from the same cell. Miniature EPSCs (mEPSCs) were recorded in the presence of 500 nM TTX and were analyzed with MiniAnalysis version 5.6 (Synaptosoft, Decatur, GA).

Action potentials were measured in the current-clamp recording mode of the patch amplifier. For threshold measurements, action potentials were generated by injecting depolarizing current (30–200 pA).

Control and experimental conditions were always performed on sibling cultures from the same litter and plating and on the same day of recording. Solitary microisland neurons were used for all electrophysiology experiments, except in experiments using neurons from AC-deficient [knock-out (KO)] animals (see Figs. 3, 6, 7, 9) in which mass cultures were used and only sucrose-evoked responses (but not spike-

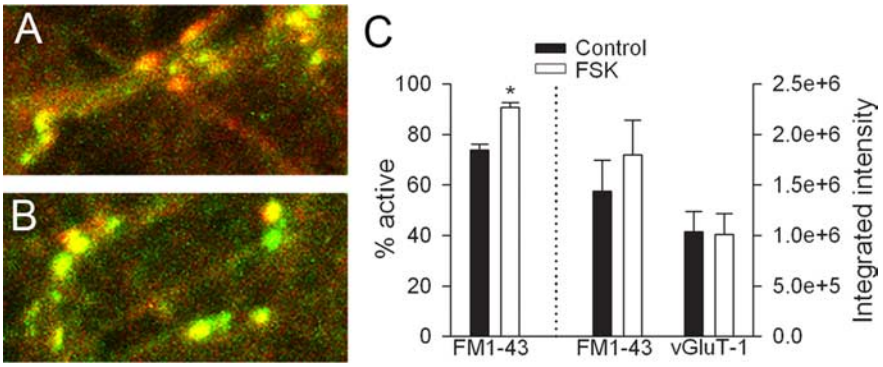

Figure 1. Effects of FSK on the percentage of active synapses, assessed optically. ***A***, Merged image of FM1-43FX stain of active synapses (green) and vGluT-1 immunoreactivity (red) to reveal all glutamatergic presynaptic terminals in a control (untreated) field. ***B***, Merge of FM1-43FX uptake and vGluT-1 staining from a field treated with 50 μM forskolin for 4 h. ***C***, Summary of percentage of active glutamatergic terminals assessed by FM1-43FX/vGluT-1 correspondence ($n = 300$ terminals from 6 experiments). *$p < 0.01$. Also shown are the average integrated fluorescence intensities of FM1-43FX and vGluT-1 staining from the same population of terminals (no statistically significant differences). Only terminals that met the criterion for being active (see Materials and Methods) were included in the FM1-43FX integrated intensity measurement.

driven EPSCs) were assayed. To isolate glutamate-mediated sucrose-driven EPSCs in AC KO animals, 25 μM bicuculline and 500 nM TTX were added to the extracellular solution.

AC1/AC8 KOs. The production of AC1 KO, AC8 KO, and AC1/8 double KO (DKO) animals has been described previously (Wu et al., 1995; Wong et al., 1999; Schaefer et al., 2000). All AC mutant lines have been backcrossed more than 10 generations onto a C57BL/6 background, with homogeneity on the C57BL/6 background confirmed by analysis of polymorphic markers between C57BL/6 and 129 mouse strains. To generate mice for these studies, we used progeny of homozygous mutants (AC1 KO, AC8 KO, and AC1/8 DKO) and WT mice bred in our colony. Wild-type C57BL/6 mice born on the same date as AC mutant litters were used as controls. Mice were maintained on a 12 h light/dark schedule with *ad libitum* access to food and water.

Reagents. All chemicals were obtained from Sigma (St. Louis, MO), unless otherwise indicated. Sp-cAMPS and Rp-cAMPS were obtained from Biomol (Plymouth Meeting, PA). KT5720 was obtained from EMD Biosciences (La Jolla, CA).

Data acquisition and statistics. pClamp software, version 9 (Molecular Devices), was used for electrophysiology data acquisition and analysis for all experiments. Data plots were created with SigmaPlot software (SPSS Science, Chicago, IL). Data are presented in the figures and the text as mean ± SEM. Paired and unpaired *t* tests were used to evaluate statistical significance between two experimental conditions. Comparisons among multiple conditions were made using one-way ANOVA tests with secondary Tukey's tests for pairwise comparisons within the group.

Results

Role of cAMP signaling and AC1/AC8 in basal and FSK unsilencing

Our own and others' previous results have demonstrated that a fraction of hippocampal presynaptic terminals are silent under basal conditions (Rosenmund et al., 2002; Altrock et al., 2003; Moulder et al., 2006; Ting et al., 2007) (Fig. 1*A*). Silent terminals are identified as presynaptic puncta that label with antibodies directed against synaptic vesicle proteins but that do not label with FM dyes, which label release-competent vesicles (Fig. 1*A*). To test whether cAMP increases can activate the basal population of silent synapses, we incubated sibling cultures in 50 μM forskolin (FSK) or control medium for 4 h, followed by brief depolarization in the presence of FM1-43FX to label the entire pool of release-competent, recycling vesicles. This live-cell phase of the experiment was followed by fixation and staining for the vesicular glutamate transporter (vGluT-1) to identify all glutamatergic presynaptic sites. Imaging of FM1-43FX and vGluT-1 was performed concurrently on the fixed cells. FSK clearly increased the percentage of active synapses, from 73.7 ± 2.4 to 90.7 ± 1.9% (Fig. 1*B*,*C*) ($p < 0.01$). In contrast, neither brief (30 min) nor 4 h FSK incubation resulted in reliable increases in FM1-43FX staining intensity when assayed with our stimulation/labeling protocols ($p > 0.06$). Therefore, we conclude that FSK effects in this assay are primarily through binary synaptic activation rather than on vesicle recruitment at already active synapses.

The FM1-43FX/vGluT-1 staining assays the recycling vesicle pool with a strong depolarization designed to recruit all release-competent vesicles, but FSK effects might not be apparent during glutamate release triggered by single action potentials. To confirm that the FM1-43FX/vGluT-1 staining assay has functional implications for spike-driven neurotransmission, we also assayed postsynaptic responses. For these studies, we used autaptic synapses to allow comparison of action-potential-evoked EPSCs with secretagogue-evoked EPSCs from the same population of axon terminals. Figure 2, *A* and *C*, shows representative action-potential-evoked autaptic EPSCs, which were enhanced by 50 μM FSK incubation for 4 h.

If FSK increases the number of active synapses, this should increase the total number of vesicles available for release. The entire population of release-ready vesicles can be assayed using brief hypertonic sucrose challenge to synapses (Rosenmund and Stevens, 1996; Moulder and Mennerick, 2005). Sucrose-evoked EPSC charge can be compared with the smaller, spike-driven EPSC charge to yield an estimate of vesicular release probability (p_r). Consistent with the effect observed in FM1-43FX/vGluT-1 staining, we found that integrated sucrose-evoked EPSCs were enhanced by FSK incubation (Fig. 2*B*,*D*,*E*). Sucrose-evoked EPSCs were increased by an average of 27.4% (Fig. 2*E*), which was similar to the increase in active synapse number (23.1% increase) (Fig. 1). We also found that Sp-cAMPS, a membrane-permeant analog of cAMP, reproduced the effects on sucrose-evoked EPSCs (36.3 ± 12.0% increase, $p < 0.03$; $n = 10$), consistent with the idea that the cAMP pathway, rather than nonspecific actions of FSK, account for the effects. The ratio of the postsynaptic charge transfer of action-potential-evoked EPSCs to sucrose-evoked responses yielded a vesicular release probability estimate of 7.7 ± 0.9% for control synapses and 10.4 ± 0.6% for FSK-treated synapses ($p < 0.05$). This result suggests that, in addition to recruiting new active synapses, FSK also increases the release probability to single spikes, similar to previous reports (Sakaba and Neher, 2001; Kaneko and Takahashi, 2004; Huang and Hsu, 2006). The observed increase in spike-driven EPSC amplitude (73.2% increase) (Fig. 2*E*) is most likely attributable to the combined effects of FSK on release probability and the number of release sites. We did not investigate the increased vesicular release probability further in the present work.

cAMP activation has also been linked to postsynaptic receptor modulation in certain situations (Greengard et al., 1991). To test that the sucrose-mediated responses were enhanced through presynaptic mechanisms, we examined the amplitude of mEPSCs in control and FSK-treated cells. We found no increase in mEPSC

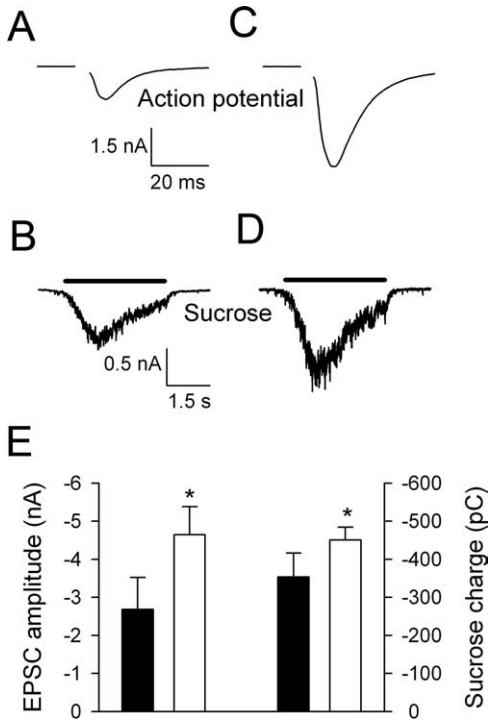

Figure 2. Effects of forskolin on action-potential-evoked EPSCs and hypertonicity-evoked EPSCs, a measure of the readily releasable vesicle pool size. ***A***, ***B***, Autaptic action-potential-driven and sucrose-driven EPSCs (AMPA receptor mediated) from a control cell. ***C***, ***D***, Action-potential-elicited and sucrose-elicited EPSCs from an FSK-treated cell (4 h, 50 μM treatment). ***E***, Summary of results for action-potential- and sucrose-driven EPSCs from control (filled bars) and FSK-treated sister cells (open bars). $n = 12$ cells in each condition. $^*p < 0.05$.

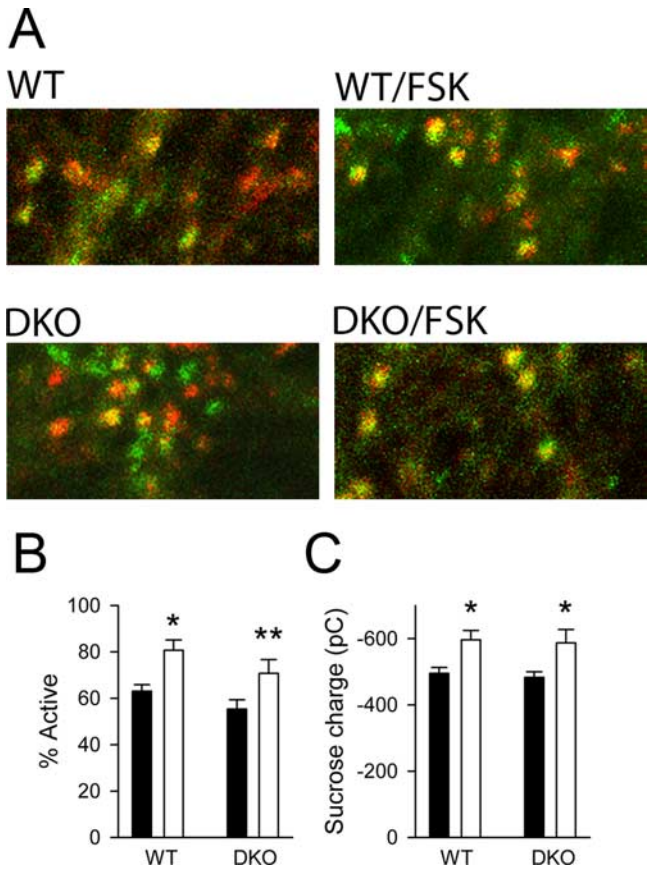

Figure 3. FSK effects on presynaptic unsilencing are unaffected by genetic deletion of the two major Ca^{2+}-sensitive adenylyl cyclase isoforms, AC1 and AC8. ***A***, The panels show merged FM1-43FX (green) and vGluT-1 (red) images in the indicated conditions. ***B***, Summary of percentage of active synapses by FM1-43FX/vGluT-1 correspondence ($n = 15$–30 fields from 3–6 experiments). $^*p < 0.01$, $^{**}p < 0.05$ compared with the control condition for each genotype. ***C***, Summary of sucrose-evoked charge from control (filled bars) and FSK-treated (open bars) WT and DKO cells ($n = 10$ cells per condition). $^*p < 0.05$ compared with the control condition for each genotype.

amplitude under our conditions (14.7 ± 0.68 vs 14.5 ± 0.74 pA; $p > 0.05$; $n = 10$ in each condition). In contrast, mEPSC frequency was significantly increased in FSK-treated cells (0.56 ± 0.06 Hz for control vs 0.82 ± 0.08 Hz for FSK-treated; $p < 0.02$; $n = 10$ in each group). In summary, electrophysiology primarily confirmed predictions from the FM1-43FX/vGluT-1 staining, although electrophysiological experiments also revealed an increase in vesicular p_r that staining studies could not detect. Both techniques demonstrated that an increase in the total number of immediately releasable vesicles underlies a large part of FSK effects.

Because the activation status of presynaptic terminals can be altered by changes in neuronal electrical activity (Moulder et al., 2006), is it possible that FSK and cAMP act upstream of neuronal depolarization? For instance, tetrodotoxin-induced electrical silencing increases the percentage of active presynaptic terminals (Moulder et al., 2006). Furthermore, cAMP signaling pathways have been reported to have direct effects on ion channels, particularly potassium conductances (Dong and White, 2003). Could FSK stimulation act by quieting neuronal spiking? Direct recordings of eight neurons treated acutely with FSK showed no significant change in excitability judged by spike number in response to a 100–200 pA, 100 ms current injection (0.2 ± 0.2 spike decrease), action potential threshold (0.5 ± 0.4 mV depolarization), action potential peak (2.6 ± 1.0 mV more positive), or action potential width (0.14 ± 0.3 ms widening). Four hour exposure to FSK also had no significant effect on action potential threshold, peak, or width ($n = 8$; spike number in response to current injection was not assessed because 4 h exposure could not be performed with an in-cell design). Thus, if electrical activity and cAMP increases are linked serially in a causal pathway, it is likely that changes in cAMP are downstream of activity changes. Our results do not exclude the possibility of parallel actions of activity and cAMP signaling.

Given the generally ubiquitous role of Ca^{2+} in the induction of activity-dependent synaptic plasticity, we explored the role of Ca^{2+}-sensitive isoforms of adenylyl cyclase, AC1 and AC8. We examined presynaptically silent synapses and releasable vesicle pools from cells deficient in both AC1 and AC8 and from cells in wild-type control mice. In the FM1-43FX/vGluT-1 assay, we found a marginally lower percentage of active synapses in DKO cells compared with wild-type controls examined in parallel. In 65 fields from 13 experiments, the percentage of active synapses in WT mouse control cultures was 69 ± 2.6%. In DKO cultures, it was 62 ± 2.4%. This difference was statistically significant ($p = 0.02$) but of modest magnitude. In experiments in which the effects of FSK on the percentage of active synapses were examined in the absence of AC1 and AC8, FSK increased the percentage of active synapses in both WT and DKO neurons (Fig. 3*A*,*B*) to a similar degree as in Figure 1. In complementary electrophysiology experiments, sucrose responses were not significantly different between wild-type and AC1/AC8-deficient cells in a sample of 10 neurons from each genotype, and 4 h FSK treatment increased sucrose responses similarly between WT and DKO cells (Fig. 3*C*) (20.5 ± 5.6% increase in WT and 21.6 ± 8.4% increase for DKO

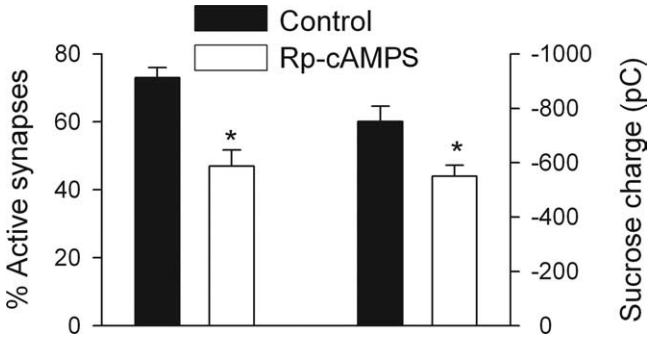

Figure 4. Pharmacological antagonism of cAMP signaling silences presynaptic terminals. The left bars summarize effects of 4 h incubation in 50 μM Rp-cAMPS on active synapses, assessed using FM1-43FX/vGluT-1 correspondence ($n = 10$ fields from 2 experiments). The right bars summarize the effects on sucrose-mediated EPSC charge ($n = 11$ cells per condition). *$p < 0.01$.

cells). The lack of change in basal sucrose EPSCs differs superficially from the small reduction in active synapses in AC1/AC8 cells observed in the FM1-43FX/vGluT-1 assay (see above). The lack of genotype effect in sucrose-evoked EPSCs likely reflects the small sample size compared with staining assays. In addition, it is important to note that absolute synapse number will be a large contributor to EPSC variability but will not contribute variability in the staining assay, because the FM1-43FX/vGluT-1 assay is inherently ratiometric. Therefore, we conclude that AC1 and AC8 are not necessary for maintaining the activation status of presynaptic terminals and are not necessary for the FSK-induced unsilencing of inactive synapses. It seems likely that other redundant and/or compensatory ACs are capable of mediating these effects in the absence of AC1/AC8.

Other measures also failed to reveal a strong effect of AC1/AC8 deletion on synaptic function. We measured synapse density along randomly selected segments of dendrite using vGluT-1 staining. We found no difference in the number of vGluT-1-positive puncta per 100 μm dendrite length. The density was 22.1 ± 2.2 puncta/μm in wild-type and 24.3 ± 3.5 puncta/μm in AC1/AC8 null neurons ($n = 10$ dendrites from each genotype). Furthermore, spike-driven EPSCs from autaptic AC1/AC8-deficient cultures did not differ from wild-type in EPSC amplitude (−1941.9 ± 198.8 nA for AC1/AC8 vs −2357.0 ± 417.4 nA for wild type; $n = 12$ each; $p > 0.05$) or the degree of paired-pulse modulation (2.9 ± 7.5% depression for AC1/AC8 vs 11.2 ± 13.4% depression for wild type; $n = 12$ each; $p > 0.05$).

AC1 and AC8 deletion had little effect on the basal percentage of active synapses. Is ongoing cAMP signaling therefore important for the basal level of synapses, or only for FSK-induced unsilencing of latent synapses? Furthermore, is the small but detectable effect of AC1/AC8 deletion on the percentage of active synapses caused by secondary (e.g., developmental) effects of AC1/AC8 loss or by a necessity of ongoing cAMP signaling for maintaining active synapses? We tested these questions with pharmacological antagonism of cAMP signaling. The membrane-permeant cAMP antagonist Rp-cAMPS (50 μM), administered to cells for 4 h, significantly reduced the percentage of active synapses in the FM1-43FX/vGluT-1 assay (Fig. 4) ($p < 0.01$). Neither the intensity of FM1-43FX-positive puncta nor of vGluT-1-positive puncta were altered by Rp-cAMPS treatment ($p = 0.08$ and 0.19, respectively; $n = 10$ fields). In parallel electrophysiology experiments, Rp-cAMPS decreased the size of sucrose-evoked EPSCs by a similar percentage (Fig. 4). These results thus suggest that ongoing cAMP signaling in hippocampal neurons is required to maintain the basal complement of active synapses and the total cellular immediately releasable vesicle pool. The results also help bolster the conclusion that AC1 and AC8 are not crucial among adenylyl cyclases for maintaining the activation status of presynaptic terminals.

Recovery from depolarization-induced silencing

We have shown previously that a wide range of depolarizing challenges, ranging from pathophysiological (Moulder et al., 2004) to physiological (Moulder et al., 2006), results in strong presynaptic silencing. Manipulation of spiking within the physiological range of activity requires several days to produce strong presynaptic silencing, whereas strong depolarization requires only hours to produce 70–80% presynaptic silencing. To avoid potential developmental confounds of manipulations (of cAMP or other pathways) that continue for days, we focused here on strong stimulation, in which manipulations can be completed in a matter of hours, thus circumventing developmental effects of prolonged drug treatment.

Similar to our previous studies, we found that 30 mM K^+ added to cultures for 4 h produced strong presynaptic silencing (Fig. 5). To test whether the unsilencing induced by cAMP stimulation can overcome this effect, we cotreated cultures with FSK during the period of depolarization. As in previous experiments (Fig. 1), we found that FSK increased the basal percentage of FM1-43FX-positive terminals (Fig. 5*A*,*B*). In addition, FSK, added simultaneously with the depolarizing stimulus, interfered with the ability of depolarization to silence presynaptic terminals (Fig. 5*A*,*B*). In parallel electrophysiology experiments, we also found that FSK impeded depolarization-induced decreases in sucrose-evoked EPSCs (Fig. 5*C*). Furthermore, the effect of FSK appeared at least somewhat selective. Although coincubation with the phorbol ester β-phorbol 12,13-dibutyrate (PDBu) (1 μM) significantly increased the percentage of active synapses in nondepolarized cultures, PDBu, in contrast to FSK, did not interfere with the depolarization-induced silencing (Fig. 5*D*). This result suggests that neither stimulation of PKC pathways (Stevens and Sullivan, 1998; Wierda et al., 2007) nor a direct effect of the phorbol on vesicle priming (Betz et al., 1998; Wierda et al., 2007) interferes with presynaptic silencing in the same way that cAMP stimulation does.

Given these results suggesting that cAMP stimulation can interfere with presynaptic silencing, we reasoned that a role for Ca^{2+}-sensitive AC1/AC8 in presynaptic unsilencing may be particularly evident during or after perturbation of the system with depolarization-induced silencing. Using sucrose-evoked EPSCs, we were unable to detect any difference between AC1/AC8 DKO animals and WT animals in the level of presynaptic silencing (Fig. 6). Therefore, loss of AC1/AC8 did not enhance the level of silencing produced during strong depolarization.

Although the Ca^{2+}-sensitive cyclases did not appear to participate in the degree of silencing produced by depolarizing challenge, we reasoned that they may participate actively during recovery from silencing. We have shown previously that, after strong, depolarization-induced presynaptic silencing, reactivation of synapses occurs over ~4 h (Moulder et al., 2004). Ca^{2+}-sensitive adenylyl cyclases may be particularly important in resetting normal synaptic functioning after removal of the depolarizing challenge, a process important for the resumption of normal neuronal signaling and for resetting appropriate balance between excitation and inhibition. To test for a role of AC1/AC8 in recovery from presynaptic silencing, we used a protocol of 16 h depolarization, which produces a level of silencing (~80%)

from which recovery is easily monitored (Moulder et al., 2004) (Fig. 7). After 3 h recovery, WT mouse synapses had significantly recovered from silencing by both FM1-43FX/vGluT-1 staining criteria and sucrose-evoked EPSC criteria (Fig. 7*A–C*). In both assays, synapses from AC1/AC8 DKO animals were strongly resistant to recovery from presynaptic silencing (Fig. 7*B*,*C*).

We also asked whether AC1 and AC8 are critical for inducing the increase in the percentage of functional synapses that is observed with chronic activity blockade. Block of action potential firing produces adaptive unsilencing over a period of days rather than hours (Moulder et al., 2006). We challenged WT and DKO neurons with combined TTX and ionotropic glutamate receptor blockers for 6–10 d to silence electrical activity and then quantified the percentage of FM1-43FX-positive terminals. Activity blockade resulted in a net increase in functional presynaptic terminals in both WT and DKO neurons (Fig. 7*D*). These results strongly suggest that, whereas AC1/AC8 isoforms play a critical role in rapid responses to activity changes (Fig. 7*A–C*), other AC isoforms can compensate over longer time periods required for TTX-induced unsilencing. This is consistent with the observation that the percentage of FM1-43FX-positive terminals is only modestly reduced under basal conditions in AC DKO neurons (Fig. 3).

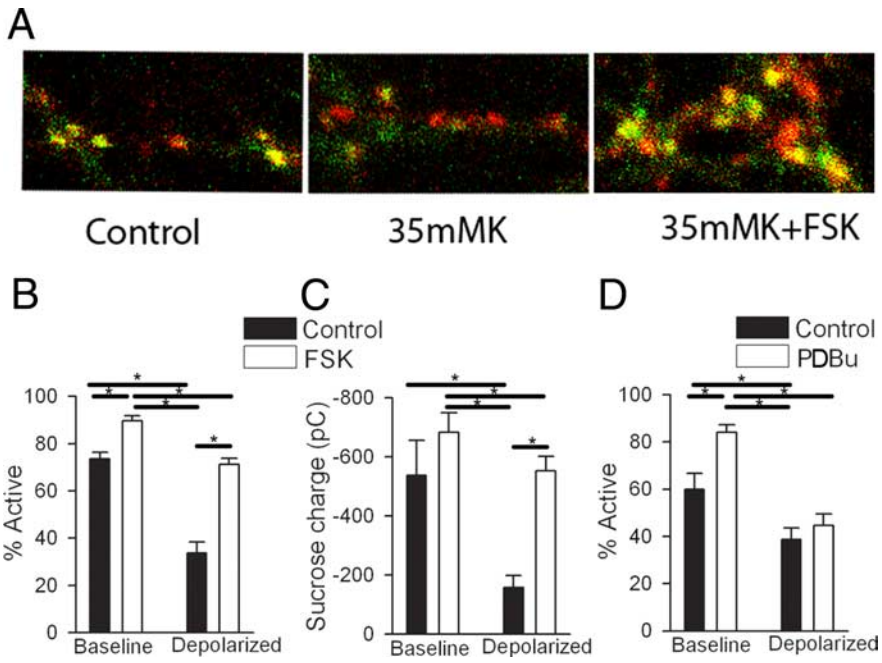

Figure 5. Depolarization-induced presynaptic silencing is occluded by FSK. ***A***, Merged images of FM1-43FX (green) and vGluT-1 (red) in control conditions, after 4 h strong depolarization (35 mM K^+), and after strong depolarization plus 50 μM FSK. ***B***, Summary of percentage of active synapses by FM1-43FX/vGluT-1 correspondence ($n = 24–25$ fields from 5 experiments). *$p < 0.05$. ***C***, Summary of sucrose-evoked charge under the same experimental conditions ($n = 12$ cells per condition). *$p < 0.05$. ***D***, Summary of active synapses after depolarization and lack of effect of the phorbol ester PDBu (1 μM present throughout the 4 h depolarizing challenge; $n = 15$ fields from 3 experiments). *$p < 0.05$. Baseline (control) and Depolarized + PDBu treatment groups are also significantly different from each other ($p < 0.05$).

As a test of whether acute actions of the cAMP pathway are likely to account for the recovery phenotype after strong depolarization, we assessed whether the DKO deficit could be rescued by the presence of the cAMP agonist Sp-cAMPS in the recovery medium. Indeed, we found that the cAMP agonist restored recovery from presynaptic silencing in DKO cells (Fig. 7*E*). These results are consistent with the idea that the acute loss of cAMP pathways is responsible for the phenotype of the DKO cells.

To compare quantitatively the effect of genetic deletion of AC1/AC8 with the effect of pharmacological inhibition of all cyclase activity, we examined the effect of Rp-cAMPS on recovery from synaptic silencing. We found a similar effect of the broad-spectrum inhibition of cyclase activity as with cells doubly deficient in AC1/AC8 (Fig. 8*A*). Therefore, we conclude that one or both of these AC isoforms are primary contributors to recovery from depolarization-induced presynaptic silencing.

The effect of Rp-cAMPS suggests involvement of PKA-dependent signaling rather than cyclic-nucleotide gated channels or the guanine nucleotide exchange factor/exchange protein directly activated by cAMP signaling pathways (Schaap et al., 1993). To verify this conclusion, we also examined the PKA inhibitor KT5720 (2 μM, 4 h). As, expected, KT5720 decreased the size of the sucrose-evoked readily releasable pool (RRP) and blocked the recovery of the RRP from depolarization-induced silencing (Fig. 8*B*). Although KT5720 apparently had a greater effect on the size of sucrose-evoked RRP than did the cAMP antagonist Rp-cAMPS (Fig. 4), ANOVA analysis with a secondary Tukey's test indicated that this difference was not statistically significant ($p > 0.05$). Therefore, two pharmacological lines of evidence are consistent with a primary role of PKA in presynaptic reactivation downstream of cAMP.

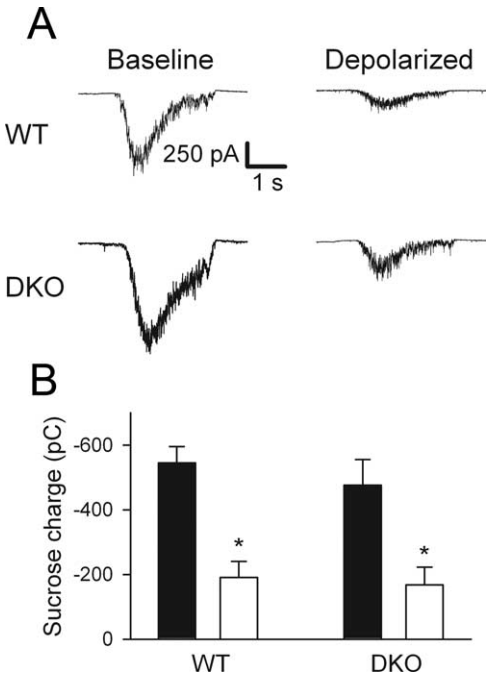

Figure 6. AC1/AC8 deletion does not affect presynaptic silencing. ***A***, Representative sucrose-evoked EPSCs from the indicated conditions. The depolarized condition was 4 h in 35 mM K^+. ***B***, Summary of sucrose effects in control (filled bars) and depolarized (open bars) neurons ($n = 10$ cells each condition). *$p < 0.05$ relative to baseline condition.

To parse possible contributions from the two Ca^{2+}-sensitive isoforms of AC, we examined recovery from presynaptic silenc-

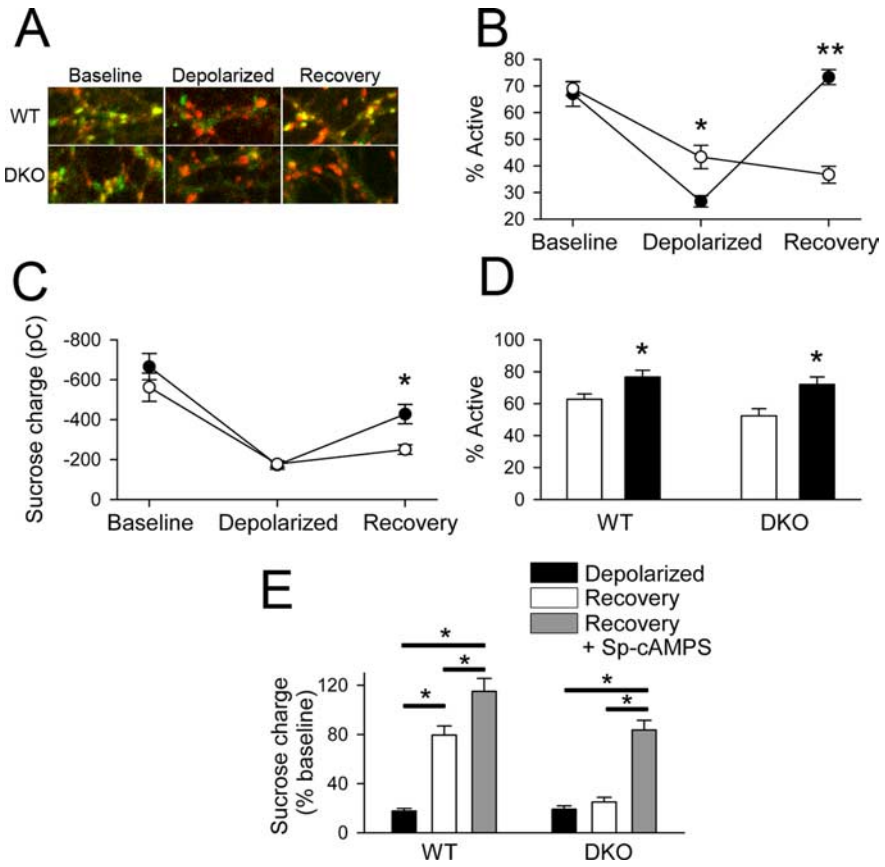

Figure 7. AC1/AC8 deletion retards recovery from adaptive presynaptic silencing. ***A***, Representative merged images from the indicated conditions. FM1-43FX is green, and vGluT-1 staining is red. The depolarization condition was 16 h in 35 mM K^+. Recovery was assessed 3 h after removal of the depolarizing stimulus. ***B***, Summary of active synapses assessed by FM1-43FX/vGluT-1 correspondence in the indicated conditions. Filled symbols represent wild type; open symbols indicate DKO ($n = 30$ fields in 6 experiments per condition). *$p < 0.01$, **$p < 0.001$, significant difference between WT and DKO. ***C***, The experimental conditions in ***B*** were repeated using the sucrose-evoked EPSC assay as a readout of presynaptic silencing ($n = 15$ cells per condition). *$p < 0.002$, significant difference between WT and DKO. ***D***, Summary of active synapses assessed by FM1-43FX/vGluT-1 correspondence in control (open bars) and TTX-treated (filled bars) neurons from WT and DKO animals ($n = 25$ fields from 5 experiments each). *$p < 0.05$, significant difference from the control condition in that genotype. ***E***, Sucrose-evoked EPSC charge was used to assess the ability of Sp-cAMPS to rescue the recovery deficit in DKO cells. Sp-cAMPS (50 μM) was added to recovery medium. *$p < 0.05$. $n = 5$ cells per condition.

ing in cultures prepared from animals deficient in individual isoforms. Consistent with the mild baseline phenotype of doubly deficient cells, we found that neither single knock-out exhibited a significantly different percentage of active synapses at baseline (without depolarizing challenge) (Fig. 9). Both single-deletion genotypes responded to depolarizing challenge with presynaptic silencing similar to control (Fig. 9). However, when we examined recovery from presynaptic silencing, AC8-deficient cells, but not AC1-deficient cells, exhibited the same strongly retarded recovery phenotype as doubly deficient cells. This result applied to both imaging assays (the FM1-43FX/vGluT-1 assay) (Fig. 9*A*,*B*) and the electrophysiology assay (Fig. 9*C*). Therefore, we conclude that AC8 is particularly important for the recovery of hippocampal presynaptic terminals from depolarization-induced presynaptic silencing.

Discussion

Our results show a critical role for cAMP signaling in maintaining presynaptic activation status. Furthermore, the results show a prominent role for AC1/AC8 in the recovery of presynaptic terminals from depolarization-induced silencing, but these same isoforms are not necessary for the maintenance of the basal ratio between active and inactive terminals. They are also not necessary for the slow adaptation produced by TTX-induced presynaptic silencing. Our experiments lend new insights into effects of the cAMP signaling pathway on basal synaptic function. In addition, our results suggest a particularly important role for Ca^{2+}-sensitive ACs in the restoration of synaptic function after periods of strong activity.

cAMP and presynaptic potentiation

The idea that the cAMP pathway increases synaptic efficacy through presynaptic mechanisms is well supported (Seino and Shibasaki, 2005). Using methods of conventional EPSC measurements, synaptosome measurements, and optical quantal analysis, previous studies have implicated cAMP formation with increased vesicle availability rather than or in addition to vesicle release probability. However, most previous studies have not clearly distinguished vesicle availability at existing, active synapses versus activation of previously inactive synapses (Huang et al., 1994; Weisskopf et al., 1994; Tong et al., 1996; Bolshakov et al., 1997; Lonart et al., 1998; Kaneko and Takahashi, 2004; Reid et al., 2004; Menegon et al., 2006) (but see Ma et al., 1999; Kohara et al., 2001). Furthermore, some studies have explicitly excluded an effect of cAMP signaling on the number of active synapses (Trudeau et al., 1996). By a combination of optical and electrophysiological measures, our work shows strong evidence that cAMP signaling activates previously silent presynaptic terminals. We and others have demonstrated previously the existence of presynaptically silent synapses under basal conditions, defined by normal antibody staining for synaptic vesicle markers but lack of FM dye labeling during brief, strong depolarization (Rosenmund et al., 2002; Altrock et al., 2003; Moulder et al., 2006; Ting et al., 2007). There is an important role for cAMP potentiation in the awakening of these basally silent presynaptic terminals.

Some work has suggested that cAMP signaling increases the size of the recycling vesicle pool at already active synapses (Kuromi and Kidokoro, 2000; Menegon et al., 2006). With neither brief 30 min FSK stimulation nor our standard 4 h FSK stimulation paradigm did we observe reliable increases in the intensity of FM1-43FX labeling of synapses that might have indicated an increase in the recycling vesicle pool. Slight differences in culture preparations or in dye protocols might explain the differences. It seems possible that the increased fluorescence observed by others at already active terminals may be related to the p_r increase that we detected in the present study with electrophysiological techniques.

The role of cAMP in synaptic potentiation has arguably been studied most thoroughly in the context of mossy fiber long-term potentiation (LTP) in the hippocampus. Although in some stud-

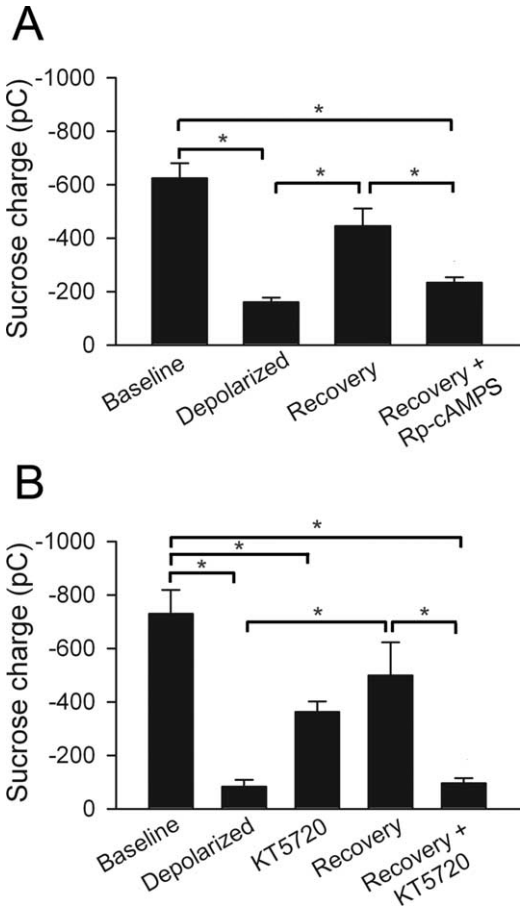

Figure 8. Pharmacological evidence for PKA involvement in the recovery effects. ***A***, Rp-cAMPS (50 μM) included in recovery medium prevented recovery from depolarization-induced presynaptic silencing. *$p < 0.05$, significant difference ($n = 15$ cells per condition). ***B***, Effects of the PKA inhibitor KT5720 (2 μM) on sucrose responses and on recovery from depolarization-induced silencing. *$p < 0.05$ ($n = 12$–15 cells per condition). KT5720 and depolarized treatment groups are also significantly different from each other ($p < 0.05$).

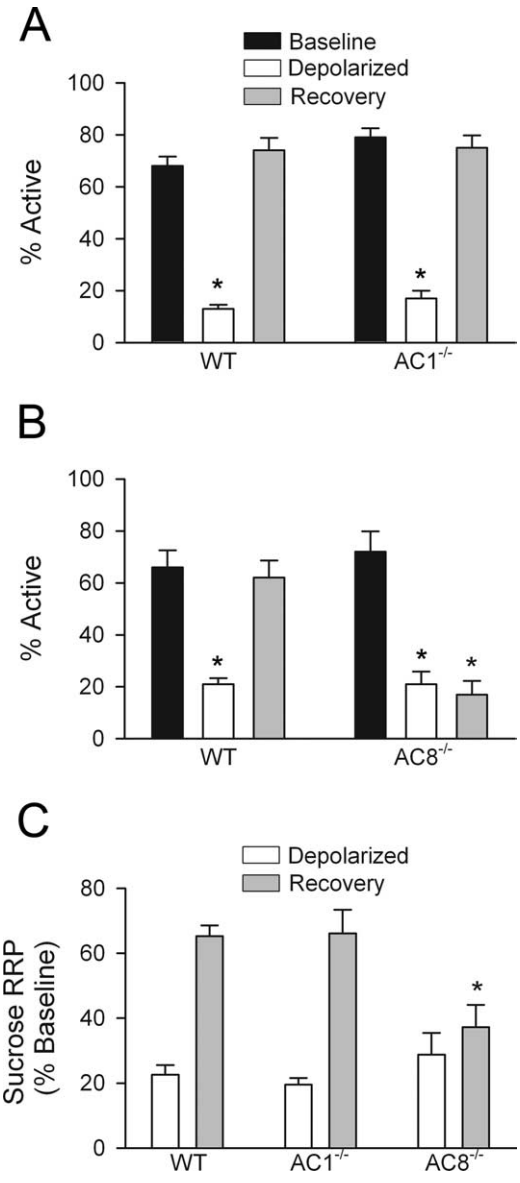

Figure 9. Effects on recovery are attributable to AC8. ***A***, ***B***, Effects of individual AC1 and AC8 genetic deletion on recovery from depolarization-induced presynaptic silencing. The percentage of active synapses was assessed by FM1-43FX/vGluT-1 staining immediately after strong depolarization (Depolarized; 16 h, 35 mM K^+) and 3 h of subsequent recovery (Recovery). For both panels, 10 fields per condition in two experiments were examined. *$p < 0.05$, significantly different from the baseline condition in the respective genotype. Recovery conditions in WT (***A***, ***B***) and *AC1*$^{-/-}$ (***A***) cultures are not statistically different from the baseline condition in the respective genotype. ***C***, Effects of single deletions on sucrose-evoked EPSC charge. Sucrose responses are expressed relative to average baseline responses from control cultures (= 100%; $n = 10$–15 cells per condition). *$p < 0.05$, significantly different from wild-type and *AC1*$^{-/-}$ recovery conditions. The depolarized conditions for each genotype are not statistically different from each other.

ies cAMP-mediated synaptic potentiation appears selective to these mossy fiber synapses (Tong et al., 1996; Lonart et al., 1998; Kohara et al., 2001), in other studies cAMP stimulation also affects other synapses, including Schaffer collateral synapses between CA3 and CA1 pyramidal neurons (Bolshakov et al., 1997; Ma et al., 1999; Wong et al., 1999). It is possible that developmental considerations or other differences in experimental conditions explain the differences among studies with regard to the selectivity of cAMP-induced potentiation. In our case, no effort was made to culture dentate granule cells selectively, the neurons that give rise to the mossy fibers. In addition, none of the presynaptic terminals we studied had the large size characteristic of mossy fiber terminals. Therefore, our studies serve to confirm the importance of cAMP pathways to synaptic function beyond the specialized mossy fiber synapse.

cAMP in the context of depolarization-induced silencing

Our work suggests a possible reason for the existence of multiple cAMP isoforms. Under conditions of basal activity, there appears to be redundancy in the operation of multiple FSK-sensitive ACs in maintaining an optimal ratio of active to inactive synapses (Fig. 3). This same redundancy appears to be sufficient to participate in slow changes in the percent of silenced terminals (Fig. 7*D*). However, during challenge to the system with strong depolarization, Ca^{2+}-sensitive ACs play a particularly prominent role in more quickly returning the system to a normal ratio of active to inactive synapses than would be available without these isoforms.

Our results demonstrating a role for AC8 in a presynaptic adaptation to excessive activity parallels a recent study showing a postsynaptic role for AC1 in adapting cortical synapses to electrical silencing (Gong et al., 2007). These compartmentalized effects of AC8 and AC1 also fit with recent evaluations of the subcellular localization of AC1 and AC8 protein. Although the domains are somewhat overlapping, AC1 and AC8 localize to primarily

postsynaptic and presynaptic compartments, respectively (Conti et al., 2007).

If these Ca^{2+}-sensitive AC isoforms are activated by Ca^{2+} influx, it may be surprising that loss of AC1/AC8 does not increase the number of synapses silenced by strong depolarization (Figs. 6, 7, 9). One possibility is that the depolarizing challenges induced a maximum number of inactivated synapses, causing a "floor" effect. This seems unlikely because we have shown that, with prolonged depolarization, all glutamate synapses are silenced. We clearly did not reach a minimum of zero in the present studies (Figs. 6, 7). Perhaps more likely is the possibility that synapses have mechanisms in place that, during the depolarizing challenge, override recovery mechanisms. In other words, strong Ca^{2+} influx may activate silencing pathways that preclude the expression of the cAMP-dependent unsilencing mechanisms. This idea has precedent in the LTP literature, in which the level of Ca^{2+} influx dictates the induction of either LTP or long-term depression (Abraham and Tate, 1997; Cormier et al., 2001). It is also possible that activity and cAMP signaling do not affect presynaptic function in a strictly linear pathway, although the ability of FSK to rescue presynaptic silencing induced by depolarization (Fig. 5) suggests that the two mechanisms are closely tied.

It is interesting that, in our hands, exogenous cAMP stimulation also increases vesicle release probability, assayed with electrophysiology. Nevertheless, AC1/AC8-dependent recovery from presynaptic silencing involves a selective recovery in activity status of terminals, without changes in p_r (Moulder et al., 2004). It is possible that the pathways involved in the p_r increase and the synaptic activation involve parallel signaling pathways downstream of cAMP formation, and the p_r pathway is suppressed by the conditions of presynaptic silencing/recovery. It is also possible that AC1/AC8 are selectively coupled to presynaptic unsilencing pathways, whereas other cyclases participate in maintaining presynaptic activation status and in increasing p_r.

Possible downstream targets

As with several other forms of cAMP-dependent synaptic plasticity, the downstream targets of cAMP in synaptic unsilencing remain to be determined. Because depolarization-induced presynaptic silencing has been fairly thoroughly characterized in our previous studies, unsilencing appears well poised for additional investigation (Moulder et al., 2003, 2004, 2006). Hallmarks of silencing include the inability of silenced terminals to release transmitter, even in response to the Ca^{2+}-independent secretagogue hypertonic sucrose. Conversely, vesicles remain morphologically docked and competent to release in response to the secretagogue α-latrotoxin (Moulder et al., 2006). By these criteria, we have proposed a specific deficit in vesicle priming at silenced terminals. Given the present results, cAMP-dependent targets known to be involved in vesicle priming emerge as strong candidates for modulation of synaptic activation state. For instance, active zone proteins of the Rim family are known PKA substrates and are involved in vesicle priming (Lonart, 2002; Calakos et al., 2004) and mossy fiber LTP (Castillo et al., 2002).

Together, our studies highlight the importance of the cAMP second-messenger pathway in maintaining synaptic activity and Ca^{2+}-sensitive AC isoforms in the recovery of presynaptic function after silencing. The studies give a mechanistic foothold by which to explore both upstream and downstream effectors of this notable form of synaptic plasticity.

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