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Hippocampal long-term depression and depotentiation are defective in mice carrying a targeted disruption of the gene encoding the RIβ subunit of cAMP-dependent protein kinase

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ABSTRACT **The cAMP-dependent protein kinase (PKA) has been shown to play an important role in long-term potentiation (LTP) in the hippocampus, but little is known about the function of PKA in long-term depression (LTD). We have combined pharmacologic and genetic approaches to demonstrate that PKA activity is required for both homosynaptic LTD and depotentiation and that a specific neuronal isoform of type I regulatory subunit (RIβ) is essential. Mice carrying a null mutation in the gene encoding RIβ were established by use of gene targeting in embryonic stem cells. Hippocampal slices from mutant mice show a severe deficit in LTD and depotentiation at the Schaffer collateral–CA1 synapse. This defect is also evident at the lateral perforant path–dentate granule cell synapse in RIβ mutant mice. Despite a compensatory increase in the related RIα protein and a lack of detectable changes in total PKA activity, the hippocampal function in these mice is not rescued, suggesting a unique role for RIβ. Since the late phase of CA1 LTP also requires PKA but is normal in RIβ mutant mice, our data further suggest that different forms of synaptic plasticity are likely to employ different combinations of regulatory and catalytic subunits.**

Little is known about the molecular mechanisms underlying homosynaptic long-term depression (LTD), an electrophysiological phenomenon thought to reflect some of the biochemical processes used in mammalian learning (1, 2). Like long-term potentiation (LTP), LTD requires an increase in intraneuronal calcium (3–5). However, in LTD a low level of calcium influx is believed to preferentially activate phosphatases (6–10), whereas in LTP a larger influx of calcium is thought to activate kinases, including (indirectly) the cAMP-dependent protein kinase (PKA) which is critical for the late phase of LTP (11–14).

In addition to mediating use-dependent changes in synaptic efficacy, PKA may be important for certain forms of learning and is specifically involved in the switch from short- to long-term memory (15). Since several isoforms of PKA exist, its involvement in synaptic plasticity and learning raises a question: Do different types of learning-related neuronal changes require specific regulatory (R) or catalytic (C) subunits? In the mouse, there are four R subunits (RIα, RIβ, RIIα, RIIβ) that bind cAMP and two C subunits (Cα, Cβ) that phosphorylate substrate proteins when released from the R subunits upon cAMP binding. In the nervous system, RIβ appears to be specific to neurons (16) and is expressed in many regions including the neocortex, the pyramidal layer of the hippocampus, and the Purkinje and granular layers of the cerebellum (17). To elucidate the role of PKA in synaptic plasticity and to determine whether specific subunits serve unique intracellular signaling functions, we used homologous recombination in embryonic stem cells to generate mice carrying a null mutation in RIβ.

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MATERIALS AND METHODS

Generation of Mutant Mice. The targeting of embryonic stem cells and the establishment of chimeras carrying the RIβ mutation have been described (18). Three of the four REK2-derived cell lines and one of the four D3-derived cell lines that produced fertile chimeras contributed to the germline. Mouse genotypes were determined by Southern blot.

RNA, Protein, and Kinase Assay. RNA was prepared by the guanidinium chloride method, and Northern blot analysis was performed essentially as described (19). Filters were hybridized with a ^{32}P-labeled antisense RNA probe synthesized from *Bgl* II-linearized RIβ cDNA (20). Western blots and kinase assays were performed as described (19, 21).

Histologic Analysis and Determination of Transgene Expression. Adult males were intracardially perfused with 4% paraformaldehyde in 100 mM sodium phosphate (pH 7.4). Brains were removed, postfixed for 2 hr, and embedded in paraffin. Coronal sections (6 μm) were cut and stained with cresyl violet. Staining of 20-μm sections of brains from pRIβ-lac mice for β-galactosidase activity was performed with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (GIBCO/BRL) and counterstained in nuclear fast red (22).

Electrophysiology. Mice were on a C57BL/6 × 129 mixed strain background, age 4–6 weeks for LTD and depotentiation experiments, or age 5–20 weeks for LTP and paired-pulse facilitation experiments. All experiments used age-matched controls and were performed blind to genotype. Details of stimulation and recording were as described (10). Stimulus intensity was adjusted to produce a response of approximately 1-mV amplitude, with an initial slope of approximately −0.5 mV/msec. Tetanic stimulation to produce CA1 LTP was 100 Hz for 1 sec, delivered either once or four times with an intertrain interval of 4 min. The stimulus to produce LTD at both synaptic loci tested was 1 Hz for 15 min. In experiments with picrotoxin, the concentrations of $CaCl_2$ and $MgSO_4$ were both 4.0 mM. For depotentiation experiments, the stimulus to produce LTP was 100 Hz for 1 sec, delivered twice with an intertrain interval of 20 sec. This was followed by a low-frequency stimulus (LFS) of 5 Hz for 3 min to produce depotentiation.

Abbreviations: EPSP, excitatory postsynaptic potential; LFS, low-frequency stimulus; LTD, long-term depression; LTP, long-term potentiation; PKA, cAMP-dependent protein kinase; R, regulatory; C, catalytic.

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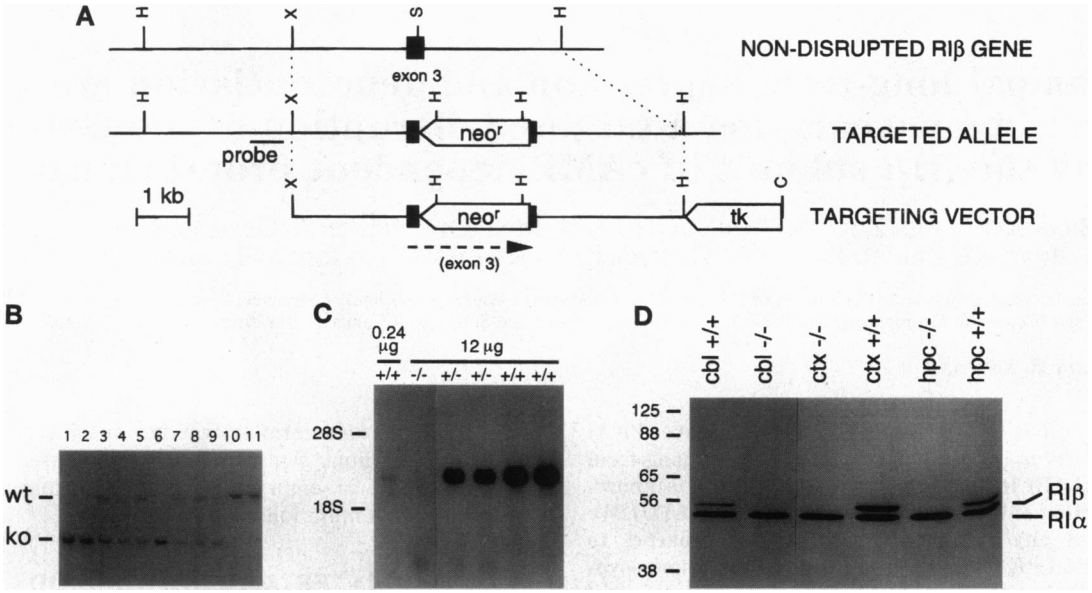

FIG. 1. Generation and analysis of RIβ$^{-/-}$ mice. (*A*) The RIβ locus and targeting vector. Targeting of the endogenous gene (shown at the top) by homologous recombination interrupts the coding region in exon 3 with the neomycin-resistance cassette (neo^r). The fragment used to make radioactive probes for genomic Southern blots is indicated. tk, Thymidine kinase. (*B*) Genomic Southern blot of offspring from heterozygote crosses. *Hin*dIII-digested DNA shows a wild-type band of 8.4 kb (wt) and/or a mutant band of 5.8 kb (ko, for knockout) when hybridized with the probe indicated in *A*. Samples shown are from wild-type (lanes 10 and 11), heterozygous (lanes 3–5 and 7–9), and homozygous mutant (lanes 1, 2, and 6) offspring. (*C*) Northern blot analysis of one homozygous mutant mouse (−/−), two heterozygous mice (+/−), and two wild-type mice (+/+). The amount of total brain RNA loaded in each lane and the migration of 28S and 18S rRNA are indicated. (*D*) Western blot analysis of protein samples (40 μg) from cerebellum (cbl), neocortex (ctx), and hippocampus (hpc) of wild type (+/+) and homozygous (−/−) mutant mice. Blot was probed with a polyclonal antiserum that identifies both RIα and RIβ protein.

RESULTS

Targeted Disruption of the RIβ Gene Yields Viable Homozygotes. Electroporation of the RIβ targeting vector (Fig. 1*A*) into embryonic stem cells, followed by positive–negative selection (23, 24), produced four germline-competent embryonic stem cell lines with a disruption in the RIβ gene (18). Heterozygous mice carrying the mutation were bred, and they produced wild-type, heterozygous, and homozygous offspring at the expected 1:2:1 Mendelian ratio, indicating that the mutation caused no embryonic lethality (Fig. 1*B*). RIβ$^{-/-}$ mice have shown normal growth and viability, and both males and females are fertile.

Analysis of PKA Expression in Brain from RIβ Mutant Mice. Northern blots showed that heterozygotes had a reduction in RIβ mRNA, whereas homozygotes had no detectable mRNA at the size of normal RIβ mRNA, ≈2.8 kb (Fig. 1*C*). Western blot analysis demonstrated that in multiple regions of the brain RIβ protein was completely absent (Fig. 1*D*).

Direct measurements of basal and cAMP-induced PKA activity in whole brain extracts from wild-type and mutant mice were very similar (Fig. 2). It is likely that kinase activity remained unchanged due to a compensatory increase in RIα subunit. By Western blot, the homozygotes showed a consistent increase in RIα protein compared with wild-type controls in both neocortex and hippocampus (E.P.B., unpublished observations). No significant changes in the quantity of any of the other PKA subunits were observed in either hippocampus or neocortex.

Histological Analysis and Identification of Neurons Capable of RIβ-*lac* Transgene Expression. Histological analysis of brain, adrenals, and testes revealed no abnormalities in the mutants. Of particular relevance, the gross neuroanatomy of the RIβ$^{-/-}$ hippocampus appeared normal, as shown in the coronal sections in Fig. 3. In the RIβ$^{-/-}$ mice, the development of neurons that would normally express RIβ might be impaired, leading to a compensatory replacement by neurons that express other regulatory subunits. To examine this possibility, RIβ null mutant mice were crossed with a previously established transgenic mouse line that expresses an RIβ promoter-driven β-galactosidase reporter gene (22). Examination of hippocampal sections from these mice revealed no differences in transgene expression in RIβ$^{-/-}$ mice compared with RIβ$^{+/+}$ transgenic littermates, indicating that neurons that normally would have expressed RIβ are not preferentially lost during development in the RIβ$^{-/-}$ mice and that the lack of RIβ protein does not dramatically affect expression from the RIβ promoter (Fig. 3).

Mutant Mice Are Deficient in LTD and Depotentiation. In wild-type mice, a LFS (1 Hz for 15 min) produced a significant depression of the field excitatory postsynaptic potential (EPSP) lasting at least 35 min (Fig. 4*A*). In contrast, the mutant mice showed a short-term depression that returned to baseline by 20 min following the LFS. It has been proposed that LTD requires inhibitory interneuron activity (25). However, we find that in wild-type slices the γ-aminobutyrate (GABA) receptor

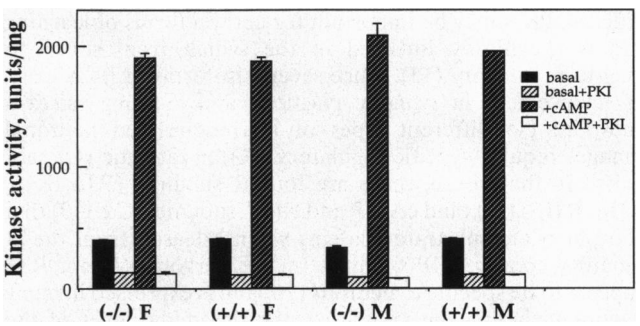

FIG. 2. Kinase assay of brain homogenates from wild-type (+/+) and mutant (−/−) mice. Phosphorylation of a peptide substrate of PKA (Kemptide) was assayed with whole brain homogenates in the presence or absence of protein kinase inhibitor peptide (PKI, 40 μg/ml) or 5 μM cAMP. Results from one male (M) and one female (F) of each genotype are shown.

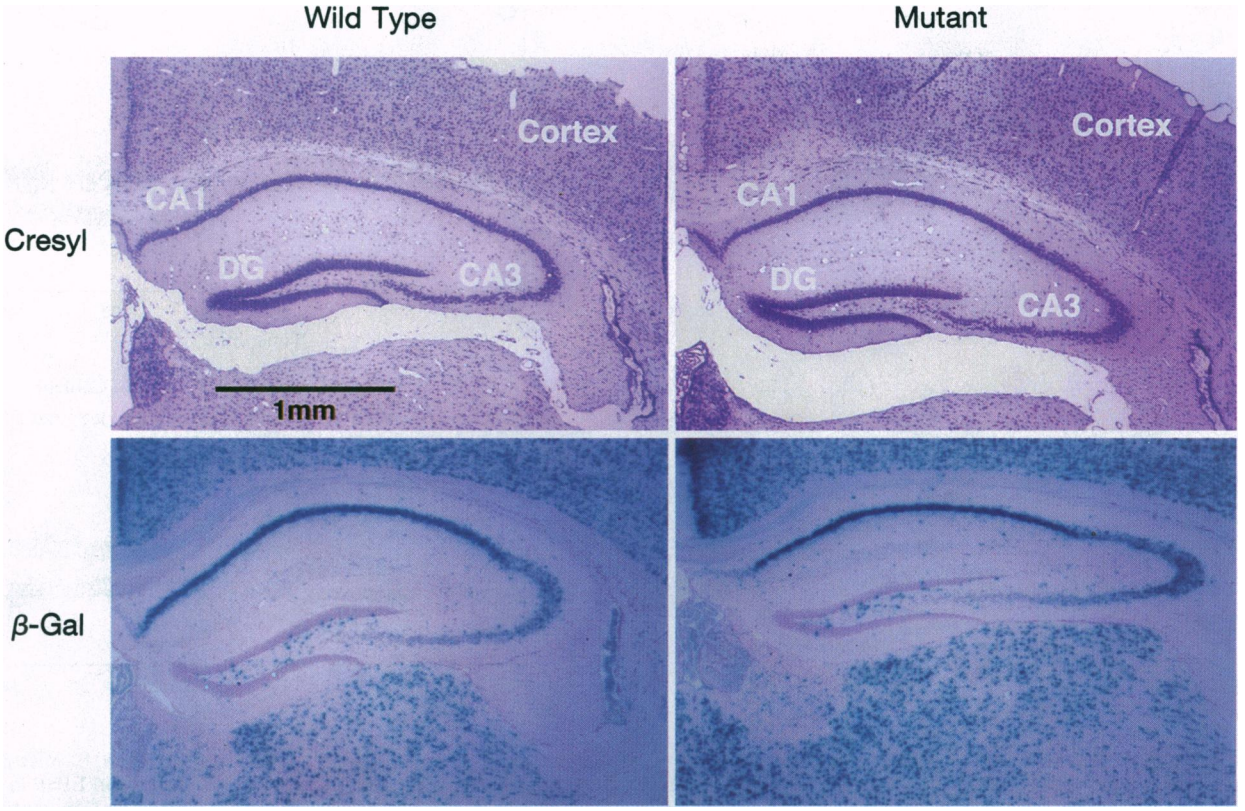

FIG. 3. Histologic analysis and expression of pRIβ-lac transgene. (*Upper*) Cresyl violet-stained sections from a wild-type (*Left*) and an RIβ mutant (*Right*) hippocampus. (*Lower*) Expression of a β-galactosidase transgene driven by the RIβ promoter in wild-type (*Left*) and an RIβ mutant (*Right*) hippocampus.

antagonist picrotoxin does not inhibit LTD induction (data not shown), indicating a lack of involvement of this inhibitory pathway in LTD. This suggests that the LTD defect in the mutants is inherent to the principal Schaffer collateral connections, and not secondary to a defect in inhibitory interneurons.

In addition to manifesting LTD, wild-type slices show a related depression called depotentiation, whereby LTP, once induced, is abolished (10, 26–29). Although slices from RIβ$^{-/-}$ mice showed the immediate phase of depotentiation, they rapidly returned to a potentiated response in <15 min (Fig. 4*B*). Thus, the deficiency in maintaining LTD correlates with an inability to exhibit stable depotentiation in slices from the mutant mice.

Pharmacologic Inhibitors of PKA Block LTD and Depotentiation. To test whether a decrease in PKA activity might account for the defects in LTD and depotentiation, we examined slices from wild-type mice in the presence of KT5720, which blocks the enzymatic activity of the C subunit of PKA. KT5720 blocked LTD (Fig. 4*C*) and also inhibited depotentiation (Fig. 4*D*), suggesting a requirement for PKA activity in both forms of synaptic depression.

RIβ$^{-/-}$ Mice Exhibit Normal LTP and Paired-Pulse Facilitation in CA1. Since PKA has been shown to be important in the late phase of LTP at the Schaffer collateral–CA1 pyramidal cell synapse (11, 14), we examined LTP of this synapse in hippocampal slices from mutant animals and wild-type controls. In a protocol that ensures robust potentiation (100 Hz stimulation for 1-sec duration delivered four times at an intertrain interval of 4 min), a similar degree and time course of LTP were observed in wild-type and mutant slices (Fig. 5*A*). Likewise, no difference in potentiation was seen when the stimulus was delivered only once (data not shown). These data suggest that the action of RIβ is fairly selective at this synapse and restricted to LTD and depotentiation.

To assure that RIβ deletion did not interfere with other aspects of synaptic transmission, we examined the maximal response that could be evoked at this synapse and found it to be similar between the two groups (7.9 ± 0.8 mV, n = 10 slices for mutants, compared with 9.1 ± 0.5 mV for wild type, n = 13 slices). Another measure of synaptic function, paired-pulse facilitation, whereby a temporally associated second stimulus pulse yields a larger postsynaptic response than the first, due to the accumulation of calcium in the presynaptic terminal, was normal at this synapse at several interstimulus intervals (Fig. 5*B*).

The LTD Defect Is Also Evident in the Dentate Gyrus of RIβ$^{-/-}$ Mice. To examine the generality of the LTD deficit observed in the CA1 region, we turned to the perforant path–dentate granule cell synapse, where LFS-induced LTD has not previously been demonstrated in the hippocampal slice preparation. We found that the same LFS (1 Hz for 15 min) that produced LTD in the CA1 region also reliably produced homosynaptic LTD of the lateral perforant path–dentate granule cell synapse. Slices from RIβ$^{-/-}$ mice were deficient in this type of LTD as well (Fig. 6), suggesting that a similar molecular mechanism requiring RIβ underlies LTD at both synapses.

DISCUSSION

PKA Plays a Role in LTD and Depotentiation. In view of the increasing evidence that PKA is involved in a variety of learning-related forms of synaptic plasticity, it is of interest to determine which specific isoforms are involved in each form of plasticity. The experiments described here specifically implicate the RIβ-containing holoenzyme of PKA in the homosynaptic LTD of two different neural pathways in the hippocampus. RIβ mutant mice are deficient in LTD at both the Schaffer collateral–CA1 synapse on pyramidal cells and the

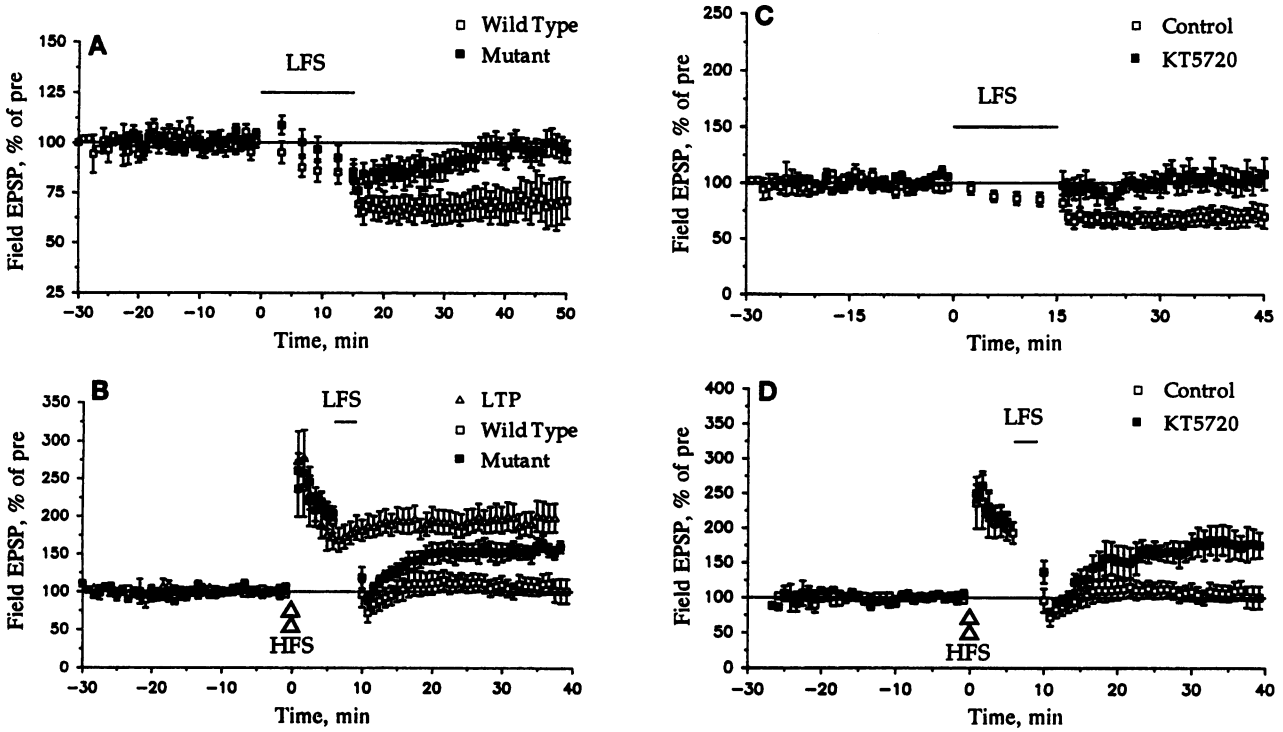

FIG. 4. Synaptic depression is defective at the Schaffer collateral–CA1 pyramidal cell synapse in hippocampal slices from RIβ$^{-/-}$ mice and in wild-type slices treated with the PKA inhibitor KT5720. (*A*) LTD. LFS (bar) produced a reduction in the initial slope of the field EPSP in slices from wild type (□; 70.3 ± 8.7%, n = 9 slices from five mice) but not mutants (■; 97.1 ± 5.7%, n = 20 slices from 9 mice). (*B*) Depotentiation. Five minutes after a high-frequency stimulus (HFS, double triangle) to produce LTP, slices were subjected to a LFS train (bar). The field EPSPs from wild-type slices (□) were depotentiated back to baseline 25–30 min following LFS (104.7 ± 14.0%, n = 7 slices from five mice), whereas mutant slices (■) were not persistently depotentiated (155.1 ± 9.0%, n = 9 slices from five mice). Wild-type slices not subjected to the LFS train showed normal LTP (△). (*C*) LFS (bar) was delivered in the presence or absence of 1 μM KT5720. KT5720 (■) did not significantly affect baseline responses (data not shown) but prevented LTD (105.3 ± 12.1%, n = 5 slices from five mice). (*D*) KT5720 also prevented depotentiation, as measured 25–30 min following LFS (176.1 ± 21.3%, n = 6 slices from six mice). Error bars show standard error of the mean.

lateral perforant path–dentate synapse on granule cells. Depotentiation, tested at the CA1 synapse, also showed a marked reduction in mutant mice. In wild-type slices, a pharmacologic inhibitor of PKA (KT5720) prevented both LTD and depotentiation at the CA1 synapse, providing further evidence for a direct regulatory role of PKA in synaptic depression.

RIβ Is Specifically Required for LFS-Induced Synaptic Depression. Our data suggest that different forms of synaptic plasticity may depend upon specific isoforms of PKA. What gives RIβ its unique role in LTD and depotentiation? Two obvious possibilities exist, and these are not mutually exclusive. First, the RIβ holoenzyme has been shown to have a lower threshold of cyclic nucleotide activation than the RIα holoenzyme (30, 31). This increased sensitivity to cAMP conferred by expression of RIβ might be critical for homosynaptic depression. Thus, RIβ holoenzyme might be expected to dissociate and become activated in response to very modest increases in cAMP. Although we were unable to detect any significant changes in the cAMP activation curves for PKA in cortical homogenates of mutant mice (data not shown), this result may be difficult to interpret. The sensitivity of the assay may be impaired by partial activation of type I kinases in response to catecholamine release at the time of death. Additionally, total PKA activity reflects the overall predominance of type II kinase in the brain, as well as the contribution of glial cells (which do not express RIβ) to the homogenate.

As a second possibility, RIβ holoenzyme may be localized to specific regions within the cell in close proximity to impor-

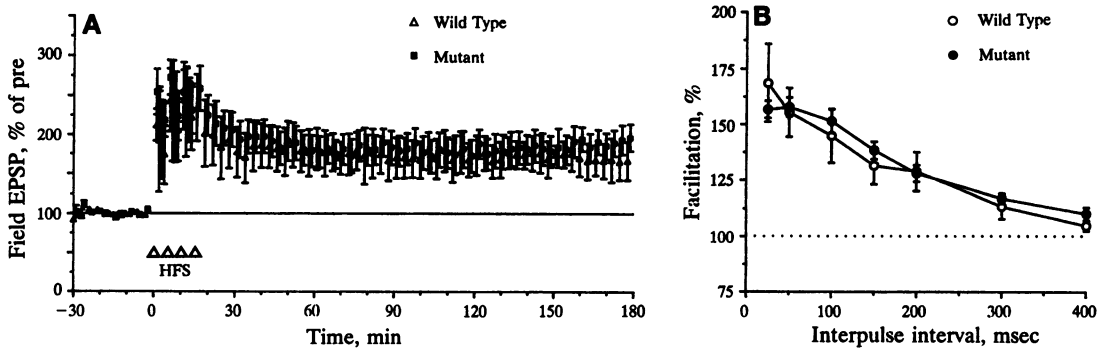

FIG. 5. LTP and paired-pulse facilitation are normal at the Schaffer collateral–CA1 pyramidal cell synapse. (*A*) As long as 3 hr after high-frequency stimulation (HFS, four triangles) the field EPSPs from wild-type (△; n = 8 slices from seven mice) and mutant (■; n = 9 slices from nine mice) slices were not significantly different. (*B*) Wild-type (○; n = 7 slices from four mice) and mutant (●; n = 15 slices from eight mice) slices also showed no significant difference in paired-pulse facilitation of the EPSP at various interpulse intervals.

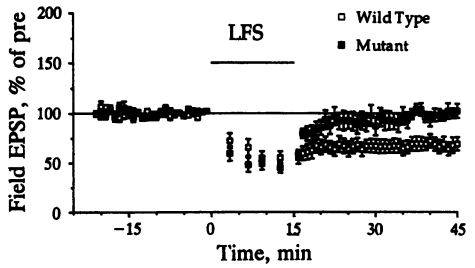

FIG. 6. LTD is absent at the lateral perforant path–dentate granule cell synapse. Twenty-five to thirty minutes after LFS (bar), average EPSP from wild-type slices (□) was reduced significantly from baseline (68.3 ± 6.8%, n = 12 slices from 6 mice) while that from mutants (■) was not (100.8 ± 6.3%, n = 12 slices from seven mice).

tant effectors or substrates used in LTD. Other R subunits, such as RIα, might not have the same affinity for specific anchoring proteins and thus fail to achieve appropriate localization within the cell. Type II PKA holoenzymes are anchored by AKAPs (A kinase-anchoring proteins) and this anchoring is required for cAMP modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptors (32) and calcium channels (33). No specific anchoring proteins for type I isozymes have been characterized, but the RIα subunit does appear to spatially localize during T-cell activation (34), indicating that subcellular localization is possible for the type I isoforms.

How Might the RIβ Holoenzyme Fit into Models of Synaptic Plasticity? The surprising findings that PKA activation is required for LTD and that mutant mice that are missing the RIβ holoenzyme of PKA cannot sustain LTD suggest that further refinement of the proposed mechanistic models of synaptic depression is necessary. The model for LTD proposed by Lisman (6) and further elaborated by Malenka (2) predicts that PKA inhibition should lead to activation of phosphatase 1 and, therefore, a possible increase in LTD. We see the opposite result: a loss of LTD when PKA is either altered by genetic manipulation of kinase isoforms or inhibited pharmacologically.

Thus, our data suggest that a molecular explanation of LTD based on calcium influx must consider the potential interactions between calcium and cAMP signaling cascades. Increased calcium can lead to changes in cAMP concentrations via either the neuronally expressed calmodulin-dependent adenylyl cyclases (types I, III, and VIII) (35) or the calmodulin-stimulated phosphodiesterases (36). Calcium can also lead to the dephosphorylation of PKA substrates by activating phosphatases such as calcineurin. For its part, PKA is known to phosphorylate the voltage-gated calcium channels (37) and glutamate receptors (38) that are expressed in neurons. Given these and other sites of crosstalk, it is premature to outline a detailed model. Perhaps the simplest idea is that LTD might result from the synergistic effects of modest increases in both intracellular calcium and cAMP, leading to simultaneous activation of calcineurin and the type Iβ (i.e., RIβ-containing) holoenzyme of PKA. By extension, LTP in the CA1 region might depend on robust activation of both calcium- and cAMP-dependent pathways. The type Iβ holoenzyme would not be required for LTP as long as the stimulus raised the cAMP concentration to a threshold capable of activating other less-sensitive PKA isoforms. Genetic manipulation of the various PKA R and C subunits by gene disruption and mutation in mice may further illuminate the role that the cAMP cascade plays in synaptic plasticity and provide mutant mice to correlate behavioral changes with electrophysiological defects.

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