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Limbic epilepsy in transgenic mice carrying a Ca^{2+}/calmodulin-dependent kinase II α-subunit mutation

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ABSTRACT **Multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMK) phosphorylates proteins pivotally involved in diverse neuronal processes and thereby coordinates cellular responses to external stimuli that regulate intracellular Ca^{2+} [Hanson, P. I. & Schulman, H. (1992) *Annu. Rev. Biochem.* 61, 559–664]. Despite extensive study, the impact of this enzyme on control of the excitability of neuron populations in the mammalian nervous system *in situ* is unknown. To address this question, we studied transgenic mice carrying a null mutation (−/−) for the α subunit of CaMK. In contrast to wild-type littermates, null mutants exhibit profound hyperexcitability, evident in epileptic seizures involving limbic structures including the hippocampus. No evidence of increased excitability was detected in mice carrying null mutations of the γ isoform of protein kinase C, underscoring the specificity of the effect of CaMK. CaMK plays a powerful and previously underappreciated role in control of neuronal excitability in the mammalian nervous system. These insights have important implications for analyses of mechanisms of epilepsy and, perhaps, learning and memory.**

The epilepsies comprise a diverse collection of disorders that affect an estimated 1% of the population (1). Many forms of epilepsy in humans and diverse species have prominent genetic determinants (2). Seventeen single-locus (and presumed single-gene) mutations are associated with epilepsy as a prominent phenotype in the mouse (3). In no instance, however, has the mutant gene been identified. While investigating molecular determinants of epileptogenesis using focal electrical stimulation of the brain (4, 5), we unexpectedly discovered that mice carrying a null mutation of the α subunit of Ca^{2+}/calmodulin-dependent kinase II (αCaMK) are epileptic.

MATERIALS AND METHODS

Stimulus-Evoked Seizures. Wild-type (+/+), heterozygous (+/−), and homozygous null mutant (−/−) mice at least 6 months of age weighing 20–36 g underwent implantation of a bipolar stimulating–recording electrode in the right amygdala using stereotaxic guidance or the right angular bundle with electrophysiologic guidance under pentobarbital anesthesia (60 mg/kg). In some animals additional electrodes were placed in the caudate, thalamus, and hippocampus. An additional recording electrode was placed on the dura overlying the left frontal cortex. After a postoperative recovery period of at least 1 week, the electrographic seizure threshold was determined by application of a 1-sec train of 60-Hz, 1.0-msec biphasic rectangular pulses beginning at a current intensity of 40 μA, followed by trains of increasing current intensity administered at 1-min intervals until an electrographic seizure of at least 3 sec was observed. Behavioral seizures were classified according to a modification of Racine (6) as follows: 1, chewing; 2, head nodding; 3, unilateral forelimb clonus; 4, bilateral forelimb clonus; 5, bilateral forelimb clonus plus falling and/or hindlimb clonus; 6, running or bouncing seizure; 7, tonic hindlimb extension; 8, tonic hindlimb extension culminating in death. After completion of the experiment, electrodes placed with stereotaxic guidance were verified to be within the targeted structures by described methods (7). Care of animals was done in accord with institutional guidelines.

Timm Histochemistry. Timm staining was done essentially as described by Sutula and coworkers (8). Subjects underwent perfusion (0.16% sodium sulfide/0.1 M sodium phosphate buffer, pH 7.4, followed by 3% glutaraldehyde in the same buffer) under pentobarbital anesthesia. Horizontal (20-μm) sections through the septal portion of hippocampus were mounted on gelatin-coated slides, developed in the dark for 90–120 min in a 5:1 mixture of 20% (wt/vol) gum arabic/2% (wt/vol) hydroquinone solution containing 3% (wt/vol) citric acid and 0.9% (wt/vol) silver nitrate. To facilitate meaningful comparison of sections of different animals, slides from wild-type and mutant animals were included in the same slide rack and exposed to the same solutions for the same time duration. To assure objectivity in data analysis, slides were subsequently coded and analyzed by an individual unaware of the genotype or treatment of the animal.

RESULTS

αCaMK Mutant Mice. A small, normally subconvulsive, electrical stimulus of the amygdala of wild-type (+/+) mice typically induced a brief electrographic seizure (Fig. 1*A*) (duration 16 ± 4 sec, mean ± SEM; n = 13 mice) usually unaccompanied by behavioral change. By contrast, an equivalent stimulus of eight −/− mice induced a prolonged electrographic seizure (duration 62 ± 11 sec, $P < 0.01$ ANOVA with post hoc Bonferroni multiple-comparsons test) associated with behaviors ranging from minimal change to subtle head nodding (class 2) to clonic and tonic contractions of limb muscles (class 5). In four additional −/− mice, a similar stimulus evoked repeated and/or prolonged electrographic seizures lasting 37–66 min associated with intense behavioral seizures culminating in tonic hindlimb extension and death (class 8) (Fig. 1*B*). Repeated electrographic seizures with intense behavioral seizures continued for 144 min before remitting spontaneously in an additional −/− mouse. A similar stimulus applied to +/− mice induced electrographic seizures (32 ± 15 sec, n = 8 mice; ANOVA, not significant)

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Abbreviations: CaMK, Ca^{2+}/calmodulin-dependent protein kinase II; αCaMK, α subunit of CaMK; PKCγ, protein kinase Cγ; LTP, long-term potentiation.

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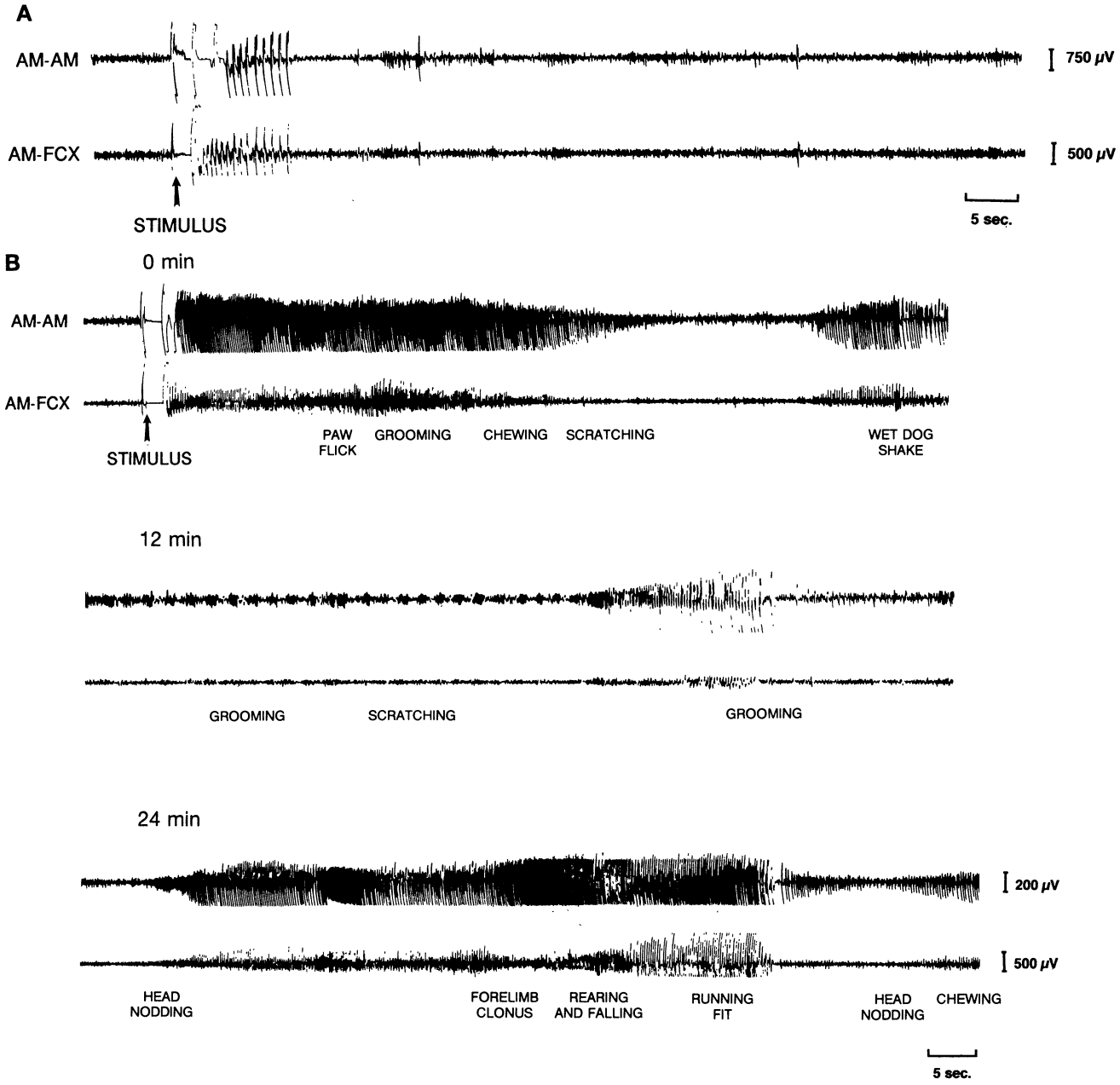

FIG. 1. Electroencephalogram after initial stimulation of +/+ and CaMK −/− mutant mice. (*A*) In a +/+ mouse, an electrographic seizure of 11 sec was recorded from the bipolar electrode in the right amygdala (AM-AM) and between electrodes in right amygdala and overlying left frontal cortex (AM-FCX) after amygdala stimulation. (*B*) A −/− mouse exhibited repeated seizures, including a class 6 seizure at 24 min and a fatal seizure at 38 min (9).

with a class 5 seizure in one mouse but minimal or no behavioral seizure in the remaining mice.

The intensity of the stimulus-evoked seizures suggested that the null mutants may be epileptic—that is, may exhibit seizures without electrical stimulation. A morphologic characteristic of epilepsy in a diversity of animal models and humans consists of pathologic sprouting and aberrant synapse formation by the mossy fiber axons of the hippocampal dentate granule cells (10, 11). This pathologic projection can be detected in the molecular layer of the dentate gyrus of the hippocampus with a Timm stain (Fig. 2). Evaluation of Timm-stained sections by an observer (J.O.M.) unaware of genotype disclosed abundant granules in the supragranular region of the dentate gyrus in all unstimulated −/− mice ($n = 6$) but in none of the +/+ mice ($n = 7$) with intermediate numbers in +/− mice ($n = 3$) (Fig. 2). Together with the hyperexcitability evident in response to subconvulsive stimulations of amygdala, this morphologic abnormality provided circumstantial evidence for the presence of epilepsy in the mutant mice.

To directly test for spontaneous seizures in unstimulated mice, intracerebral electrodes were implanted in multiple sites and video and electroencephalogram recordings were taken for 60 consecutive hr. At least one spontaneous seizure was detected in each of three −/− mice but in none of three +/+ mice. Electrographic seizures were evident initially in electrodes in either the amygdala or hippocampus with subsequent propagation to other structures (Fig. 3); in no instance was electrographic seizure detected first in either thalamic or cortical sites. The behavioral features consisted of clonic motor seizures, essentially class 4 or 5.

Protein Kinase Cγ (PKCγ) Mutant Mice. To determine whether null mutations of genes encoding other protein kinases expressed in the limbic system also resulted in increased excitability, we studied PKCγ transgenic mice carrying null

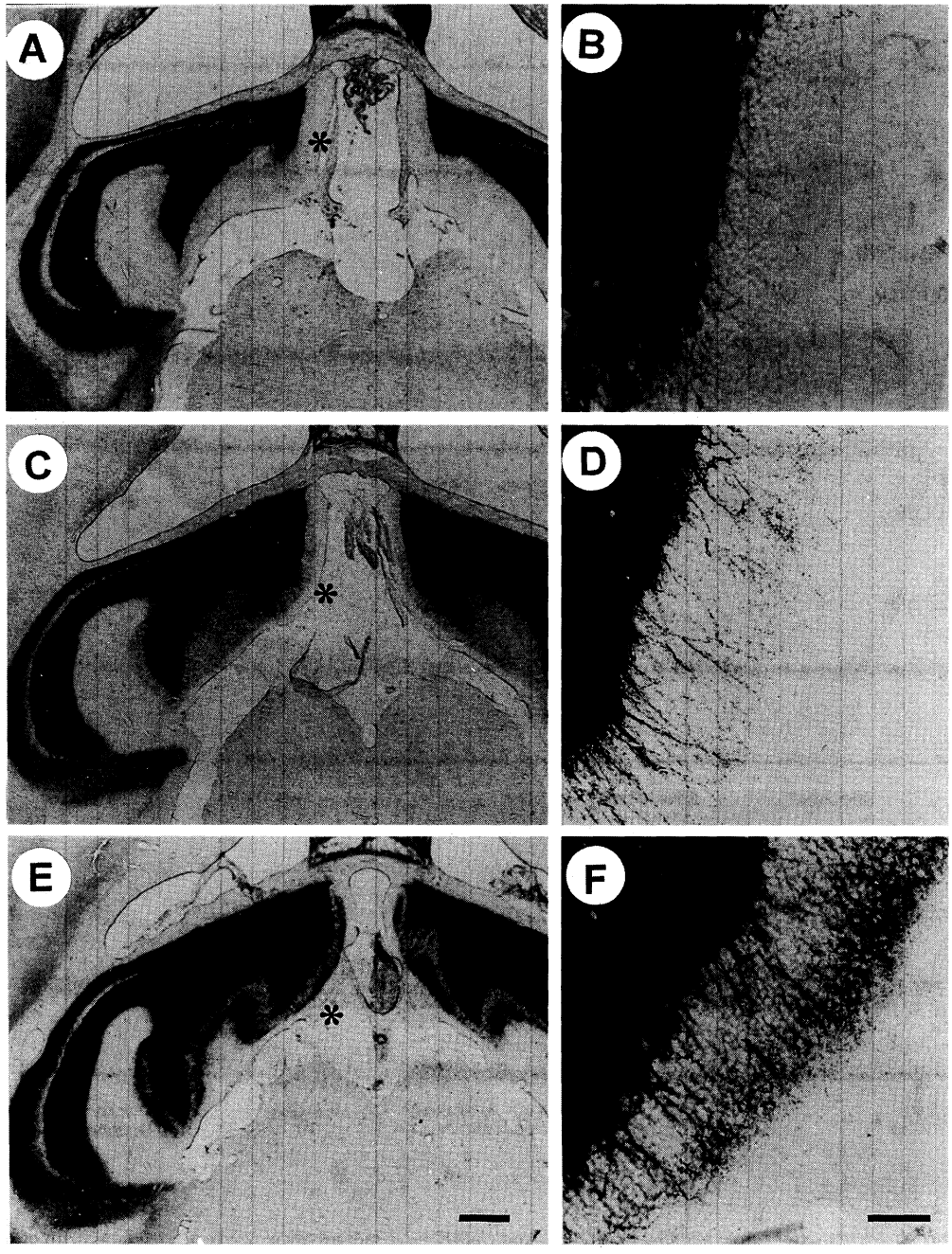

FIG. 2. Horizontal sections of unstimulated +/+ (*A* and *B*), +/− (*C* and *D*), and CaMK −/− (*E* and *F*) hippocampus examined with Timm histochemistry at the septal region. The area near the asterisk in *A*, *C*, and *E* is shown at higher magnification in *B*, *D*, and *F*, respectively. The Timm method stains neural elements containing heavy metals and stains mossy fibers in particular because of their high zinc content (12). The Timm granules are prominent in the −/− mouse but not in the +/+ mouse; intermediate numbers of Timm granules are present in the +/− mouse (13). [(*A*, *C*, and *E*) bar = 500 μm; (*B*, *D*, and *F*) bar = 20 μm.]

mutations of the γ isoform (14). A small, low-intensity electrical stimulation of the angular bundle elicited brief electrographic seizures in both wild-type (duration, 25 ± 4 s, mean ± SEM; $n = 8$ mice) and PKCγ mutants (duration 32 ± 4 s, mean ± SEM; $n = 9$ mice), which did not differ significantly (data not shown). To determine whether unstimulated PKCγ mutant mice exhibited mossy fiber sprouting evident in the CaMK mice, Timm histochemistry of the hippocampal formation was examined. The Timm staining pattern of both the +/+ and PKCγ null mutants was similar to that observed in normal mice; essentially no Timm granules were detectable in the supragranular region of the dentate gyrus, and no differences were detected between the +/+ ($n = 5$) and −/− ($n = 6$) mice (data not shown).

DISCUSSION

Two principal findings emerge from this study. (*i*) Unexpectedly, CaMK mutant mice exhibit a striking increase in brain excitability evident in epileptic seizures involving the hippocampus and other limbic structures. (*ii*) The phenotype is specific to CaMK, in that null mutation of another protein kinase expressed in the limbic system, PKCγ, is not associated with increased excitability.

Linking an epileptic phenotype to an identified gene adds to current insights into the genetic bases of the epilepsies. Seventeen distinct single-locus mutations in the mouse have been associated with an epileptic phenotype, but in no instance has the responsible gene been identified (3). An association has

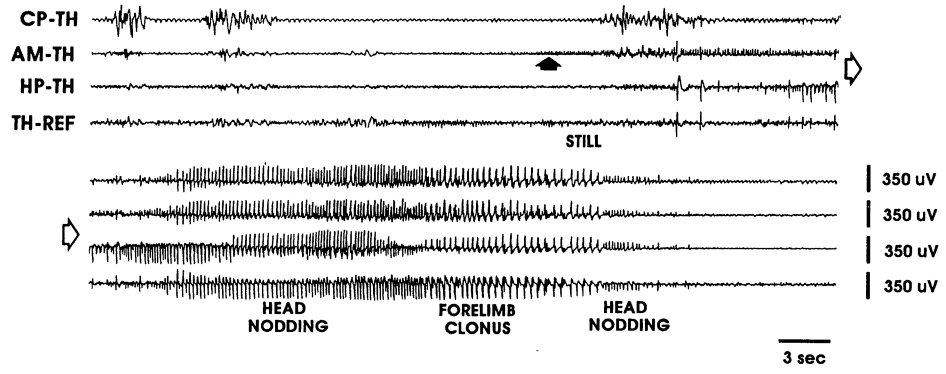

FIG. 3. Electroencephalogram recorded during a spontaneous seizure (class 4) of a CaMK null mutant. Intracerebral electrodes were placed under stereotaxic guidance into right caudate–putamen (CP), amygdala (AM), hippocampus (HP), thalamus (TH), or secured to skull overlying left frontal cortex (REF). Filled arrowhead denotes electrographic seizure onset evident in AM–TH recording. uV, μV.

been identified between a partial duplication of the ceruloplasmin gene and limbic epilepsy in the EL mouse, a disorder with multiple genetic determinants (15); whether the ceruloplasmin gene itself contributes to the enhanced seizure susceptibility is presently unclear. A point mutation in a mitochondrial gene encoding a $tRNA^{Lys}$ has been linked to a disorder in humans [myoclonic epilepsy and ragged-red fiber disease (MERRF)] characterized by myoclonic seizures, ataxia, dementia, as well as degeneration of skeletal muscle (16). In contrast to MERRF, the epileptic phenotype in the CaMK mutant mice occurs without detectable abnormalities in organs other than the central nervous system, presumably because the expression of CaMK is confined to the central nervous system (17).

The mechanism by which a null mutation of the gene encoding αCaMK produces this hyperexcitability is unclear. The possibility that the critical factor is the absence of the αCaMK gene product during development, rather than its absence in the mature brain, cannot be excluded. However, induced expression of an inhibitor of CaMK in the nervous system of the mature *Drosophila* is sufficient to produce enhanced repetitive firing in response to low-frequency stimulation of the motor neuron (18), a pattern of hyperexcitability linked to epileptic discharge in the mammalian nervous system (19). Because reduced CaMK enzymatic activity is evident in hippocampal membranes of the null mutants (20), decreased enzyme activity in the mature mouse brain may be sufficient to produce this hyperexcitability.

Although many forms of epilepsy exist in multiple species including >40 types in humans alone (21), in no instance are the cellular and molecular mechanisms fully understood (2, 19). In contrast to most existing models in which epilepsy is produced by some insult or electrical stimulation of the brain and a myriad of mechanisms could be responsible (2, 19), the link to a single, identified gene focuses the search for the underlying mechanisms. The extensive studies of CaMK provide a defined list of candidate substrates with identified functions (22). Understanding the mechanisms in this model may shed light on mechanisms of acquired forms of epilepsy in other models and in humans. Our findings also render CaMK a candidate gene for analogous forms of inherited epilepsies in humans (23).

A.J.S. and S.T. previously reported impaired acquisition of a memory task, the Morris water maze, which correlated with defective formation of long-term potentiation (LTP) of an excitatory synapse (Shaffer collateral–CA1 pyramidal cell) studied in hippocampal slices isolated from the CaMK mutant mice (20, 24). Formation of LTP has been hypothesized to underlie acquisition of learning and memory (9). One interpretation was that defective activation of CaMK in the mature brain limited formation of LTP, which in turn caused impaired memory evident in the behavioral task. Because seizures evoked in rats *in vivo* inhibit formation of LTP at the Shaffer collateral–CA1 synapse *in vitro* (12), the occurrence of epileptic seizures in the null mutants could contribute to the impaired LTP previously reported. However, the CaMK null mutants also exhibit defective formation of long-term depression (13); thus the defective LTP cannot be explained by prior induction of LTP caused by seizure activity that simply saturated levels of potentiation. Nonetheless, our findings underscore the need for additional studies aimed at elucidating the causal relationships among hyperexcitability, defective LTP, and defective memory in the CaMK null mutants.

Note Added in Proof. After acceptance of this manuscript, a report appeared describing epilepsy in mice lacking 5-HT_{2C} (5-hydroxytryptamine type 2C) receptors (25).

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