Enriching the environment of αCaMKII^{T286A} mutant mice reveals that LTD occurs in memory processing but must be subsequently reversed by LTP

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αCaMKII^{T286A} mutant mice lack long-term potentiation (LTP) in the hippocampal CA1 region and are impaired in spatial learning. In situ hybridization confirms that the mutant mice show the same developmental expression of αCaMKII as their wild-type littermates. A simple hypothesis would suggest that if LTP is a substrate for learning, then enriching the environment should cause learning-dependent changes in wild-type mice that have LTP. Such changes would not be seen in LTP-deficient αCaMKII^{T286A} mutants. Excitatory synaptic currents in CA1 neurons, recorded with patch clamp in brain slices, revealed that enrichment induces an increase in glutamate release probability and a decreased miniature current amplitude. Confocal microscopy also showed dendritic spine density to be reduced. However, contrary to the hypothesis above, these enrichment-induced changes occur only in the mutant mice and are not detectable in wild-type littermates. We suggest that enrichment induces αCaMKII-independent changes in both wild-type and mutant mice. Such changes may be subsequently reversed in wild-type animals via αCaMKII-dependent mechanisms, such as LTP. Reversal of plasticity has long been hypothesized to be essential for the hippocampus to maintain its role in memory processing. The inability to reverse plasticity in αCaMKII^{T286A} mutant mice would then result in impairment of spatial learning.

More than half a century ago, Donald Hebb presented a cellular hypothesis for learning and memory, suggesting that coordinated activity between pre- and postsynaptic neuronal partners should change the synaptic strength between them (Hebb 1949). Long-term potentiation (LTP) (Bliss and Lomo 1973) and long-term depression (LTD) (Ito et al. 1982) are experimental paradigms in which it is now well established that such changes in synaptic strength can be induced, and considerable progress has been made toward understanding their respective mechanisms. However, the question still remains whether synaptic activity, similar to that needed to induce these experimental phenomena, occurs physiologically and whether LTP and/or LTD are processes that underlie learning. If so, when an animal forms memories, LTP and/or LTD would be expected to be induced within the relevant areas of the brain in vivo, resulting in changes in the relative strengths of synapses. Thus, an animal that lacked LTP and failed to learn would show differences in synaptic transmission compared to an animal which had intact learning mechanisms. However, various mice with different mutations that result in impairments in the induction of LTP and spatial learning have been reported to show no gross differences in properties of basal synaptic transmission in the hippocampus and cortex, when compared to their wild-type (WT) littermates (e.g., Silva et al. 1992; Giese et al. 1998; Takahashi et al. 1999; Wong et al. 1999; Huang et al. 2000). This could imply that LTP does not occur physiologically, but there are several other possible explanations. It may be that changes in synaptic weight are masked by compensatory changes occurring at other synapses in order to maintain the excitability of the neuron (Turrigiano et al. 1998; Turrigiano and Nelson 2004). In this case, relative changes in synaptic weight might occur but would not be detected in the field recordings that are generally used for such tests. Alternatively, net increases or decreases in synaptic strength could be masked by changes in synapse number. Another reason for the lack of detectable synaptic differences might be related to the fact that standard laboratory housing conditions may not provide sufficient opportunity for the formation of hippocampus-dependent memories to result in measurable changes in synaptic strengths (Wurbel 2001). Indeed, various differences in the properties of hippocampal synapses have been observed when animals are raised in enriched environments compared to those raised in standard laboratory cages (for review, see Van Praag et al. 2000; Fernandez-Teruel et al. 2002), an indication that Hebbian-like processes may, indeed, take place physiologically in response to environmental stimulation.

In the present study, we have used the mutant mouse developed by Giese et al. (1998) in which a point mutation (T286A) in the α-subunit of CaMKII prevents autophosphorylation at this site. In vitro, T286 autophosphorylation enables the kinase to remain autonomously active in the absence of calcium/calmodulin (Hanson et al. 1989; Bayer et al. 2006). T286 autophosphorylation and thus calcium/calmodulin-independent activity is lost in the αCaMKII^{T286A} mutant mice. While autonomous activity of the enzyme may be required for the maintenance of LTP (Lisman et al. 2002), more recent experimental evidence supports the notion that T286 autophosphorylation itself, rather than increased levels of kinase activity, may

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underlie the important roles of CaMKII in LTP (Lengyel et al. 2004; Robison et al. 2006). The mutation results in mice deficient in LTP at Schaffer collaterals to CA1 pyramidal neuron synapses. It is putatively due to this lack of LTP that the mutant mice are also substantially impaired in spatial learning tasks.

We have investigated the physiological role of LTP by comparing the effect of environmental enrichment on synaptic parameters of αCaMKII^{T286A} mutant mice with their WT littermates at the level of single or very few synapses. We recorded from individual CA1 neurons using patch-clamp techniques in hippocampal slices and investigated excitatory synaptic transmission, recording both minimally evoked synaptic responses and miniature postsynaptic currents (mEPSCs). In addition, we compared spine density in both their apical and basal dendrites.

Results

The distribution of the wild-type versus mutant αCaMKII mRNA over development

Before assuming that the role of a protein in physiology can be clarified by a mutation, it is necessary to investigate the effect of the mutation on that protein's developmental distribution. Overall, the subtlety of the T286A mutation, which leaves the calcium-dependent function of the kinase unchanged, decreases the likely compensation via up-regulation of other proteins. Previous studies in the αCaMKII^{T28A} mouse have shown there to be no measurable up-regulation of β-CaMKII, which was the most likely compensation for a change in α-subunit function. Moreover, in adult brains the distribution of the protein is unchanged (Giese et al. 1998).

To check for possible differences in developmental distribution, we have used in situ hybridization techniques to compare αCaMKII mRNA in the mutant mice to that in their wild-type littermates at various stages from postnatal day 1 (P1) to adult. No differences were detectable between the distribution and qualitative levels of αCaMKII transcripts between WT and mutant mouse brain (Fig. 1A). Thus, the calcium/calmodulin-dependent functions of the enzyme would be expected to be similar in both groups. Moreover, Giese et al. (1998) also showed comparable levels of Ca/CaM-dependent kinase activity in hippocampal tissue from the adult WT and mutant mice. Thus, the T286A mutation appears to affect only the autophosphorylation-dependent function of the α-subunit.

An interesting additional observation is that the mRNA for the α isoform of the enzyme is strongly expressed in the hippocampus from birth. A previous study was only able to detect αCaMKII in the CA2 hippocampal sub-region of P1 animals (Bayer et al. 1999). Our findings are consistent with this study in that the highest levels of mRNA are seen in CA2. However, we also detect a strong signal within CA3, plus lower levels in CA1 and the inner blade of the dentate gyrus. Outside of the hippocampus, αCaMKII expression is clearly present in the entorhinal cortex, the striatum, and the olfactory bulb in the P1 mouse brain. In P8 animals, αCaMKII levels in the CA1 region have reached those seen in CA2 and CA3, and the expression in the brain appears to have a similar overall pattern to that seen in the adult (data not shown).

Effects of environmental enrichment on unitary evoked synaptic transmission

Environmental enrichment was used to increase spatial exploration and learning. This would be expected to maximize any differences seen between genotypes, but more importantly, it allows a comparison of changes within either genotype that are most likely due to learning. Mice of both genotypes were raised under the same standard laboratory conditions until weaning (P21) and were then either transferred for 3–5 wk into an enriched environment or returned for the same period to standard laboratory cages. Hence, both enriched and nonenriched groups were raised in the same environment during the primary period of development. Although all groups were compared (Analysis of Variance, ANOVA), it is of particular interest to investigate differences in mice of the same genotype raised in the two housing conditions. This allowed observation within each genotype of changes induced only by environmental enrichment, largely avoiding differences in development that might occur between the genotypes considering that αCaMKII is present from P1.

We recorded synaptic parameters in acute hippocampal slices comparing results from the four groups (male mice, 6–8 wk old): WT and mutant (M) mice in standard environment (SE) versus enriched environments (EE) (primarily WTSE vs. WTEE; MSE vs. MEE). We used minimal extracellular stimulation (in the presence of the $GABA_A$ receptor antagonist SR9553, 6 μM) (see Materials and Methods) to evoke a single or very few axons (referred to as unitary synaptic currents). The same method of stimulation was used throughout, and all analyses here and subsequently were performed blind to genotype and environment.

Before considering the effects of enrichment, it is interesting to note that, in agreement with previous studies (Giese et al. 1998), no significant differences were found between mutant and WT mice raised in standard housing conditions (Fig. 1B,C) (WTSE vs. MSE). There was a trend for mutants to show a lower amplitude of unitary EPSC (two-tailed *t*-test, *df* 20; $P = 0.067$), but this was not accompanied by changes in any other variable measured.

Considering findings from previous studies on environmental enrichment (e.g., Foster and Dumas 2001), we predicted that we would see differences between the WT animals raised in the two environments. If such environmentally induced changes were dependent on LTP, or on other processes mediated by the autophosphorylation of αCaMKII, we would not expect to see them in the T286A mutants.

Contrary to expectations, we were unable to detect any significant differences, for any of the parameters tested, between WT mice after exposure to standard versus enriched environments (WTSE vs. WTEE). However, differences were present in almost all measured parameters between the two groups of mutant mice (MSE vs. MEE) (Figs. 2–4). In mutant mice, the mean amplitude of unitary EPSCs (including failures) was significantly higher in mice raised in an enriched environment than those raised in standard laboratory cages (MEE vs. MSE) (Fig. 2A). This seemed to be largely due to an increase in release probability as it was accompanied by a decrease in failure rate (Fig. 2B) and a decrease in paired-pulse ratio (Fig. 2C). While a decrease in failure rate (MSE: 62.5% ± 2.5%, $n = 11$; vs. MEE: 44.6% ± 3.6%, $n = 10$) could additionally reflect a decrease in silent synapses (Edwards 1991; Isaac et al. 1995; Liao et al. 1995), this would not be expected to affect the paired-pulse ratio.

For all three measurements, there was a significant difference between the two mutant groups (MSE vs. MEE) but no significant difference between WT mice (WTSE vs. WTEE). For EPSC amplitude and paired-pulse ratio, ANOVA showed no overall effect of genotype or environment separately, but there was a significant interaction between the variables ($P < 0.05$) such that the enriched environment only affected the mutant and not the WT mice. In contrast, for probability of failures, the effect of environment was highly significant across the groups ($P < 0.005$), while genotype showed no significant effect. There was a strong trend to interaction between the variables, although this did not reach statistical significance ($P = 0.065$). Thus, overall the results indicate an increase in release probability in the

mutant mice due to enrichment, which is not seen in the WT mice.

Frequency and amplitude of miniature synaptic currents

The frequency and amplitude of miniature EPSC were recorded in the presence of tetrodotoxin (1 µM) and SR9553 (6 µM). As the signal-to-noise ratio is low, some currents would have been lost in the noise. To avoid any possibility of bias, the parameters for detection were clearly defined (see Materials and Methods) and all analysis was again carried out blind. The detection was set on the side of missing some real events rather than inclusion of artifacts. Again, no significant changes were seen between the WT mice in different environments. However, there was also no evidence in mutant mice for the increase in frequency of mEPSCs that would have been predicted from the observed increase in evoked release probability. In fact, any trend was in the opposite direction with a tendency toward a decrease in frequency in the enriched mutant mice compared to the mutant mice from standard environments (MSE vs. MEE) (Fig. 3A,B). In contrast to frequency, the mean of the median amplitude of mEPSCs showed a highly significant effect of environment overall ($P < 0.001$) but no significant effect of genotype ($P = 0.4$). Again, the only individual group significantly affected were the mutants with a small (14%) but highly significant decrease in mEPSC amplitude after environmental enrichment (Bonferroni post-test, $P < 0.01$, MSE vs. MEE) (Fig. 3C). A trend to a decrease in mEPSC amplitude is also seen in the enriched WT animals when compared to those raised in standard environments, but the mean decrease (9%) did not reach significance ($P \sim 0.07$). Note that because of the low signal-to-noise ratio of mEPSC recordings a decrease in miniature amplitude could mask an increase in frequency. Moreover, the observed decrease in mEPSC amplitude would be underestimated if there were more small miniature currents disappearing into the noise. This problem would also apply to the evoked currents. However, as evoked unitary EPSCs are associated with a stimulus and can be averaged, their detection is less dependent on baseline noise. Importantly, there was neither a correlation between baseline noise and miniature frequency nor any significant effect of either genotype or environment on background noise level.

Thus the results clearly indicate a small enrichment-induced decrease in mEPSC amplitude in mutant mice that may result in the apparent trend toward a decrease in mEPSC frequency. Although an increase in true frequency cannot be ruled out, the results suggest that the environmentally induced increase in evoked release probability observed in the mutant mouse experiments is not accompanied by an increase in mEPSC frequency. As mEPSCs are derived from synapses impinging throughout the dendritic tree, the lack of an overall increase in mEPSC frequency could also be due to compensatory changes in release properties

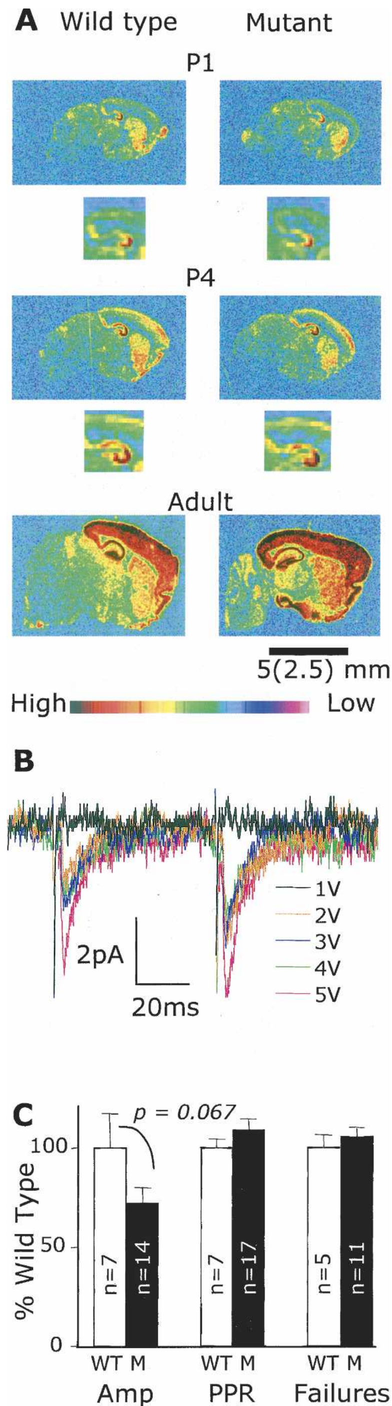

Figure 1. Wild-type (WT) and mutant mice exhibit no differences when raised in standard housing conditions. (*A*) In situ hybridization reveals that the distribution of mRNA for αCaMKII is unchanged in mutants compared to WT mice throughout development. A clear signal is already present from postnatal day 1 (P1) in both genotypes. Larger panels show comparable parasagittal slices of whole brain, while zooms show detail of the hippocampal region. Identical results were confirmed using a second antisense oligonucleotide complementary to a different region of the mRNA molecule (data not shown). (*B*) Raw data showing the all or nothing nature of minimal stimulation. Recordings were made at room temperature. Experiments and all subsequent analyses were performed blind to genotype throughout. Whole-cell patch-clamp recordings were made in CA1 pyramidal cells in acute slices using a CsCl-based intracellular solution in the presence of SR95531 (6 µM) to block $GABA_A$ receptors. Minimal stimulation was performed by placement of a patch electrode containing 1 M NaCl extracellularly in the Schaffer collateral pathway. Stimulus voltage was gradually increased until a synaptic current was detected. The voltage was further increased until an increase in mean synaptic amplitude was detected (averaging 20 consecutive events)—indicating the recruitment of a second axon—and then returned to the middle of the stimulus voltage range at which synaptic currents were first detected (i.e., minimal stimulation). Synaptic failures were only measured if the signal–noise ratio was sufficient to detect individual currents unambiguously. For amplitude and paired pulse ratio measurements, consecutive responses were averaged allowing use of all stable recordings. (*C*) Mean data from WT and mutant mice raised in standard housing conditions reveal that there are no significant changes in EPSC amplitude, failures rates, or in paired pulse ratio. There is a trend toward smaller amplitudes in the mutant mice, but this does not reach significance. Error bars in these and subsequent bar charts represent the standard error of the mean. Statistics were performed by ANOVA.

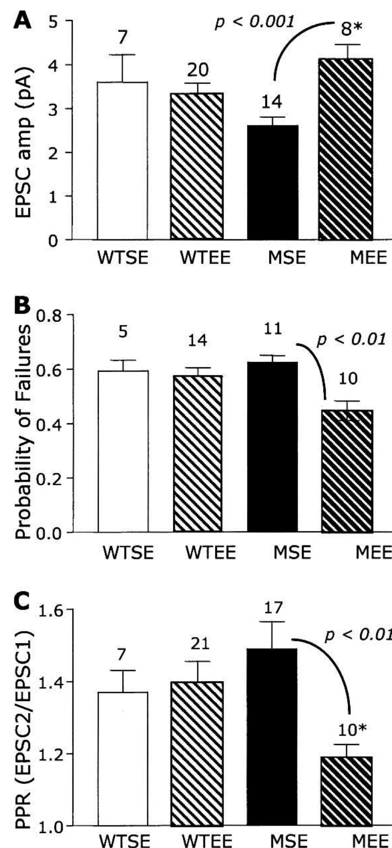

Figure 2. Enriching the environment increases release probability of the Schaffer collateral synapses in mutant but not in wild-type (WT) mice. (*A*) In mutant mice, the mean amplitude for evoked EPSCs is significantly higher in animals raised in enriched environments compared to mutants raised in standard conditions. There are no significant differences between other mouse groups. (*B*) Enrichment results in a significant decrease in the probability of failures in the mutant mice. No change is seen in WT mice. (*C*) Enrichment results in a significant decrease in the paired pulse ratio in the mutant mice, with again no change seen in WT mice. Thus, in all measures the only significant differences are between mutant mice raised in the two housing conditions. Statistics were performed using ANOVA. Where there was an overall significant effect of housing condition, Bonferroni post-tests were performed to test for effect of enrichment in WT and mutant mice individually. Significance levels on figures refer to post-tests.

occurring elsewhere in the neuron or to an overall change in the number of active synapses.

Dendritic spine density

In order to investigate the possibility of a change in the number of synapses, we compared spine density in the region of the dendrites that is thought to receive synaptic input primarily from the Schaffer collaterals. We thus filled individual neurons with Alexa 594 by including it in a patch electrode and imaged the dendrites at high resolution with a confocal microscope. The analysis procedures are detailed in Materials and Methods. There was a 20% decrease in spine density in the most proximal 150 µm of the apical dendrites in CA1 cells from enriched mutants compared to those raised in a standard environment (Fig. 4). Similar to the changes in miniature amplitudes, ANOVA confirmed a highly significant effect of environment ($P < 0.005$) with no significant effect of genotype ($P = 0.74$). Moreover, the difference between the mutant groups (MSE vs. MEE) was again statistically significant (Bonferroni post-test, $P < 0.05$), while the similar trend in the WT mice did not reach significance ($P \sim 0.1$). No changes in spine density were detected in the basal dendrites (data not shown). The simplest interpretation of such a decreased spine density is that environmental enrichment causes a decrease in the number of synapses in the proximal apical dendrites of CA1 neurons in αCaMKII^{T286A} mutant mice.

Discussion

The choice of the αCaMKII^{T286A} mutant mouse

Many different LTP-null mutants could have been chosen for this study. The T286A point mutation of αCaMKII is the mutation of choice for a variety of reasons. First, the essential role of the autophosphorylation of αCaMKII in the induction of LTP is

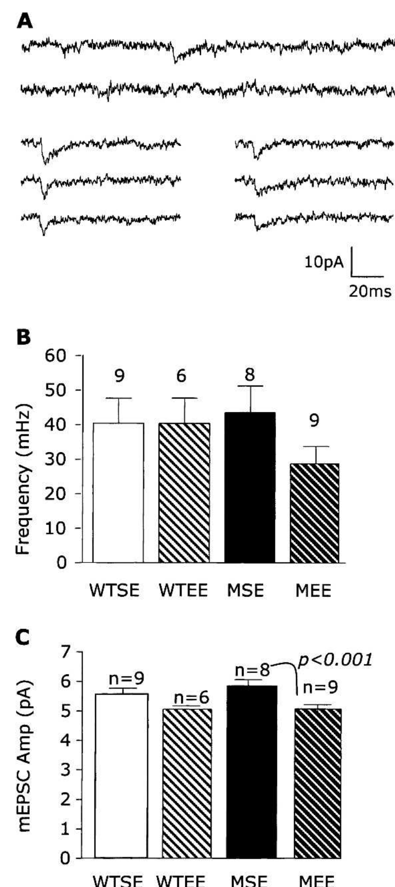

Figure 3. Enriching the environment results in a decrease in amplitude but not in frequency of mEPSCs in mutant mice. (*A*) Raw data showing typical miniature synaptic currents in the presence of TTX and SR95531. The currents shown are from a mutant mouse raised in a standard environment. The first panel demonstrates the infrequent nature of the miniatures; total time, 485 msec. (*Below*) Individual traces are shown of other miniatures detected in the same cell. Very small amplitude mEPSCs will be lost into the noise; the detection threshold was 4pA. (*B*) The mean median mEPSC amplitude is decreased in the mutant group as a result of enrichment. Although not reaching statistical significance, a similar trend is seen in the wild-type (WT) mice, and there is an overall significant effect of environment across the genotypes. (*C*) There is no difference in the mean mEPSC frequency between the groups, although a trend toward a decrease in frequency is evident in the enriched mutant mice. This could be as a result of the decreased amplitude causing more mEPSCs to be lost into the baseline noise. Statistics were performed using ANOVA and Bonferroni post-tests. Where there was an overall significant effect of housing condition, Bonferroni post-tests were performed to test for effect of enrichment in WT and mutant mice individually. Significance levels refer to post-tests.

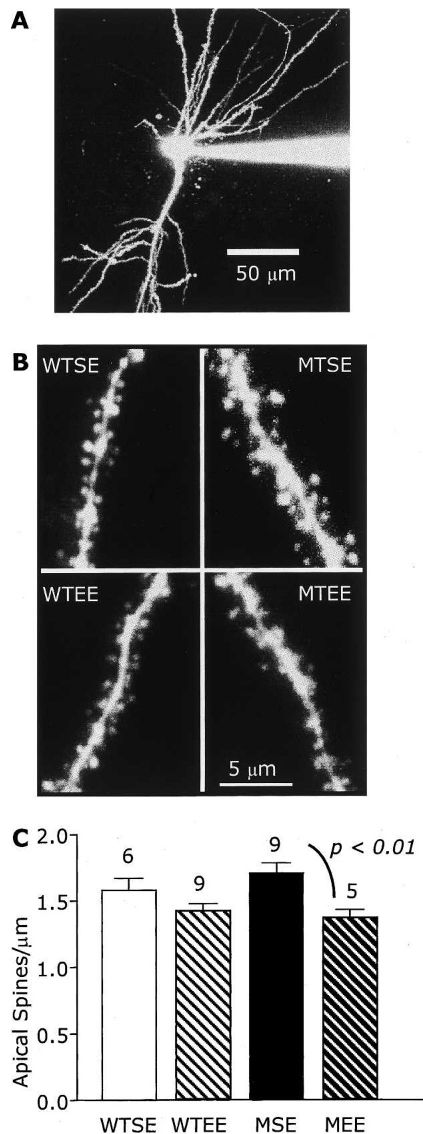

Figure 4. Spine density is significantly decreased by enrichment in mutant mice in the secondary and tertiary apical branches of CA1 neurones. The dendritic segments analyzed were restricted to a 150-μm radius from the soma. (*A*) A typical CA1 neuron filled with the fluorescent dye Alexa Fluor 594. All imaging was carried out on living cells, using a 60× water immersion objective (numerical aperture 0.9) with the patch electrode still connected, as shown. (*B*) Examples of spines used for spine density analysis. Note that all analysis was carried out blind. (*C*) Mean spine densities for the apical dendrites of CA1 cells from the four animal groups. Enrichment caused a decrease in apical spine density in the mutant animals; no changes were observed in wild-type (WT) mice. Statistics were performed using ANOVA. Where there was an overall significant effect of housing condition, Bonferroni post-tests were performed to test for effect of enrichment in WT and mutant mice individually. Significance levels refer to post-tests.

widely accepted (for reviews, see Lisman et al. 2002; Griffith 2004). Second, the apparent lack of compensation by up-regulation of closely related proteins and the preservation of distribution of the mRNA throughout development in the mutants suggest that the effects seen are directly due to the mutation. Furthermore, the "dys-regulation" of αCaMKIIautoP has also been directly implicated in Angelman's mental retardation syndrome, a human learning disorder (Weeber et al. 2003). We cannot discount the possibility that there may also be unknown αCaMKIIautoP-dependent processes influenced by increased levels of exercise. However, normal performance of the T286A mutants in the visible version of the Morris water maze (Giese et al. 1998; Need and Giese 2003) suggests that the point mutation does not result in vision, motor coordination, or motivation impairments that might affect the activity levels of the mutant mice or the consequent activity-linked effects on brain neurochemistry.

αCaMKII^{T286A} mice lack NMDA receptor-dependent potentiation as soon as it can be measured in the hippocampal CA1 region (Giese et al. 1998). They have also been reported to have unstable place cells (Cho et al. 1998) and no spatial learning in the Morris water maze (Giese et al. 1998; Need and Giese 2003). Interestingly, they also lack LTP in the barrel cortex and plasticity induced by whisker removal (Hardingham et al. 2003), as well as impaired ocular dominance plasticity in V1 following monocular deprivation (Taha et al. 2002). The unchanged levels or properties of basal synaptic transmission and connectivity in mutants compared to WT controls in normal environments is consistent with these previous studies. Moreover, it has been demonstrated that enrichment of the environment does not rescue the learning deficit in the Morris water maze (Need and Giese 2003). This is in contrast to the memory deficits in the CA1-restricted NMDA receptor null mutants (Rampon et al. 2000).

Hence, this mouse is ideal for studying the effect of a lack of LTP without detectable compensations to override the deficit in learning and memory.

Overall effects of environmental enrichment

Enrichment-induced changes occurred in the mutant mice and were not reflected in their wild-type littermates. This observation was entirely consistent across almost all variables tested. The decrease in spine density and increase in release probability observed in enriched mutants would have opposite effects on miniature frequency, which is consistent with the lack of overall change observed in this variable. It is interesting to note that in the environmentally induced changes observed between mutant groups, both the decrease in miniature amplitude and decreased spine density showed trends in the same direction in the wild-type animals. In contrast, the increase in release probability, whether observed as an increase in evoked amplitude, decreased failure rate, or decreased paired pulse facilitation, suggested absolutely no trends to change in the data from the wild-type groups.

The fact that throughout this study all statistically significant changes seen were between the mutant mouse groups was entirely contrary to our original prediction that effects of enrichment would primarily occur in WT mice and be absent in the mutants. Thus, if LTP were the substrate for experience-dependent changes resulting from our enrichment paradigm, such changes were not detectable in WT mice in any of the parameters measured. In contrast, in the mutant mice that have impaired LTP in the CA1 region, enrichment caused significant increases in release probability at Schaffer collateral synapses and a decrease in spine density restricted to the apical dendrites, as well as a decrease in the median mEPSC amplitude.

How can a mutation that impairs the function of an enzyme result in changes that are not detectable when the enzyme (and LTP) is intact? One possibility would be that the function of the kinase is inhibitory and prevents the effects of an enrichment-induced process in WT animals. Thus, in the mutants, where such inhibitory actions would be impaired, the effects of enrichment are revealed, that is, disinhibited. If this were the case, it would imply that autophosphorylated αCaMKII would result in the prevention of plasticity. However, everything that is known

about the actions of autophosphorylated αCaMKII suggests that it is required for LTP (e.g., Malenka et al. 1989; Malinow et al. 1989; Silva et al. 1992; Pettit et al. 1994; Lledo et al. 1995; Mayford et al. 1995; Giese et al. 1998). In addition, CaMKII has been linked with enhanced dendrite arborization and synapse formation (Fink et al. 2003), plus the activity-dependent growth of filopodia and spine formation (Jourdain et al. 2003). Indeed, autophosphorylation of CaMKII clearly promotes rather than inhibits synaptic plasticity (Lisman et al. 2002), making disinhibition a highly unlikely explanation for the observed changes in the mutant mice.

A second possibility would be that LTP and LTD contribute equally to enrichment-induced changes in the WT mice, resulting in no measurable change in mean synaptic parameters or spine density. This would result in an equal number of synapses being potentiated or depressed in WT mice, while mutant mice would only show depression. However, if this were the case, the EWT mice, while showing no change in mean current amplitude or in the mean values of other synaptic parameters measured, would have the widest range of synaptic amplitudes, ranging from depressed to potentiated. They would thus be expected to show an increase in variance of the synaptic parameters measured both compared to non-EWT mice and compared to EMT. This was not the case for EPSC1 amplitude, failures, paired-pulse ratio, or miniature current amplitude (data not shown).

A third hypothesis that is, however, compatible with all the data and with the known functions of CaMKII is that a reversible process is in operation. If the initial changes caused by enrichment were αCaMKII-independent, they would occur in both WT and mutant mice. However, if such effects were then reversed by an αCaMKIIautoP-dependent mechanism, the original change would remain in the mutant mice but would not be detectable in WT mice. Based on this concept, we propose the hypothesis that an αCaMKIIautoP-dependent form of plasticity, like LTP, is essential for the active reversal, or "clearing," of temporary memory circuits in the hippocampus.

As the hippocampus is not thought to be the final storage place for memory but rather a processing unit, it has long been suggested that any memory trace stored in the hippocampus should be time-limited (Squire 1992) to avoid saturation of memory processing. Thus, individual changes in synaptic strength should be returned to control levels once the memory is more permanently stored elsewhere. Our data would be consistent with the hypothesis that an αCaMKIIautoP-independent form of activity-dependent plasticity (possibly LTD) occurs during enrichment and the laying down of the memory trace. For long-term memory storage, the information would then be passed on from the hippocampus, presumably to appropriate areas in the cortex. This is in agreement with previous evidence that LTD may be required for the decoding of environmental novel object–place associations in the hippocampus (Kemp and Manahan-Vaughan 2004). An αCaMKIIautoP-dependent mechanism, such as LTP, may then be responsible for returning the depressed synapses to baseline strengths. Without this clearing of the temporary memory circuits, hippocampal function would rapidly be saturated and the ability of animals to learn new tasks impaired.

This hypothesis is also consistent with the observations from several groups working in behaving animals, including Xu et al. (1998) and more recently Abraham et al. (2002). These groups both used electrodes implanted in freely moving rats and demonstrated that LTP could be reversed when animals were exposed to novel or enriched environments, respectively (for review, see Xu et al. 1998). In these cases, the αCaMKIIautoP-dependent plasticity (i.e., LTP) was experimentally induced but was then reversed by experience-dependent plasticity. The present data suggest that these processes would occur in the opposite sequence, under physiological conditions. Hence the experience-dependent plasticity (perhaps LTD) would be the active process occurring as a result of exposure to an enriched environment with the α-CaMKIIautoP-dependent plasticity then reversing the changes in WT mice but not in the T286A mutant mice.

In this context, we suggest that the effects of enriching the environment observed in the CaMKII mutant mice result from the experience-dependent depression of synaptic transmission. This depression, reflected in the decrease in miniature EPSC amplitude and decrease in spine density, may occur first as a direct result of enrichment. Moreover, these two effects may be linked as AMPA receptor endocytosis (Carroll et al. 1999; Beattie et al. 2000; Brown et al. 2005) as well as spine retraction (Nägerl et al. 2004) and synapse elimination (Shinoda et al. 2005) have all been shown to occur as a result of experimental stimuli that produce LTD.

It is interesting to note that, while in all cases changes were only found to be statistically significant in the mutant mice, trends in the same direction were seen for the decreases in both miniature amplitude and spine density in the WT littermates. Moreover, for both these variables, environment was seen to have a statistically significant effect overall. This would be consistent with enrichment effects that occurred in both genetic groups but had been largely reversed in the WT mice by an αCaMKIIautoP-dependent mechanism, such as LTP. In the enriched mutant mice, the observed increases in release probability resulting in increased evoked EPSC amplitude and decreased paired pulse facilitation may, therefore, be compensatory changes that occur as a result of the persistent depression, rather than primary effects of enrichment. This would be consistent with the observation that these effects are only seen in the mutant mice with no similar trends in their WT littermates.

We suggest, in agreement with Manahan-Vaughan and Braunewell (1999), that LTD (or another form of αCaMKIIautoP-independent plasticity) (Stevens et al. 1994; Kirkwood et al. 1997) is a likely molecular mechanism for the processing of at least some forms of hippocampus-dependent memory. In this case, LTP may be the phenomenon that clears the memory trace from the hippocampus after consolidation elsewhere in the brain, thus allowing the re-use of the same hippocampal circuitry for future plasticity.

We cannot assess from the present data whether the opposite process may also occur in parallel, that is, the experience-induced strengthening of synapses followed by subsequent reversal by synaptic depression or de-potentiation. We would not expect to be able to find evidence of reversible experience-induced changes that were initiated by LTP-like processes using an LTP-null mutant. Such changes would not be detected in either group, having been reversed in the WT mice and not occurring in the first place in αCaMKII^{T286A} mutant mice. Two studies, published since the submission of this manuscript, have suggested that LTP may also occur in some parts of the hippocampus as a result of context-related shock-induced learning paradigms (Pastalkova et al. 2006; Whitlock et al. 2006). Interestingly, no effect of the LTP reversing peptide "ZIP" could be observed in baseline recordings, suggesting that no previously occurring LTP remained in the general circuit, despite previous experience (Pastalkova et al. 2006). Hence, it is possible that LTP and LTD act together to allow the temporary storage of memory and that each synapse must be returned to baseline by an opposing process, after the memory trace is consolidated elsewhere.

Finally, the question arises as to why we detect no significant changes with enrichment in wild-type mice when changes have been reported in many previous studies (for review, see, e.g., Nithianantharajah and Hannan 2006). First, this is in itself

compatible with our hypothesis. If the strength of synaptic transmission is constantly being determined by the exact balance between LTD and subsequent LTP, the measured level at any time point would be dependent on the enrichment paradigms used. Hence, in many studies (e.g., Foster et al. 1996; Gagné et al. 1998) animals were placed in an enriched environment for 1–6 h per day for 4–5 wk. In contrast, in the present study, animals were in enriched environments for 24 h per day for 3–5 wk. In the former case, depending on the timing of testing after removal from brief exposure to enriched environments, it is possible that full reversal would not have had time to occur, whereas in the present case, enrichment and reversal would be happening continuously. Testing the effects of short periods of enrichment followed by withdrawal over various time periods is clearly a direction for future study of this hypothesis. Another difference is that the majority of previous studies have been carried out in rats, and this could present a different balance from that seen in mice. However, the fact that similar trends were seen in the wild-type mice suggests that, if numbers were much larger, significant differences might have arisen. The important point for the present hypothesis is that, even if there were real changes in the wild-type mice, the changes in the mutant mice were clearly greater or more consistent.

In summary, by the use of environmental enrichment, study of the αCaMKII^{T286A} mutant mouse has uncovered evidence of physiological plasticity. This plasticity probably occurs during the processing of memory in the mouse brain and resembles experimental LTD. Statistically significant changes are not seen with environmental enrichment in WT mice, but trends toward changes in the same direction suggest that this form of plasticity may be continually reversed by αCaMKIIautoP-dependent processes. In the hippocampus, such reversal of plasticity would be expected in order to allow further learning to occur.

Materials and Methods

All experiments were performed at room temperature. The experimenter was blind to genotype, and all analysis of data was performed blind to both genotype and housing condition. All experiments were carried out according to the conditions of a UK Home Office license.

Animals and housing conditions

Male αCaMKII^{T286A} homozygote mutant mice and wild-type littermates (Giese et al. 1998) were weaned into either standard or enriched housing conditions and exposed to a 12-h light/12-h dark cycle with food and water provided ad libitum from the cage lid. The standard housing condition was a standard Plexiglas mouse laboratory cage (length $\times$ width $\times$ height: 35 $\times$ 20 $\times$ 14 cm) lined with wood shavings and supplied with a small amount of tissue paper as bedding material. Mice were housed in groups of three to five. The enriched housing condition was a large Plexiglas laboratory cage (length $\times$ width $\times$ height: 35 $\times$ 43 $\times$ 21 cm) that contained various inanimate objects that were changed at least five times per week; the combination and arrangement of items were never repeated. Mice in the enriched housing condition were housed in groups of five to 15. Typical objects included various purpose-built rodent houses, plastic and cardboard tubes, running wheels, ladders, seesaws, ropes, and various assortments of bedding materials (e.g., sawdust, shredded paper, various grades of wood chippings, rodent bedding wool, paper bags, and sheets of paper roll).

In situ hybridization

Mice (of ages indicated) were decapitated and their brains carefully removed and frozen quickly on dry ice. Cryostat brain sections (15 µm) were thaw-mounted onto silan-coated slides (3′-aminopropyltriethoxysilan; Sigma). Sections were fixed with 4% paraformaldehyde in PBS (pH 7.4), dehydrated, and stored in ethanol or frozen until used. Sense or antisense oligonucleotides corresponding to the murine αCaMKII mRNA nucleotide sequence 5′-ATCCCAGCCCTAGTTCCCAGCCTAAAGCCTCG CCTGCCTGCCCAGTGCCA-3′ (probe A, nucleotides 80–129) were 3′-end-labeled with [^{35}S]dATP (Dupont/NEN; 1200 Ci/mmol). Parasagittal brain sections were hybridized overnight at 42°C in 100 µL of hybridization buffer containing 4 $\times$ 10^5 cpm (~2–5 pg/µL) of either the labeled antisense or sense probe (used as control of unspecific labeling). To show the specificity of the antisense oligonucleotide, a competition control was performed by adding a 500-fold excess of unlabeled antisense oligonucleotide to the hybridization mixture (control). A second antisense oligonucleotide, corresponding to a different region of the αCaMKII mRNA sequence (probe B, nucleotides 1074–1120), was also used and produced comparable results to those shown. After hybridization, slides were washed twice for 20 min at room temperature in 1$\times$ SSC containing 50 mM β-mercaptoethanol, 30 min at 57°C in 1$\times$ SSC, 5 min at room temperature in 1$\times$ SSC, and 5 min at room temperature in 0.1$\times$ SSC. Specimens were subsequently dehydrated, air-dried, and exposed to Kodak Biomax X-ray film (Eastman Kodak) for 21 d. Films were imaged with a monochrome CCD camera and digitized using M5+ software (Imaging Research Inc.; courtesy of C. Yeo, University College London). Using M5+, the original grayscale autoradiograph digital images were converted to a linear pseudocolor scale. The color scale corresponds to increasing optical densities. The solutions for in situ hybridization were PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4; STOP buffer: 0.14 M NaCl, 0.02 M Tris-HCl (pH 7.5), 0.025 M EDTA, and 0.1% SDS; hybridization buffer: 50% formamide, 10% dextran sulfate, 50 mM DTT, 0.3 M NaCl, 30 mM Tris-HCl, 4 mM EDTA, 1$\times$ Denhardt's solution, 0.5 mg/mL denatured salmon sperm DNA, and 0.5 mg/mL polyadenylic acid; 1$\times$ SSC: 0.15 M NaCl + 0.015 M Na citrate, adjusted to pH 7 using HCl.

Hippocampal slice preparation and whole-cell recordings

Animals were killed by decapitation under British Home Office License and the head immediately submerged into a dissecting dish filled with ice-cold slicing solution for dissection and removal of the hippocampal formation. The slicing solution was 85 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 0.65 mM $CaCl_2$, 4 mM $MgCl_2$, 25 mM glucose, 75 mM sucrose, 0.005 mM NBQX, and 0.005 mM 7-chlorokyneurenate, equilibrated with 95% O_2 and 5% CO_2.

Transverse hippocampal slices (350 µm) were prepared as previously described (Edwards et al. 1989) from 6–8-wk-old male homozygote wild-type, or mutant mice using the Integra-slice fitted with a ceramic blade (Campden Instruments). Slices were placed in a submerged recording chamber containing slicing solution (33°C), and after completion of slicing, the solution was gradually exchanged with normal artificial cerebrospinal fluid (ACSF) over ~30 min and then returned to room temperature (22°–24°C). The composition of ACSF was 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 25 mM glucose, equilibrated with 95% O_2 and 5% CO_2. The microelectrode internal solution was 140 mM CsCl, 2 mM $CaCl_2$, 5 mM HEPES, 10 mM EGTA, and 2 mM Mg-ATP.

Microelectrodes (thick-walled borosilicate glass; World Precision Instuments; 4–5 MΩ) filled with a CsCl-based internal solution were used to record whole-cell EPSCs in CA1 pyramidal neurons using an Axopatch 1D patch-clamp amplifier (Axon Instruments); holding potential −70 mV. Series resistance was assessed for stability but remained uncompensated throughout, and all synaptic currents were recorded at a bandwidth of 10 kHz (4-pole Bessel filter).

In order to evoke action potentials in Schaffer collateral axons, electrical stimuli were delivered via a microelectrode electrode (4–5 MΩ; filled with 1 M NaCl) placed within the stratum radiatum. Stimulus voltage was gradually increased until a synaptic current was detected (20 consecutive events were averaged

at each voltage). The voltage was further increased until an increase in mean synaptic amplitude was detected—indicating the recruitment of a second axon—and then returned to the middle of the stimulus voltage range at which synaptic currents were first detected (i.e., minimal stimulation). CA1 evoked responses were recorded via an Axopatch 1D amplifier, filter setting 10 kHz, in the presence of 6 µM SR95531, and were sampled directly onto computer (10 kHz) through a 2-kHz low-pass filter (8-pole Bessel; Frequency Devices) with the computer program WinWCP (kindly supplied by J. Dempster, University of Strathclyde, Electrophysiology Software: http://www.strath.ac.uk/Departments/PhysPharm/ses.htm). Analysis of the EPSC waveforms was also performed using WinWCP.

Spontaneous miniature synaptic currents were recorded, as above (in the presence of 6 µM SR95531 and 1 µM tetrodotoxin) and digitized at 32 kHz (CED1401, Cambridge Electronic Design, Ltd.) onto DAT tape (Model VDAT2; Vetron Technology Inc.). They were subsequently re-sampled onto computer (through a 2-kHz low-pass filter, as above) using the program WinEDR (also supplied by J. Dempster, University of Strathclyde, Electrophysiology Software: http://www.strath.ac.uk/Departments/PhysPharm/ses.htm). The detection criteria were set to identify events that crossed a −3-pA threshold for at least 0.7 msec, and 102-msec stretches of recording, each containing a detected event, were exported into WinWCP for waveform analysis. The detected events were then further selected according to criteria developed using comparison of data with control experiments in which glutamate currents were blocked with NBQX (10 µM). Thus detected events were included as currents if:

- the decay time was slower than the rise time;
- the current returned to a stable baseline;
- the peak amplitude was ≥4 pA;
- the current remained below baseline for at least 10 msec.

Laser-scanning confocal microscopy

For dendritic spine visualization, CA1 pyramidal cells were whole-cell patch-clamped with recording microelectrodes (as above) supplemented with the fluorescent dye Alexa Fluor-594 (0.2 mg/mL). Images were obtained using an Olympus Fluoview confocal microscope (generously supplied by Olympus) mounted on an upright Olympus BX50WI microscope using a 60× water-immersion objective (Olympus; numerical aperture, 0.9). Confocal images were acquired as a series of optical z-sections (step-size 0.2 µm). Spine counts were made using Image J software (U.S. National Institutes of Health). Spine densities (spines per micrometer of dendrite; total number of spines counted along a section of dendrite divided by the length of the section) were assessed by studying the individual *xy* composite images of a z-series; owing to resolution difficulties, spines directly above or below the dendrite were excluded from the density calculations. Thus, the density calculation is an underestimate but comparable between groups. The apical spine counts were calculated from dendritic images of the secondary branches (those that arose directly from the primary apical dendrite) and tertiary branches (arising from secondary dendrites).

Statistics

All data were analyzed across genotype and housing condition using analysis of variance (ANOVAR) with the Bonferroni post-test. Data were considered significant if $P < 0.05$. The Kolmogorov-Smirnov test was used to test for normal distributions. In the case of miniature amplitude distributions, the data would not be expected to be normally distributed. Hence, the median was taken for each set of data. As the medians would be expected to be normally distributed across animals within any group, the medians were then used for parametric analysis as above. The histograms for miniature amplitude distributions represent the means of the medians for each group.

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