

# A Pharmacogenetic Inducible Approach to the Study of NMDA/ $\alpha$ CaMKII Signaling in Synaptic Plasticity

Masuo Ohno,<sup>2</sup> Paul W. Frankland,  
and Alcino J. Silva<sup>1</sup>

Departments of Neurobiology, Psychiatry,  
and Psychology  
Brain Research Institute  
University of California, Los Angeles  
Los Angeles, California 90095-1761

## Summary

We recently introduced an inducible pharmacogenetic approach where pharmacological manipulations can be used to reveal recessive mutant phenotypes in a temporally controlled manner [1]. This approach takes advantage of synergisms between pharmacological and genetic manipulations to alter the function of specific signaling pathways. For example, mice heterozygous for a point mutation (T286A) in the  $\alpha$ -calcium/calmodulin-dependent kinase II ( $\alpha$ CaMKII) gene show normal learning and memory. However, a concentration of an NMDA receptor antagonist (CPP) that does not affect learning in wild-type (WT) littermates, reveals learning deficits in this heterozygote ( $\alpha$ CaMKII<sup>T286A+/-</sup>) [1]. Here, we show that pretetanic application of a concentration of CPP (0.1  $\mu$ M) ineffective in WT hippocampal slices induced deficits in  $\alpha$ CaMKII<sup>T286A+/-</sup> slices in hippocampal long-term potentiation (LTP), a mechanism thought to be involved in learning and memory. Importantly, posttetanic application of CPP (0.1  $\mu$ M) had no effect on the expression or maintenance of LTP in hippocampal slices from  $\alpha$ CaMKII<sup>T286A+/-</sup> mice. Thus, this pharmacogenetic approach allowed us to demonstrate that NMDA receptor-dependent autophosphorylation of  $\alpha$ CaMKII is required during the induction but not maintenance of LTP. This ability to temporally induce recessive mutant phenotypes could be applicable to a broad range of problems and genetic systems.

## Results and Discussion

Recently, we showed that subthreshold doses of drugs that disrupt specific signaling components upstream or downstream of genetically targeted molecules can be used to reveal the effects of recessive mutations in a temporally controlled manner [1]. This ability to temporally control the phenotypes of mutations is critical for experimental design and interpretation. For example, it helps to address concerns that the cognitive effects of certain mutations are not due to the disruption of adult processes but to undetected developmental deficits. With this pharmacogenetic approach, we previously showed that NMDA receptor-dependent activation of  $\alpha$ CaMKII during training (but not afterward) is critical for

learning [1]. These findings are consistent with accumulating evidence suggesting a close structural and functional link between the NMDA receptor and  $\alpha$ CaMKII [2–6].

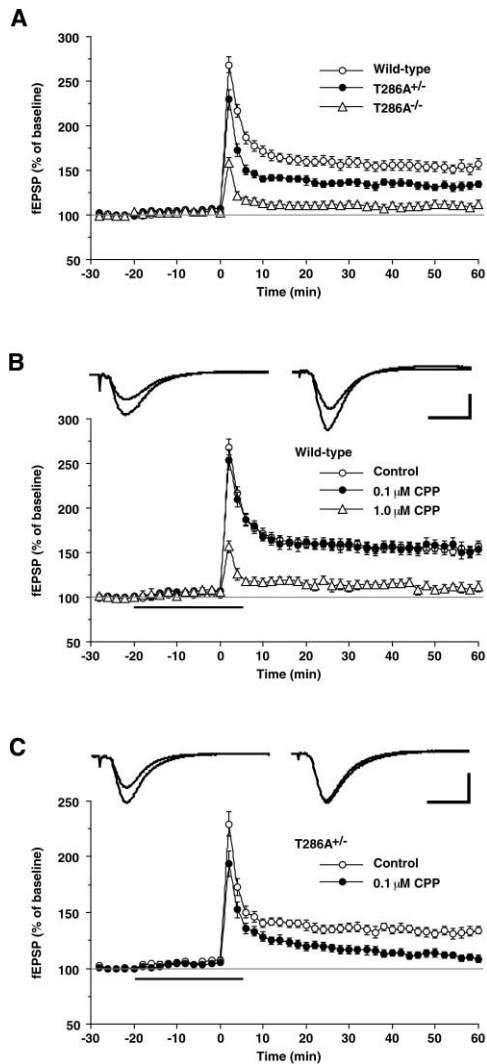
The autophosphorylation of  $\alpha$ CaMKII at Thr 286 by  $Ca^{2+}$  influx through NMDA receptors [7, 8] switches the kinase into a calcium/calmodulin-independent active state [6, 9] and is a critical step underlying hippocampus-dependent learning and memory [10]. Consistent with our previous findings [10], mice homozygous for a point mutation that blocks the autophosphorylation of  $\alpha$ CaMKII at Thr286 ( $\alpha$ CaMKII<sup>T286A-/-</sup>) showed severe deficits in hippocampal LTP at Schaffer collateral-CA1 synapses, while the heterozygous mutation ( $\alpha$ CaMKII<sup>T286A+/-</sup>) only attenuated the magnitude of LTP ( $F_{2,29} = 11.6$ ,  $p < 0.05$ ) (Figure 1A). Although these results indicate that the autophosphorylation of  $\alpha$ CaMKII is required for the induction of LTP, it is unclear whether the NMDA receptor-dependent autophosphorylation/activation of  $\alpha$ CaMKII is required for the maintenance and/or expression of LTP.

As it has been previously shown in rats, pretetanic application of the NMDA receptor antagonist CPP blocked the induction of LTP in hippocampal slices from WT mice ( $F_{2,31} = 10.4$ ,  $p < 0.05$ ) (Figure 1B), confirming the importance of NMDA receptor-dependent signaling in the induction of hippocampal CA1 LTP. To test the role of NMDA receptor-dependent activation of  $\alpha$ CaMKII in LTP, we used the pharmacogenetic approach outlined above. Although pretetanic application of 0.1  $\mu$ M CPP did not affect hippocampal LTP in WT slices, the same concentration of CPP induced severe LTP deficits in slices from heterozygous  $\alpha$ CaMKII<sup>T286A</sup> mutants ( $F_{3,46} = 16.9$ ,  $p < 0.05$ ) (Figures 1B and 1C). It is important to note that the  $\alpha$ CaMKII<sup>T286A</sup> mutation does not affect NMDA currents [10], suggesting that the effects revealed by CPP in the heterozygous mutants are due to lower levels of kinase activation. Interestingly, the residual LTP observed in  $\alpha$ CaMKII<sup>T286A</sup> heterozygous slices under 0.1  $\mu$ M CPP ( $110.2\% \pm 2.8\%$ , mean  $\pm$  SEM,  $n = 13$ ) was similar to the magnitude of LTP in  $\alpha$ CaMKII<sup>T286A</sup> homozygous slices ( $110.6\% \pm 4.5\%$ ,  $n = 6$ ) and indistinguishable from the magnitude of LTP present in WT mice after full NMDA receptor blockade (1  $\mu$ M CPP;  $110.5\% \pm 4.6\%$ ,  $n = 6$ ). These results suggest that the LTP left in the heterozygous mutants under 0.1  $\mu$ M CPP is NMDA receptor independent. Taken together, these findings suggest that the autophosphorylation of  $\alpha$ CaMKII is required for the induction of NMDA receptor-dependent LTP in area CA1 of the hippocampus and that decreasing NMDA signaling reveals LTP deficits in  $\alpha$ CaMKII<sup>T286A</sup> heterozygous mutants.

It is also important to note that 0.1  $\mu$ M CPP had a negligible effect on posttetanic potentiation measured 30 s after a tetanus in  $\alpha$ CaMKII<sup>T286A</sup> heterozygous slices ( $194.1\% \pm 11.5\%$ ,  $n = 13$ ) (Figure 1C), as compared with the effects of either 1  $\mu$ M CPP in WT slices ( $156.6\% \pm 6.6\%$ ,  $n = 6$ ) (Figure 1B) or the  $\alpha$ CaMKII<sup>T286A</sup> homozygous mutation ( $158.9\% \pm 5.7\%$ ,  $n = 6$ ) (Figure

<sup>1</sup> Correspondence: silvaa@ucla.edu

<sup>2</sup> Present address: Department of Physiology, Northwestern University Medical School, Chicago, Illinois 60611-3008.

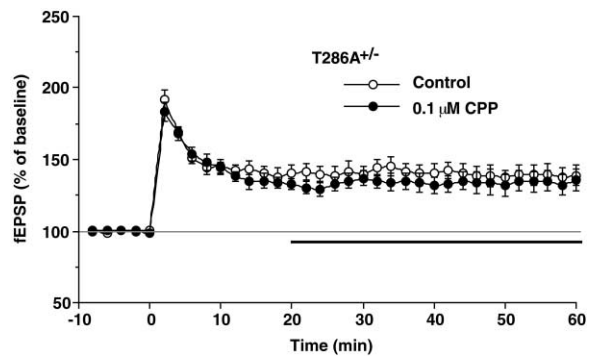


**Figure 1. A Subthreshold Concentration of CPP Triggers Hippocampal LTP Deficits in  $\alpha$ CaMKII<sup>T286A</sup> Heterozygous Mutants**

Each point indicates the field EPSP slope (mean  $\pm$  SEM) normalized to the average baseline response before the tetanus (delivered at time 0). (A) Effects of the  $\alpha$ CaMKII<sup>T286A</sup> mutations on hippocampal LTP at Schaffer collateral-CA1 synapses. T286A<sup>+/-</sup> and T286A<sup>-/-</sup> designates the  $\alpha$ CaMKII<sup>T286A</sup> heterozygous and homozygous mutants, respectively. (B) Hippocampal slices from WT mice were exposed to pretetanic application of CPP (either 0.1 or 1.0  $\mu$ M; indicated by the bar). Traces from representative experiments were shown above the graph (control, left; 0.1  $\mu$ M CPP, right). They were obtained by averaging the fEPSPs recorded during baseline and 50–60 min after tetanization. Calibration bars: 10 ms, 0.5mV. (C) LTP induction in hippocampal slices from  $\alpha$ CaMKII<sup>T286A</sup> heterozygous mutants were tested under the presence or absence of CPP. The traces above the graphs (T286A<sup>+/-</sup> control, left; T286A<sup>+/-</sup> + 0.1  $\mu$ M CPP, right) were averages of fEPSPs recorded during baseline and 50–60 min after tetanus. Calibration bars: 10 ms, 0.5mV. CPP (0.1  $\mu$ M) induced LTP deficits in  $\alpha$ CaMKII<sup>T286A+/-</sup> slices but not in WT slices.

1A). In contrast, all three manipulations block LTP to the same extent. These findings indicate that posttetanic potentiation is far less dependent on NMDA receptor-activated  $\alpha$ CaMKII than LTP.

Posttetanic (20 min) application of 0.1  $\mu$ M CPP, how-



**Figure 2. Posttetanic Application of CPP Had No Effect on Established LTP in  $\alpha$ CaMKII<sup>T286A</sup> Heterozygous Mutants**

Each point indicates the field EPSP slope (mean  $\pm$  SEM) normalized to the average baseline response before the tetanus (delivered at time 0). Hippocampal slices from  $\alpha$ CaMKII<sup>T286A+/-</sup> mutants were exposed to posttetanic application of 0.1  $\mu$ M CPP (indicated by the bar).

ever, did not affect the expression of established hippocampal LTP in  $\alpha$ CaMKII<sup>T286A</sup> heterozygous slices (Figure 2). Consistent with our findings, it was previously shown that application of CaMKII peptide inhibitor into the postsynaptic cell blocked the induction of LTP but did not affect LTP maintenance in the hippocampal CA1 region [11]. These results indicate that NMDA receptor/ $\alpha$ CaMKII signaling is required for the induction of hippocampal LTP rather than the expression or maintenance of LTP.

These hippocampal LTP results parallel our previous findings with the hippocampus-dependent learning task [1]. First, the  $\alpha$ CaMKII<sup>T286A</sup> heterozygous mutants show nearly normal hippocampal LTP and hippocampus-dependent learning. Second, decreasing NMDA receptor signaling revealed LTP and learning deficits in these heterozygotes. Third, our pharmacogenetic findings suggest that NMDA receptor-dependent autophosphorylation of  $\alpha$ CaMKII is specifically required for the induction of behavioral and synaptic plasticity in the hippocampus but not for the stability of these processes.

Recent pharmacological experiments indicate that CPP administered after LTP induction blocks the subsequent decay of LTP at perforant path-dentate gyrus synapses, a result suggesting that LTP maintenance is a persistent process, and its eventual decay is an active process mediated by NMDA receptor activation [12]. Interestingly, they also demonstrate that posttraining administration of CPP at doses that enhance LTP longevity also enhance retention of spatial memory [12]. The inducible pharmacogenetic approach introduced here could be applied to the study of NMDA receptor-dependent activation of CaMKII in LTP decay and memory extinction.

In conclusion, our results demonstrate that NMDA receptor-dependent autophosphorylation of  $\alpha$ CaMKII is required for the induction of LTP. The pharmacogenetic approach used here combines the temporal flexibility that pharmacological manipulations offer, with the molecular specificity of genetic disruptions. It is also important to note that since the pharmacogenetic approach

uses drugs at lower concentrations that are ineffective in WT controls, the nonspecific effects of these drugs should be reduced. This approach should be applicable to a broad range of biological problems and genetic systems.

#### Experimental Procedures

##### Mice

Starting with the  $\alpha$ CaMKII<sup>T286A+/−</sup> chimeras (contributing to 129 background) [10], this mutation was backcrossed five to six consecutive times into the C57Bl/6 genetic background. The  $\alpha$ CaMKII<sup>T286A+/−</sup> and  $\alpha$ CaMKII<sup>T286A−/−</sup> mice used in the experiments were F2 progeny derived from a cross between these heterozygotes. At 4–5 weeks postnatally, the mice were weaned, and their genotypes were determined with PCR analysis of tail DNA samples. All experiments were done with mice 3–7 months old, and a similar number of males and females was used.

##### Electrophysiology

Transverse hippocampal slices (400  $\mu$ m thick) were maintained in a submerged recording chamber perfused with ACSF equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 30°C. The ACSF contained (in mM) 120 NaCl, 3.5 KCl, 2.5 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 D-glucose. Extracellular field EPSPs were recorded with a Pt/Ir electrode (FHC, Bowdoinham, ME) from the stratum radiatum layer of the area CA1, and the Schaffer collaterals were stimulated with a bipolar electrode (FHC). The intensity of stimulation (100  $\mu$ s duration) was adjusted to give field EPSP  $\sim$ 33% of maximum. LTP was induced by a tetanic stimulation (100 Hz, 1 s) delivered at the test intensity. After the responses were monitored at least for 20 min to ensure a stable baseline, [ $\pm$ ]-3-[2-carboxypiperazin-4-yl]propanephosphonic acid (CPP; Sigma, St. Louis, MO) was applied for 25 min starting 20 min before the tetanus or for 40 min posttetanically (20 min after tetanization). Experiments were conducted with the experimenter blind to the drug treatments as well as the genotype of mice. To determine whether the magnitude of LTP differed significantly between the groups, responses from the last 10 min block of recordings (50–60 min) were compared by a one-way analysis of variance followed by post-hoc Newman-Keuls test when *F* ratios reached significance (*p* < 0.05).

#### Acknowledgments

We thank N.B. Fedorov and K.P. Giese for helpful discussions; and R. Chen and M. Lacuesta for help with genotyping. M.O. was partially supported by a research fellowship from the Uehara Memorial Foundation for Life Sciences. This work was funded by a grant from the National Institutes of Health (AG13622) to A.J.S.

Received: January 11, 2002

Revised: February 13, 2002

Accepted: February 13, 2002

Published: April 16, 2002

#### References

1. Ohno, M., Frankland, P.W., Chen, A.P., Costa, R.M., and Silva, A.J. (2001). Inducible, pharmacogenetic approaches to the study of learning and memory. *Nat. Neurosci.* 4, 1238–1243.
2. Husi, H., Ward, M.A., Choudhary, J.S., Blackstock, W.P., and Grant, S.G. (2000). Proteomic analysis of NMDA receptor-adhesion protein signaling complexes. *Nat. Neurosci.* 3, 661–669.
3. Kennedy, M.B. (2000). Signal-processing machines at the post-synaptic density. *Science* 290, 750–754.
4. Leonard, A.S., Lim, I.A., Hemsworth, D.E., Horne, M.C., and Hell, J.W. (1999). Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. *Proc. Natl. Acad. Sci. USA* 96, 3239–3244.
5. Silva, A.J., and Giese, K.P. (1998). Gene targeting: a novel window into the biology of learning and memory. In *Neurobiology of Learning and Memory*, J. Martinez and R. Kesner, eds. (San Diego: Academic Press), pp. 89–142.
6. Lisman, J. (1994). The CaM kinase II hypothesis for the storage of synaptic memory. *Trends Neurosci.* 17, 406–412.
7. Fukunaga, K., Soderling, T.R., and Miyamoto, E. (1992). Activation of Ca<sup>2+</sup>/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. *J. Biol. Chem.* 267, 22527–22533.
8. Ouyang, Y., Kantor, D., Harris, K.M., Schuman, E.M., and Kennedy, M.B. (1997). Visualization of the distribution of auto-phosphorylated calcium/calmodulin-dependent protein kinase II after tetanic stimulation in the CA1 area of the hippocampus. *J. Neurosci.* 17, 5416–5427.
9. Hanson, P.I., and Schulman, H. (1992). Neuronal Ca<sup>2+</sup>/calmodulin-dependent protein kinases. *Annu. Rev. Biochem.* 61, 559–601.
10. Giese, K.P., Fedorov, N.B., Filipkowski, R.K., and Silva, A.J. (1998). Autophosphorylation at Thr286 of the  $\alpha$  calcium-calmodulin kinase II in LTP and learning. *Science* 279, 870–873.
11. Otmakhov, N., Griffith, L.C., and Lisman, J.E. (1997). Postsynaptic inhibitors of calcium/calmodulin-dependent protein kinase type II block induction but not maintenance of pairing-induced long-term potentiation. *J. Neurosci.* 17, 5357–5365.
12. Villarreal, D.M., Do, V., Haddad, E., and Derrick, B.E. (2002). NMDA receptor antagonists sustain LTP and spatial memory: active processes mediate LTP decay. *Nat. Neurosci.* 5, 48–52.