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# GENE TARGETING: A NEW APPROACH FOR THE ANALYSIS OF MAMMALIAN MEMORY AND LEARNING

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## INTRODUCTION

Throughout the history of modern biological science, genetics has been an amazingly powerful tool. Biochemistry, cellular biology, immunology, and developmental biology have all blossomed through the use of such techniques. In neurobiology, however, the use of genetics has remained restricted largely to the analysis of lower organisms - invertebrates such as flies, nematodes, etc. The neurogenetics of more complex organisms such as ourselves, *Homo Sapiens*, or even other mammals has been nearly non-existent.

This situation is changing now, however, primarily because of a technique invented by others but applied by groups like ours to the brain. This technique, known as embryonic stem cell gene targeting, allows us to remove a specific single gene from an organism and then infer the function of that protein by the resulting phenotype. Because of this technique, it is now possible to analyze the complex mammalian brain by a genetic approach.

The object of our interest has been the hippocampus (Fig. 1). Although long-term potentiation in the hippocampus has been widely studied as a candidate mechanism for at least some types of learning and memory, the actual evidence for this notion is far from complete. The main support for the hypothesis comes from the analysis of rats' behavior when LTP has been eliminated by inhibitors for NMDA receptors, a subset of glutamate receptors. It has been shown that rats treated with these NMDA antagonists have an impairment in what is called spatial learning. The problem with this set of results, however, is that blockade of the NMDA receptors disrupts synaptic function within the hippocampus, and therefore may potentially interfere with the computational ability of the hippocampal circuits. Thus the failure of learning may derive not from a deficit in LTP, but rather simply from the incorrect operation of the hippocampal circuits in the absence of NMDA receptor function.

We have sought an alternate strategy to allow us to more specifically study the relationship between LTP and learning. One obvious approach would be to use the embryonic stem cell gene targeting technique to produce mice with a mutation in individual non-NMDA receptor components likely to be involved in the regulation of

LTP. We decided to apply this technique to produce a strain of mutant mice which lack the activity of an enzyme called the alpha form of calmodulin dependent protein kinase type II, or  $\alpha$ -CaMKII for short.

Regarding the analysis of the  $\alpha$ -CaMKII mutant mice, we conclude the following:

1. LTP in the CA1 region is markedly impaired in the  $\alpha$ -CaMKII mutant mice.
2. The loss of  $\alpha$ -CaMKII does not seem to affect ordinary synaptic transmission in CA1, but does impair LTP.
3. Mice lacking the  $\alpha$ -CaMKII can learn tasks that depend upon "single-association" cues, but not on "multiple spatial" cues.
4. This learning deficit does not appear to be due to a deficiency in motivation, motor control, or vision.

#### KNOCKING OUT ALPHA CAMK II

We chose to apply the ES knock-out technique to the  $\alpha$ -CaMKII enzyme for a variety of reasons. First, it has been implicated in the induction of LTP by inhibitor studies. Second, it is expressed postnatally and therefore may not be essential in development. This is an important consideration since mutation of a gene whose function is essential for neurodevelopment may result in the production of a non-viable or grossly crippled animal. Finally, this protein is not only neurospecific, being present both pre- and post-synaptically, but it is also enriched in the post-synaptic densities of the hippocampus and cerebral cortex, two structures which have been shown to be essential for complex learning.

The procedure of the ES gene knock-out technique is shown in (Fig. 2). Embryonic stem cells are isolated from a mouse and cultured. A mutated form of the gene of interest is introduced into the cells by microinjection or electroporation transfection. A small number of cells will take up the DNA for the target gene, undergo a recombination reaction and integrate the mutant gene into their genome in place of one of the two wild-type copies of the gene. These cells are selected biochemically, and then injected back into an intact blastocyst from normal (wild-type) mice. The blastocysts are placed into the uterus of a pseudo pregnant mother and are allowed to develop. Progeny will have some of its cells carrying a copy of the mutant gene and some other cells carrying only the normal wild type genes (i.e. a chimera). If the mutant gene is transmitted into the germ line, this will be manifested as a mutation heterozygous progeny when a chimeric mouse is crossed to a wild-type mouse. Interbreeding of heterozygotes gives homozygous offspring which then express only the mutant form of the targeted gene.

Western blots of tissue obtained from mice produced by this knock-out procedure for the  $\alpha$ -CaMKII protein show that the alpha form of the protein is completely missing





Figure 1.

(Fig. 3). The isoform known as the beta form of CaMKII is present, however, confirming that the knock-out technique is highly specific for the targeted gene.

#### RESULTS OF THE KNOCK-OUT OF ALPHA CAM KINASE II: ANATOMICAL, PHYSIOLOGICAL

The mutant mice are remarkably normal, at the gross levels. They gain weight normally, and they can suckle, sniff, and mate. However, they are distinctly jumpy compared to wild-type mice. For example they try to avoid human touch rather frantically long after weaning which is not the case with normal mice. Limited anatomical analysis of the hippocampus has revealed no gross alterations. At the light microscopic level (Plate 1 in color figure section) the dentate granule cells and CA fields within the brains of mutant mice appear identical anatomically to those of the normal mice. It is necessary to caution, however, that a more detailed analysis may yield more subtle anatomical differences and further study is clearly necessary.

We carried out extensive analyses of the mice, electrophysiologically characterizing the synapses of the hippocampal circuits. First, we set out to determine if the mice had normal unpotentiated transmission at hippocampal synapses. Studies carried out in collaboration with Dr. Chuck Stevens of the Salk Institute revealed no difference between the NMDA or non-NMDA components of transmission in normal versus mutant mice.

The production of LTP in these same synapses was altered, however. In normal synapses, delivery of a tetanus increases the slope of the EPSP induced by a subsequent test stimulus. In the mutant mice, however, tetanic stimulation failed to have the same effect (Fig. 4). Although in some cases it was possible to induce LTP in the mutants as measured either in single cells using the whole cell patch clamp technique or in groups of cells measured with field potentials, the number of trials in which a tetanus failed to

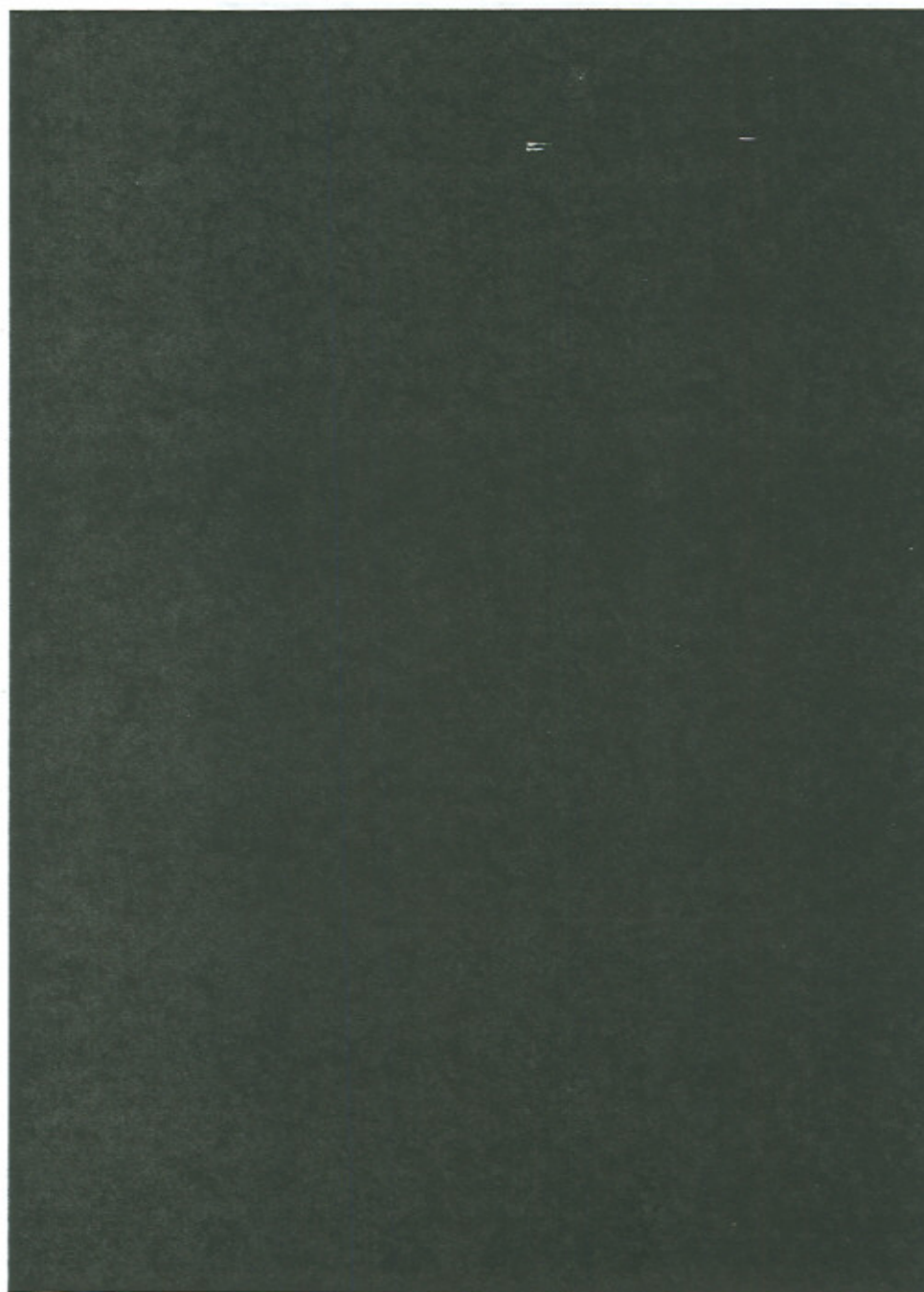


Figure 2. Generation of mouse mutants by cell-gene targeting.



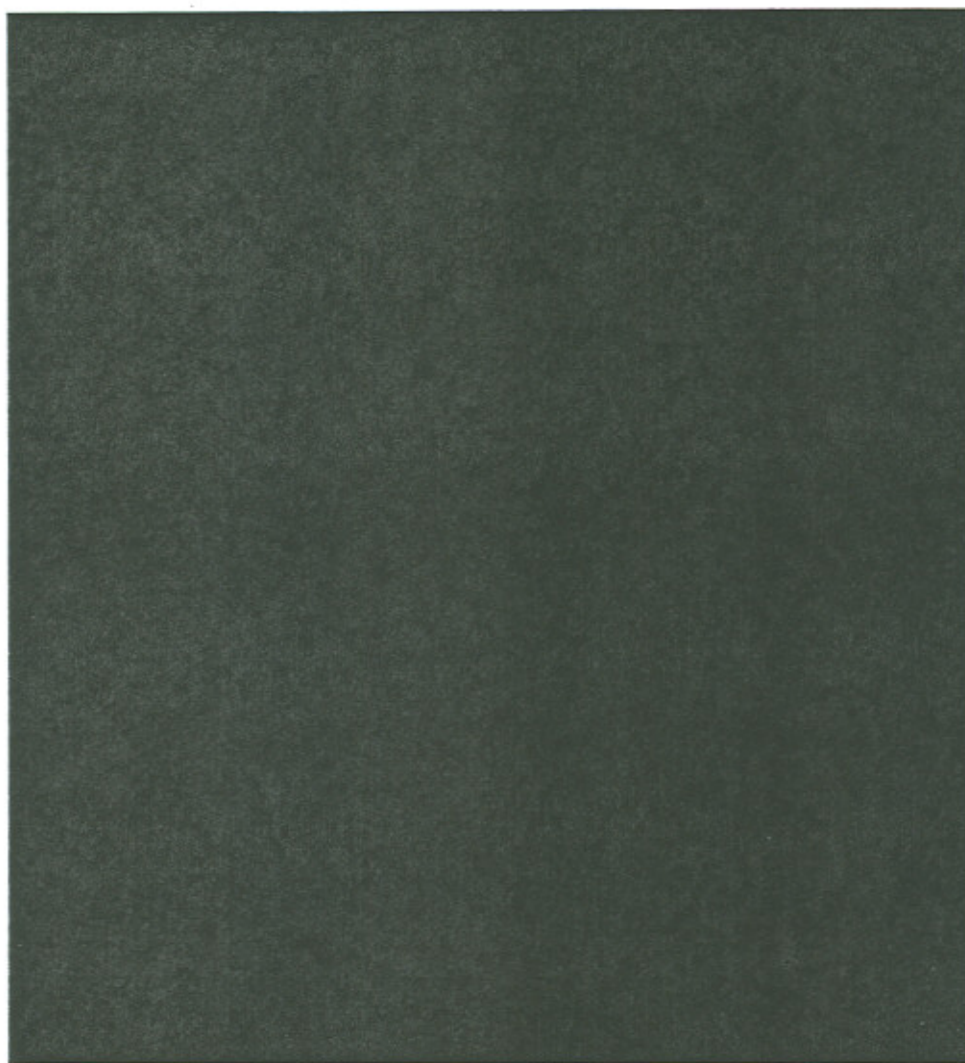


Figure 3. Western with the a and b subunit antibodies.

induce the potentiation was significantly increased over the controls. This inability to induce LTP was not due to a higher threshold for stimulation needed to establish potentiation, as increasing the number and frequency of tetanic stimuli delivered to the slice did not alter the establishment of LTP (Fig. 5). *A. Alt*

We interpret the incomplete inhibition of LTP by the  $\alpha$ -CaMKII knock-out to be consistent with its postulated role as a modulator of the phenomenon. Redundancy of the kinase's function or some activity due to the beta subunit may allow the slice to

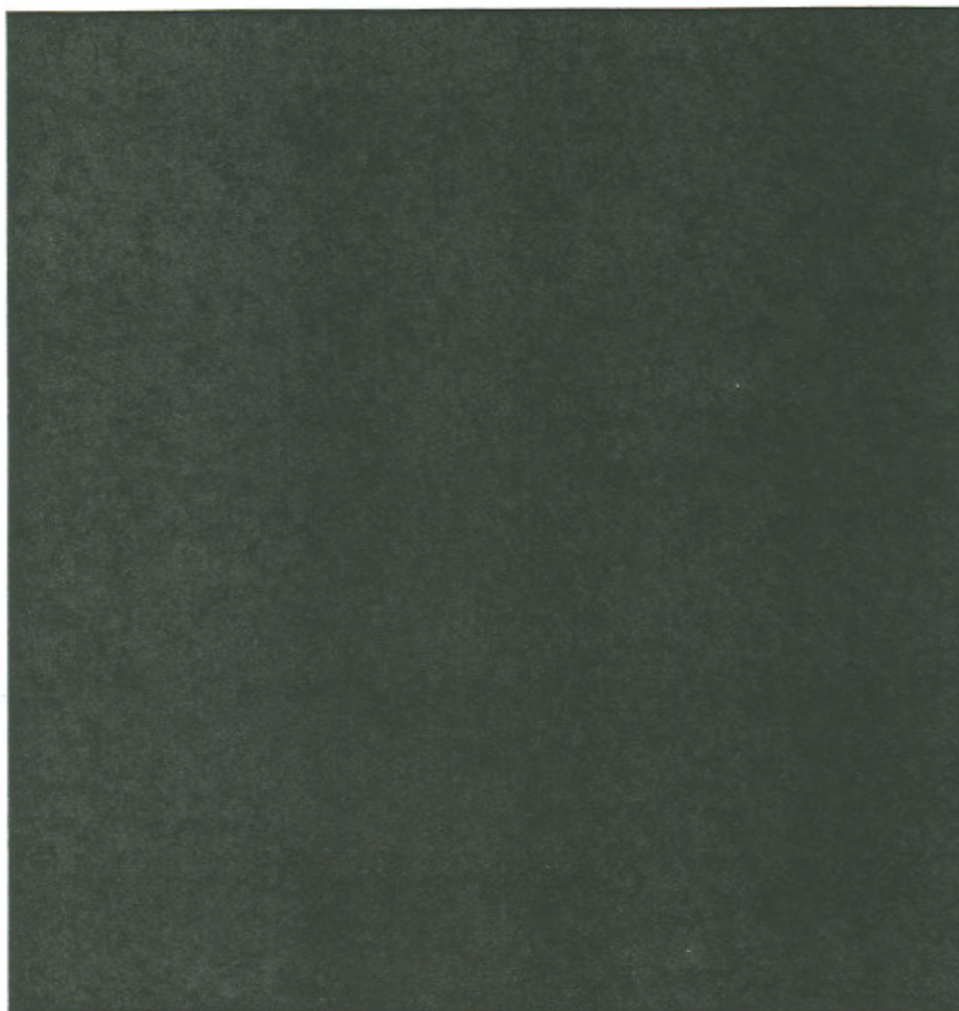


Figure 5a. Typical field potentials: Means and S.E.M.

occasionally pass the threshold needed to establish LTP. We note that in the cases in which LTP was induced in the mutant slice, the potentiation seemed to behave just like the wild-type form.

#### BEHAVIOR OF MUTANT MICE

With collaborator Jeanne Wehner at the University of Colorado, we studied the spatial learning ability of the mutant mice in a Morris water maze task. There are two versions of this paradigm, called the hidden and visible platform tasks, which test different types of learning and memory. In both versions, the platform is submerged just





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Figure 5b. LTP: Mutants vs wild type.

below the surface of an opaque liquid that fills the tub. In the visible platform task, the platform is marked with a conspicuous white flag. The mouse is placed in the liquid beginning from a random site, and then it will swim to the platform. The location of the platform and the flag is altered from one trial to another. The test is tried repeatedly for a single mouse, and the results of the swimming time required to reach the platform are plotted relative to the number of trials.

Mice mutant for the  $\alpha$ -CaMKII gene initially take longer to find the platform than do wild-type mice, but with repeated trials both mice improve in performance and eventually the two mouse groups find the platform equally quickly (Fig. 6). Morris had shown previously that the critical cue used by mice in this experiment is the simple association of the flag with the platform. Surrounding cues on the walls of the room have no relevance in this case as the platform and flag are moved around in dissociation with these objects. Thus, both the mutant and wild type mice are capable of learning this simple association task.

If we alter the paradigm to the hidden platform task, however, in which there is no flag, and the location of the platform is kept constant with respect to the surrounding objects, then a distinct difference in the ability to perform the task appears between the mutant and wild-type mice (Fig. 7). Both mice are able to improve in the task with multiple trials, but the mutant mice never catch up to the speed of the wild-type mice. In fact the mutant mice appear to take about twice as long to find the platform after the performance reaches the plateau. For the wild-type mice, it had previously been shown that they use spatial cues to find the platform – allowing them to swim directly to it after some numbers of trials. This mapping of the relative location of the platform to the objects surrounding it in space is called spatial learning and is a rather complex task. The mutant mice appear to be doing something similar, but with less proficiency than the wild-type mice.

In order to determine the differences in platform finding strategy used by the two groups of mice in the hidden platform task, we altered the paradigm slightly. In the test, called the probe trial, we first trained mice on the hidden platform task and then removed the platform. When the mice were placed in the pool without a platform they were allowed to swim for a fixed period of time (one min.) while a video camera recorded their movements. We then scored the amount of time that each mouse spent swimming in the four equal quadrants of the pool – the one quadrant which originally contained the platform and the other three which did not. As would be expected for mice which have memorized the platform by spatial strategy, wild-type mice spend a significantly greater amount of time in the training quadrant than in any of the others (Fig. 8). This is not the case with the mutant mice, however. They seem to spend approximately equal amounts of time in each of the four quadrants, suggesting that they use a different strategy to find the platform.

This difference in the strategies used by the two groups of mice was confirmed by yet another paradigm called the random platform test. In this case mice are also trained on the hidden platform task with the platform consistently located in one position. Then the mice are put into the pool again, but this time the platform is located either in the original position or else in one of seven new locations. As expected, the wild-type mice do very well at finding the platform when it is in position to which they have been trained, but have great difficulty finding it in a new position (Fig. 9). The mutant mice, however, are about equally as good locating the platform in either the trained or the new position.

We interpret the results as follows: as we move the platform around the pool it changes randomly with respect to the surrounding objects, making them useless as cues, but it is always a constant distance from the edge of the pool. We already know that the mutant mice spend equal time in the four quadrants of the pool if there is no platform, thus it appears that they swim until they can perform a simple association task – the association of a certain distance from the edge of the pool with the ability to escape from the water.





Figure 6. A Stronger tetanus did not compensate for the LTP deficiency in the mutants.



Figure 7. Visible-platform version of the Morris Maze.



Figure 8. Hidden-platform version of the Morris Maze.

#### THE BEHAVIORAL DEFICIT IS NOT DUE TO A PROBLEM WITH VISION, ATTENTION, OR SIMPLE ASSOCIATION

In order to conclude that the mice really are deficient in spatial learning, however, we must check at least three different things. First, we need to prove that the mice can see the distant objects surrounding the pool, for if they could not see as well as the wild-type mice, they would not be able to use the spatial strategy, even if they are not deficient in learning a spatial task. Second, one needs to prove that they can attend to the distant objects, as that ability is probably different from learning itself. And third, one has to show that they have the ability to associate the objects with the platform. Again this association may be performed by a mechanism independent from spatial learning itself.

The test we have employed to check these abilities is called the plus maze swimming test, devised by the Wehner group. Again the mice are placed in a pool, but in this case the pool is covered by a large piece of Plexiglas leaving open pathways in the shape of a cross. When the mice start swimming, the paths they can follow are thus very restricted as are the directions in which the mouse can see. The platform is located in one of the four arms of the cross, and the mouse is started in one of the others. The





Figure 8. Quadrant searching: the probe trial.

platform is hidden in this task, but can be associated with the object on the wall of the room which the mouse can see through the end of the arm. When the mouse is placed in one of the arms, he swims to the center and then must make a decision of whether to go right, left, or straight. At first he makes a random decision, but he will have to learn to associate the distant object on the wall with the arm containing the platform to increase the frequency of the right decision as the trials are repeated. Thus, this task requires only a simple association for learning, and also requires the mouse to see the distant object, since that's the only cue that they have, and they must attend to it. Mutant mice do not differ statistically in their ability to perform this test from the wild-type mice (Fig. 10) thus suggesting that indeed the mice can see, attend to and associate

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Figure ~~10~~<sup>10</sup>. Random vs. original location test.

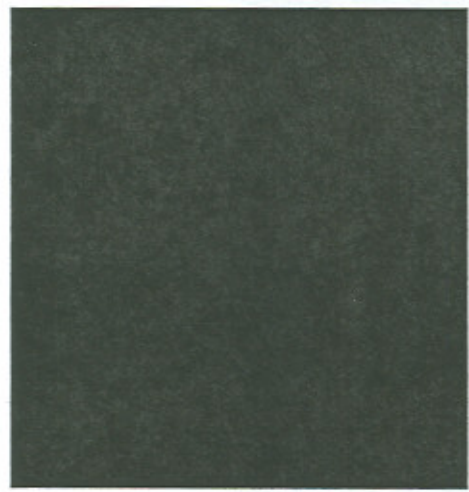


Figure ~~11~~<sup>10</sup>. Plus maze.







Figure ~~12~~<sup>11</sup>. Kindling the Alpha/CaMkII Mice.



Figure ~~13~~<sup>12</sup>.

distant objects with escape. Thus, we seem justified in concluding that their difficulty in the hidden platform task is due to a deficiency in spatial learning. Since the  $\alpha$ -CaMKII mutant mice are deficient in hippocampal LTP, but apparently have intact NMDA receptor function, the only other component of LTP whose deficiency was previously linked to spatial learning deficiency, our data considerably strengthen the notion that hippocampal LTP is indeed the basis for spatial learning.

### FUTURE STUDIES

We are beginning to extend our analysis to see if these mice are deficient in other behaviors. One phenomenon which we have looked at is that of kindling in the amygdala – another location known to be rich in  $\alpha$ -CaMKII. Kindling is a mouse model of epilepsy and the possibility of a role for LTP in inducing it has been discussed in literature. We did indeed have difficulty inducing kindling in our mutant mice, (Fig. 11). This relation is being pursued at this time.

Figure 12 shows a diagram from Kandel, Schwartz, and Jessel's Principles of Neural Science depicting the suspected components of LTP. We have been encouraged by our results examining the effects of  $\alpha$ -CaMKII on this system, and are interested in applying the gene knock-out technology to other pieces of this picture. So far, we have also knocked out the gene for the neurospecific gamma form of Protein Kinase C. The mutants are currently being analyzed. We also have knocked out one of the subunits of the NMDA receptor. Our hope is that the analysis of these mutant mice will continue to be useful for understanding the learning process.

Finally, I would like to remind you again that the gene knock-out is a technique that is useful beyond the analysis of learning and memory. For instance, we have also produced several mutants by knocking-out various T cell receptor genes. These mutants have been extremely useful for the analysis of the immune system including immunity in the cutaneous system.