Knockouts Shed Light On Learning

Mice missing certain kinase genes lack a process in the brain called long-term potentiation and have difficulty remembering spatial information

You are a mouse tossed by some scientist into a murky pool—you're keeping your head above water, but you just can't seem to remember how to get out. Maybe you've lost your kinase.

That seems to be the case with a novel type of laboratory mouse that made its debut at the Cold Spring Harbor Symposium on The Cell Surface last month—and which seems likely to become a hot new research tool for studying learning and memory. It's a "knockout" mouse, so called because it was grown from an embryo in which a single gene was "knocked out," or inactivated. In this case the missing gene is one that normally codes for a particular kinase enzyme. Although the mouse looks and acts almost completely normal, the results of the knockout are rather specific and profound: It lacks cellular responses in the brain thought to be responsible for learning, and it's not so good at remembering how to get out of that pool of water.

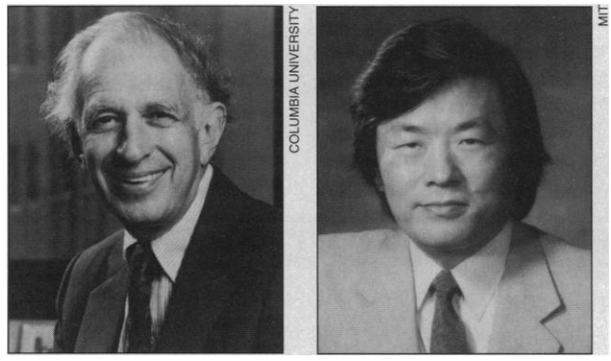
When two independent research teams—one headed by Eric Kandel and Phil Soriano, of the Howard Hughes Medical Institutes at Columbia University and Baylor University, respectively, and the other headed by Susumu Tonegawa of the Howard Hughes Medical Institute at the Massachusetts Institute of Technology—announced these results at the Cold Spring Harbor meeting, they caused quite a stir. The two teams, which used mice with different kinase genes knocked out, have produced the strongest evidence yet for a direct link between the activity of certain kinases, a process in the brain called long-term potentiation, or LTP, and learning. (The Kandel team's results are not yet published; those of the Tonegawa team appear on page 201 and 206 of this issue of *Science*.)

Knockout technique

But it's not just these specific results that have excited neuroscientists: They're just as enthusiastic about the potential impact of the technique itself on the field. Knockout mice have already made their mark in immunology—Tonegawa's area of research for many years, in which he won a Nobel Prize in 1987—and in developmental biology (*Science*, 5 June, p. 1392). But this is their first application to the study of learning and memory in mammals. And the mice could be a welcome new tool in a field where researchers have been forced to rely on drugs and inhibitors, which have the annoying problem of affecting more than just the enzyme or process at which they are targeted.

"It's an incredibly fascinating approach," says Cold Spring Harbor neuroscientist Ron Davis, who heard both talks at last month's meeting. "Genetics is going to be the best way to couple molecules to LTP to organismal behavior." Neuroscientist Paul Greengard of Rockefeller University agrees: "Showing you can analyze a phenomenon as complex as LTP in mammals via this knockout approach is very exciting."

But virtually all who commented on the work were quick to warn that the mice are not without their own problems: "With the knockout, in theory you have specificity," says Robert Malenka, a University of California, San Francisco, neuroscientist who has used inhibitors to study LTP. That's because in the knockout mouse, only a single gene and its protein product are removed. "But the bad news," adds Malenka, "is that you've affected everything during development that is dependent on [that gene]."



The kinase connection. Eric Kandel *(left)* and Susumu Tonegawa led independent groups that took similar routes.

That concern is not lost on the investigators. "It's very complicated moving from a single gene to a behavior," says Kandel. "There are cautions that need to be exercised by everyone along the way....The results should not be oversold."

The Tonegawa and Kandel groups are using the knockout mice to tackle a hotly pursued and difficult question: how the brain stores memories. For nearly two decades, investigators studying this problem have focused their attention on long-term potentiation, a strengthening of the connections between nerve cells in certain parts of the brain associated with memory. They have dwelt on LTP in part because it is one of the few long-term changes known to occur in mammalian brains, says Charles Stevens of the Salk Institute. But, Stevens adds, the direct evidence linking LTP to memory storage in mammals has been "pretty scanty," limited to studies in which rats injected with inhibitors of LTP were unable to pass tests that depend on spatial learning (*Science*, 29 June 1990, p. 1605). Though the studies were elegant, the worry has loomed that the inhibitors may be doing more in the animals' brains than just blocking LTP.

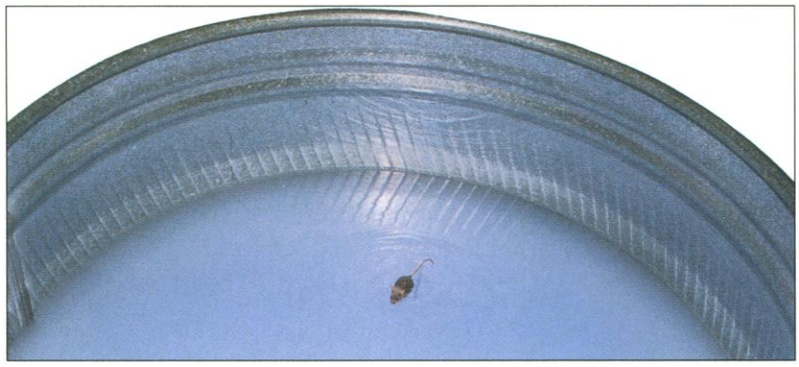
Similar inhibitor worries have plagued those trying to understand LTP at a biochemical level. Researchers know from laboratory studies that when certain nerve cells from the hippocampus and some other brain areas receive multiple signals at the same time, changes occur in the cell. Special receptors called NMDA receptors are activated, and calcium rushes in. The end result is potentiation—the neuron becomes sensitized and will show a bigger response to the next signal that comes along.

How that calcium influx causes the neuron to turn up its response is a mystery, but it probably involves the activation of kinases—enzymes that trigger changes in cells through the simple act of adding phosphate to proteins. Using drugs that block kinase activity, researchers have found several kinases that seem to be players in the LTP process, including alpha calcium/calmodulin kinase II (CAM kinase)—an obvious candidate, since its activity is triggered by calcium, and it is found in high concentrations in the parts of the neuron that receive signals. But how much stock can be placed in these inhibitor studies? "It's debatable the extent to which [any one kinase] is involved in LTP," says Rockefeller's Greengard, whose lab discovered CAM kinase, because "it's not clear that the inhibitors were specific enough."

Enter the mice. Alcino Silva, a postdoc with Tonegawa, decided several years ago that the way to test the role of CAM kinase in learning and LTP was to make and study mice that lack only that one kinase. Silva knew that such mice, if they were deficient in

LTP, would provide an independent way to test the relationship between LTP and spatial learning.

Once he had bred the mice and convinced himself that they were missing the alpha form of the CAM kinase, Silva sent some of the animals to Stevens and postdoc Yanyan Wang at the Salk Institute, who used electrodes to stimulate neurons in slices of their brains, and found that the cells were deficient in LTP. Other mice went to Jeanne Wehner and postdoc Richard Paylor of the Institute for Behavioral Genetics at the University of Colorado, Boulder, who found that the mice performed worse than their normal littermates in a water maze. The test requires them to swim around in murky, opaque water until they can find and climb out onto a hidden escape platform.



Learning disabled. Mice with impaired learning have difficulty using visual cues to locate an escape platform submerged in murky water.

The water test was designed 10 years ago by Richard Morris of the University of Edinburgh, Scotland, specifically to test spatial learning—the type of learning that seems to be controlled by the hippocampus. Normal mice learn to use visual cues from the surroundings to remember where the platform is, but the mice that were missing the CAM kinase were unable to learn to use the spatial cues to find the platform. (Other tests confirmed that their vision was fine, and that they wanted to get out of the water.) That finding linked the enzyme not just to LTP, but to spatial learning itself. "One of the significant things we have shown," says Silva, "is that the alpha calcium/calmodulin kinase II is definitely involved in spatial memory formation."

Meanwhile, Kandel, a leading researcher in the field of learning and memory, had taken a similar approach, with a different kinase, and reached a similar conclusion. Kandel and postdocs Seth Grant and Thomas O'Dell had recently found that inhibitors of tyrosine kinases, an important class of kinases involved in intracellular signaling, block LTP in brain tissue. "We wanted to define biochemically which kinases were involved," says Kandel. So they teamed up with developmental geneticist Soriano and his postdoc Paul Stein to test LTP in knockout mice made by Soriano and Stein that were missing various tyrosine kinases. Mice lacking the kinases Yes and Src had normal LTP, says Kandel, but those missing another kinase called Fyn had deficient LTP.

"Showing you can analyze a phenomenon as complex as LTP via this knockout approach is very exciting."

–Paul Greengard

Kandel says the team hasn't finished with its behavioral studies on the Fyn knockout mice. Their findings so far seem similar to those of the Tonegawa group but with an interesting twist. Soriano has bred the Fyn mutation mice with two genetic backgrounds. On both backgrounds the mice have reduced LTP. However, on one background, the mice are unable to use spatial cues to find the platform, whereas on the second background they seem to learn more normally. That, says Kandel, raises the possibility that some other genes that differ between in the two backgrounds are influencing how the mice learn.

Nevertheless, the experiments, taken together, seem to provide additional evidence linking kinases to LTP, and LTP, in turn, to actual learning. And they have already suggested answers to other nagging questions about learning and memory. For example: Which kinases are truly important in forming LTP? And how important are they? The similar drops in LTP in knockouts of two different kinases support the idea that "a number of proteins, including [several] kinases, play a role in the induction [of LTP]," says Stanford University neuroscientist Howard Schulman, "and that each one provides a certain contribution, and there is some redundancy."

Indeed it seems that neither Fyn nor CAM kinase is needed absolutely, since in both types of knockout mice, LTP was greatly reduced but not eliminated completely. That supports the notion that "there may be no one essential regulatory element," says Mary Kennedy, a California Institute of Technology neuroscientist who studies CAM kinase. "It seems more likely," she adds, "that there is a network of interacting regulatory mechanisms," the sum of which determines the likelihood of LTP.

Intriguing as the findings are, Cold Spring Harbor's Davis warns that the work "is quite preliminary as it stands right now....There may be a developmental defect which underlies the behavior," he adds. "These things are difficult to rule out with the genetic approach." What if, for example, the absence of a kinase causes the brain's wiring to be scrambled during development, and it is that scrambling that causes the mouse's learning defects, rather than the change in LTP?

Both teams acknowledge the risk of indirect developmental defects clouding their results. Stevens takes heart in the fact that the brains of the CAM kinase knockout mice look normal, but he acknowledges that is only to a first approximation. And Kandel notes that his team's mice do show signs of a developmental defect—there seem to be more neurons than normal in certain parts of the hippocampus, the part of the brain under study. "That makes one worry," Kandel says, but he notes that his group has traced hippocampal nerve fibers, and they seem to go to the right places. And both groups point out that the individual nerve cells they are studying seem normal in everything but LTP. "What you could say is that LTP is missing in these animals, and that there is some other abnormality of wiring that causes them not to learn," says Stevens, but "I think that's very unlikely."

Versatile beasts

Both groups have a full agenda for these mice and others they plan to make, from biochemical studies aimed at understanding the effects of the missing kinases to more behavioral studies, and the analysis of other types of memory that may depend on LTP and so also may be missing. The mice may even be useful for studying epilepsy, says James McNamara, director of the epilepsy program at Duke University medical school. McNamara studies kindling, a phenomenon in mice which resembles epilepsy, and says that LTP may be one of the mechanisms underlying kindling. McNamara has begun a collaboration with Tonegawa and Silva. "We're trying to kindle the [knockout mice] to see if one can correlate the development of kindling and the development of LTP."

But Stanford's Schulman says that perhaps the greatest value of the kinase knockout mice may be that they will get neuroscientists thinking about other questions in the field that knockout mice may help to answer. "It often takes a major strike like this one to get lots of people motivated to do similar things on their favorite proteins," he says.

–Marcia Barinaga