Memories Might Be Made of This

Closing in on the biochemistry of learning

By CAROL EZZELL

A blue-and-yellow sea snail sits complacently in its dark test chamber — a plastic trough atop an electronic shaker-table at the bottom of a jury-rigged refrigerator. Suddenly, bright light shines down on the snail and the chamber floor shakes mildly in a simulation of ocean turbulence. The snail instinctively anchors itself in place by tensing the muscular "foot" running along the underside of its body. Seconds later, the cycle of light and shaking repeats, once again prompting the snail to contract its foot.

After 150 such "training" cycles, the snail finds itself in a second refrigerated chamber, now with the eye of a video camera staring at it from below. This time, when bursts of light flash, an interesting thing happens behind the closed refrigerator door: The animal tenses its foot — without being shaken.

"Ordinarily, light alone would never cause that response," says Daniel L. Alkon, chief of neural systems at the National Institute of Neurological Disorders and Stroke (NINDS) in Bethesda, Md. But if the light appears repeatedly just before and during shaking, this snail, called *Hermissenda crassicornis,* eventually learns to contract its foot when light flashes, "just as Pavlov's dog would salivate when the bell occurred, as if the smell of meat were there," Alkon says.

For the past 20 years, Alkon and a number of other researchers have been studying the nervous systems of marine snails — and those of rats and rabbits — in a quest for the molecular mechanisms of memory. Their searches have led them to a molecule called protein kinase C (PKC) in the surface membranes of nerve cells.

PKC exists in all animal cells, where it plays a role in such diverse physiological processes as growth, blood clotting and the action of hormones. The molecule was first discovered in the early 1970s by a Japanese scientist; in 1979, researchers found that it acts by tacking a phosphate group onto specific sites on other molecules. The added phosphate changes the function of those molecules, increasing or decreasing their level of activity.

One of the first direct clues that PKC might underlie learning and memory came in 1986. Joseph Farley of Princeton (N.J.) University observed that injections of PKC, or of a chemical known to activate PKC, excite light-receptor nerve cells in the eyes of *H. crassicornis.* This excitation mimics that induced by the light-and-shake regimen: The nerve cells open pores in their membranes that absorb calcium, and close other pores that expel potassium. Using tiny electrodes to track this process, Farley observed that it reversed the normal negative charge inside the cells.

Six years earlier, Alkon had shown that the electrochemical current in neurons changes as an animal learns. He and Joseph Neary, now at the University of Miami, went on to demonstrate that a protein requiring calcium is involved in learning. Because chemicals like PKC mimic the cellular changes of learning, Alkon and Farley launched separate studies investigating PKC as the agent behind those learning-induced current alterations. They proposed that PKC contributes to learning by somehow closing potassium pores, priming the neurons to react more strongly to a new stimulus.

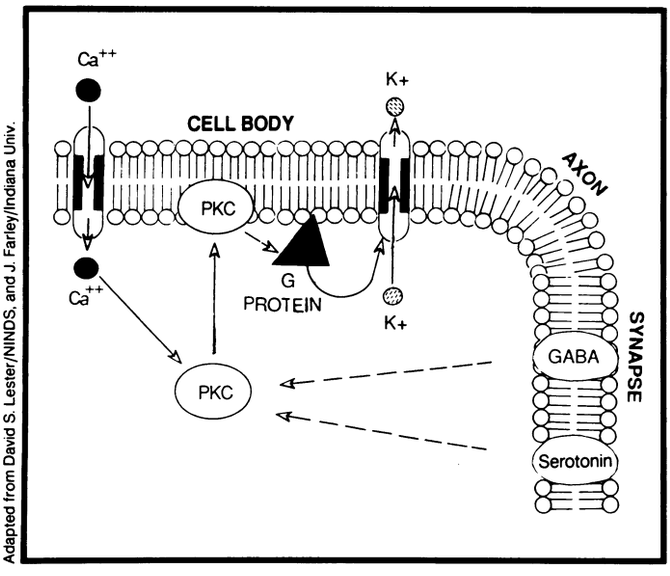
Alkon and Farley reasoned that if a single molecule was responsible for learning and memory, its appearance, disappearance and reappearance should coincide with learning, forgetting and remembering. The molecule might also bring about structural changes in neurons so that they branched to communicate with other neurons in different ways. Moreover, the learning agent would likely prove active in only one region of a neuron at a time, so that one neuron would have the capacity to hold multiple memories.

Over the past 18 months, evidence has piled up in support of the theory that PKC orchestrates neuronal functions necessary for learning and memory. "I have no doubt PKC is central to learning and memory in the models we have looked at," says Alkon, because "we've used so many different measures, and have so many different pieces of evidence that are consistent" with PKC's important role.

Farley, now at Indiana University in Bloomington, agrees. The most compelling evidence, he says, comes from experiments with marine snails.

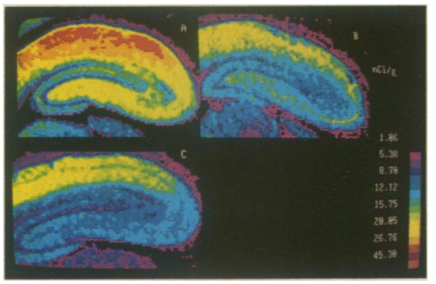
"I think it's reasonably clear you can mimic learning [in these snails] using PKC," Farley says, noting that "inhibitors of PKC will block those changes." Moreover, his data suggest that "ongoing PKC activation is also necessary for the maintenance of memory in *H. crassicornis.*"

Terry J. Crow, who also works with the colorful marine snail, says the link between PKC and learning is gaining acceptance among other neuroscientists.



Adapted from David S. Lester/NINDS, and J. Farley/Indiana Univ.

Hypothetical sequence of events in a sea snail's neuron during learning. Flash of light causes calcium to rush into the neuron. Shaking causes the release of GABA or serotonin, which binds to the neuron membrane. When light and shaking occur together, PKC is activated, linking the two events in memory.



Olds/NINDS

Computer images of PKC distribution in the hippocampus of rabbit brains. One rabbit (top left) was trained to associate a tone with a mild electrical shock. Another (top right) received shocks but heard tones only randomly. The bottom image is from a control rabbit that sat out the experiment. The larger reddish-orange zone in the trained rabbit's brain signifies higher levels of PKC in the nerve cell membranes.

"All of the things we have done here suggest that PKC is sufficient to get the neural changes involved in learning going," says Crow, a neurobiologist at the University of Texas Medical School at Houston.

A year ago, he reported studies demonstrating that a chemical that inhibits protein synthesis also prevents sea snails from remembering a training cycle for more than one hour. Crow's experiments differed from Alkon's because these snails received only one flash of light, immediately followed by an injection of serotonin—a neurotransmitter that Crow believes is also crucial to memory.

"These results indicate that synthesis of proteins during or shortly after training may be a critical step in the formation of long-term memory," write Crow and James Forrester, also from the University of Texas at Houston, in the June 1990 PROCEEDINGS of the NATIONAL ACADEMY OF SCIENCES (Vol.87, No.12).

Crow has also shown that chemicals that block PKC prevent short-term, but not long-term, memory in *H. crassicornis*. He says this suggests PKC may only be important in linking two stimuli together for short periods—perhaps a few hours—and that another mechanism may be responsible for memories lasting days, months or years. In the March 1991 JOURNAL OF NEUROSCIENCE, Crow reports evidence that short-term and long-term memory involve excitation of two different light-receptor cells in the eyes of *H. crassicornis*.

Alkon interprets the findings differently. Even though Crow and Farley have evidence that the neurons feeding signals to the light receptors in the eyes of sea snails contain serotonin, Alkon remains unconvinced that serotonin plays a primary role in learning. He has never found serotonin in cells adjacent to sea-snail light receptors, and he contends that the serotonin detected by Crow and Farley must arise elsewhere in the brain.

Alkon suspects that a different neurotransmitter — called GABA, for gamma-aminobutyric acid — provides half of the one-two punch that causes *H. crassicornis* to link two events such as light and shaking.

Neurophysiologists Juan V. Sanchez-Andres and René Etcheberrigaray, working in Alkon's laboratory, have used two different techniques to measure the effects of GABA on light-receptor cells taken from *H. crassicornis* eyes. They found that GABA causes the receptor cells to close their potassium pores, exciting them in a manner that mimics the effects of learning.

Other researchers in Alkon's lab have detected GABA in the tiny, hair-bearing cells of statocysts — organs used by *H. crassicornis* to sense motions such as shaking. In addition, says Alkon, they have demonstrated that chemicals that inhibit PKC also block the memory-mimicking effects of GABA.

"A lot of people may be excited by this," says Alkon. In previous studies, he says, GABA had been shown to inhibit neurons, not to excite them. He and senior coauthor Louis Matzel will present the new results in BRAIN RESEARCH.

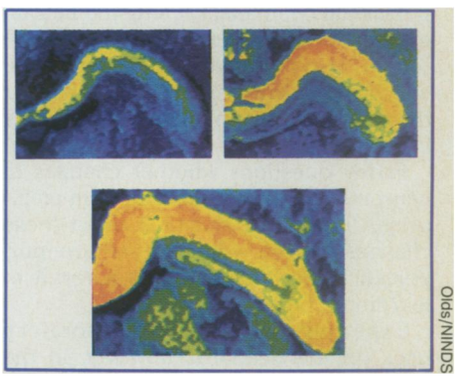
On the basis of his group's GABA findings, Alkon has constructed a theoretical model of the sequence of events occurring in the outer membranes of a snail's light-receptor nerve cells when the animal learns to connect light flashes with being shaken. According to his model, an incoming light flash triggers a series of impulses in the neuron, accompanied by an influx of calcium. Then the shaking causes the snail's statocyst to release GABA, which binds to the neuron. Both events cause PKC stored inside the cell to move to the cell membrane, where the PKC shuts down the potassium channels by acting through one member of a class of membrane agents called G proteins. When the potassium channels close, the neuron becomes more excitable, so that light later produces the same behavioral response as shaking.

Crow and Farley have developed a similar model to explain snail learning, except that it substitutes serotonin for GABA. They believe serotonin acts indirectly upon PKC, which in turn moves into the neuron membrane to shut down its potassium channels. With the potassium channels closed, the neuron becomes trigger-happy and fires after receiving another stimulus — be it light or a shot of serotonin. After the snail has learned, "the same light then gives you a different response" in the form of foot tensing, explains Crow.

Farley challenges Alkon's assertion that a G protein is necessary to help PKC close potassium channels and keep a neuron excited for learning. "In our model, PKC acts on the channels directly. . . . There's no need for a G protein," Farley says.

Crow, Farley and Alkon do agree, however, that PKC is responsible for the increased excitability of neurons that have learned. "The PKC-induced changes in learning last for many days, and sometimes even for weeks," notes Alkon.

But how do the relatively short-term effects of PKC get translated into long-term memories?



SDNIN/SPIO

Before baby rabbits can open their eyes, PKC — denoted by orange tint — resides in two tracks flanking the nerve cell bodies (top left). After their eyes open and the rabbits begin to learn, PKC moves into one track in the nerve cell dendrites (bottom). In the top right image, taken on the day the rabbits opened their eyes, PKC was in flux and did not show up in the membrane staining.

Alkon's team is tracing the longer-term effects of PKC to find out. In the January 1990 PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (Vol.87, No.1), Thomas J. Nelson from Alkon's lab reports that neurons from trained snails contain elevated levels of messenger RNA (mRNA), the chemical intermediary through which DNA makes protein. Alkon and Nelson also showed that the extra mRNA was the result of learning, and not just the experience of being under bright light or being shaken: Snails that experienced random shaking and lighting did not learn and did not show elevated amounts of mRNA.

More recently, Nelson has uncovered evidence that the G protein cited by Alkon exerts control over mRNA. "It turns out that this G protein regulates the turnover of mRNA or the readout of mRNA," Alkon says. "That's very exciting, because it suggests the G protein not only has effects on the [potassium] channels, but also affects the synthesis of

proteins." Nelson and Alkon are now preparing to publish the finding.

What does protein synthesis have to do with learning?

Alkon thinks long-term memory depends upon "hard-wiring" changes that strengthen some connections between neurons while reducing others. These structural changes occur in the dendrites, the branching fingers through which neurons receive incoming electrical impulses. To change their branching pattern, neurons must manufacture new proteins.

In Alkon's scenario, learning activates a neuron's PKC, which in turn activates a G protein. The G protein then closes the potassium channels, keeping the neuron excited for short-term memory. The G protein also regulates protein synthesis, which "hard-wires" the memory over the long term by changing the neuron's branching structure.

Farley questions whether changes in neuron branching build long-term memories. "I'm a little skeptical about these changes in cell volume that are purported to be happening [as a result of learning]," he says.

Experiments at Alkon's laboratory back up this scenario, however. In the February 1990 PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (Vol.87, No.4), Alkon and several co-workers report the results of injecting dye into light-receptor cells of trained and untrained snails. The dye revealed that the neurons of snails trained to associate light and shaking had fewer and more condensed branches than did those of untrained snails, suggesting that learning could reroute a nerve cell's branching.

Such rerouting should be reflected in fluctuations in the shipment of new cell membrane from the body of the cell to the branch tips, Alkon reasoned. Last year, Simon Moshiach from Alkon's lab, together with Nelson and Sanchez-Andres, demonstrated just such a change. With one squirt of G protein extracted from a snail, they slowed the flow of new protein globules along the axon—the long "arm" that transmits outgoing messages—of a large nerve cell taken from a crab.

Although Alkon contends that long-term memory probably requires changes in neuron structure, he also finds evidence that PKC is involved. Working with Matzel, now at Rutgers University in New Brunswick, N.J., he turned up evidence that PKC's effects can persist for weeks after *H. crassicornis* has learned.

Matzel trained the snails with just enough paired cycles of light and shaking that they learned to associate the two stimuli. He did not repeat the cycles over and over to reinforce the animals' memories, however. After about one week, the snails forgot the association: Light alone no longer caused their feet to tense. But when Matzel retrained the animals after waiting two weeks, he found that they could relearn the association after just a handful of trials.

"Even though they had forgotten, they retained some memory, because they relearned much more quickly," Alkon says.

To see if PKC had a hand in this effect, Matzel studied light-receptor cells taken from the eyes of snails that had forgotten their training. If he jolted the cells with a shot of calcium, they clamped down their potassium channels just as they would have if they had learned. But if he also added chemicals that blocked PKC, the potassium channels stayed open. "That's good evidence for long-lasting involvement of PKC in learning," Alkon argues.

With increasing evidence linking PKC to learning and memory in snails, Alkon's team began looking for a similar link in higher animals.

In 1988, Alkon's co-worker Barry Bank, with colleagues from NINDS and Yale University, used a radioactive stain to trace the activation of PKC in rabbits. They found that rabbits trained to associate a particular tone with a mild electrical shock near their eyes eventually learned to drop a protective membrane over their eyes whenever they heard the tone. The stain revealed that the trained rabbits harbored increased levels of PKC in the hippocampus of the brain. Previous studies involving animals and humans with hippocampal injuries had shown that this area of the brain is crucial to maintaining memory for many days.

James L. Olds of NINDS went one step farther by precisely tracing the movement of PKC throughout nerve cells in the rabbit hippocampus. He found that PKC levels increased in the cell bodies of the neurons one day after the rabbits had undergone training, and that the PKC moved to the dendrite membranes three days after training. These results support the finding in sea snails that after learning, PKC moves from the body of a nerve cell and into its membrane.

Working with David Olton of Johns Hopkins University in Baltimore, Olds has also trained rats swimming through a tank of water to distinguish unstable platforms from those stable enough to allow the animals to climb out and dry off. Rats given cues about which platforms were stable had lower hippocampal levels of PKC than did rats that learned the difference through trial and error, the researchers report in the November 1990 JOURNAL OF NEUROSCIENCE.

Recently, Olds and Sanchez-Andres have begun to investigate the PKC changes that occur early in an animal's development. By staining brain slices from baby rabbits not yet old enough to have opened their eyes, they have discovered that most of the PKC in the hippocampal neurons resides in two tracks flanking the cell body. In contrast, the hippocampal neurons of older rabbits have a single, diffuse track of PKC situated around the dendrites. When the baby rabbits open their eyes and begin exploring their environment at about 10 days of age, their PKC moves into the dendrites, perhaps programming their memories, Olds says.

He speculates that very young rabbits hoard PKC near the cell bodies because they haven't yet had a chance to learn anything about their surroundings. Then, when they open their eyes, their brains are flooded with new information worth remembering—a change that kicks the PKC memory pathway into action.

Olds notes that many other researchers have now implicated PKC in the development of neurons. "I'm trying to figure out the role of PKC in learning and postnatal development," he says. "I'm convinced that similar molecular mechanisms are involved."

Others are investigating PKC's involvement in human disorders, such as Alzheimer's disease, that erode both memory and learning capacity. A group led by Tsunao Saitoh at the University of California, San Diego, reports in the July 1990 JOURNAL OF NEUROSCIENCE that the brains of 11 deceased Alzheimer's victims contained only half as much PKC as the brains of seven people who had died of other causes. The results confirm previous findings by Saitoh's team (based on a less specific method for measuring PKC), which had hinted that PKC levels drop in Alzheimer's patients.

Saitoh and his colleagues ruled out the possibility that the reductions in PKC resulted from the overall death of neurons in Alzheimer's patients by measuring the PKC levels of connective-tissue cells in the autopsied brains. These cells also showed lower-than-expected amounts of PKC, they found.

Researchers who are studying the humble sea snail find encouragement in the discovery that PKC is linked to human learning and memory. It proves they aren't chasing down blind alleys in their search for the molecular Holy Grail of memory. At the same time, they're cautious about attributing the entire orchestration of human learning to PKC.

"We don't know about other molecules, such as G proteins, that are likely to be involved in learning [in humans and other higher animals]," says Alkon. "Molecular pathways are very complex, and there are undoubtedly hosts of other actors in this drama that we haven't yet met."

Adds Farley, "There's little doubt that PKC is involved in an important way, but it's certainly not the entire story." □