AMAZING PAPERS IN NEUROSCIENCE
Discovering Memory: Using Sea Slugs to Teach Learning and Memory

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Research on the sea slug *Aplysia californica* has played a key role in unraveling the molecular mechanisms for learning and memory. In this system, synapses exhibiting long-term potentiation provide an ideal experimental platform for uncovering conserved principles. This review will discuss a 1997 study published in the journal *Cell* which explored the means by which synapse-specific long-term potentiation occurs and its reliance on local protein synthesis. This study, conducted by Kelsey Martin and colleagues working in the Kandel laboratory, also explored synaptic capture: the mechanism by which a stimulated synapse recruits proteins from another, such that both undergo long-term potentiation. The authors discovered that synaptic capture does not require local protein synthesis, which led to further research on this mechanism. This study introduces undergraduates to a variety of research methods. Additionally, educators may use this paper as an introduction to the body of work produced by the Kandel laboratory and the field of learning and memory more generally. Advanced analyses of this research by upper level undergraduates may provide insights into competing theories for cellular mechanisms of long-term memory, presenting the opportunity to discuss disagreements within the scientific community.

Key words: Aplysia californica*; long term potentiation (LTP); synaptic capture; learning and memory; controversy*

The brain's ability to form memories has profound effects on animal life. Memories allow animals to use their past experiences to respond to their present and thus adapt to their environment. Neuroscience researchers have searched for the mechanisms by which the brain learns and remembers to better understand this crucial function. In 1949, Donald Hebb postulated in his book *The Organization of Behavior* that learning occurs at the synapse and that coordinated activity of pre- and post-synaptic neurons would lead to increased strength and efficacy of the synapse, thus mediating learning (Hebb, 1949; Siegelbaum and Kandel, 2013). In 1973, research by Bliss and Lømo appeared to support this theory by demonstrating that long-term potentiation (LTP) – a prolonged increase in amplitude of excitatory post-synaptic potentials (EPSPs) – increases synaptic efficacy and may play a role in long-term memory (LTM) formation (Bliss and Lømo, 1973). These events helped launch the study of molecular mechanisms underlying memory and laid the foundation for research in the laboratory of Eric Kandel on the sea slug *Aplysia californica* (Asok et al., 2019). This includes a 1997 paper published in the journal *Cell* by Kelsey Martin and colleagues (Martin et al., 1997). Here I review this paper and highlight its unique strengths as a vehicle for teaching neuroscience.

Kandel began his groundbreaking research on *Aplysia* in the 1960s. He chose this organism for numerous reasons: *Aplysia* has relatively few cells in its nervous system (~20,000); its cells are relatively large and thus easy to manipulate and record; and its nervous system contains just nine ganglia, which simplifies the identification of specific loci of neural modifications. Despite the simplicity of this organism, Kandel found that it had significant learning capabilities. For example, Kandel observed the gill-withdrawal reflex, in which the slug will protectively withdraw its gill when its adjacent water siphon is touched. If the siphon is touched repetitively with an innocuous stimulus, the *Aplysia* habituates to the stimulus over time, progressively diminishing the reflex, and thus demonstrating a form of short-term memory (STM). If the siphon receives a shock after a period of habituation, the *Aplysia* becomes sensitized again to the innocuous stimulus and more vigorously withdraws its gill. A slug receiving one shock will exhibit an exaggerated reflex for several minutes, while a slug receiving multiple shocks will exhibit sensitization lasting for several days, demonstrating a form of LTM (Kandel, 2006). Kandel and his colleagues discovered that this form of LTM was facilitated by LTP at sensory-motor synapses, which was isolated and studied in further experiments (Kandel, 2012). Due to its simple neuroanatomy and easily-observed learning behaviors, *Aplysia* is a valuable model organism that allowed researchers to unravel the molecular basis of learning using targeted, reductionist strategies to delineate the pathways by which LTP occurs.

Aplysia researchers in the Kandel laboratory studied two forms of synaptic modulation: short-term potentiation (STP) and LTP. Short-term potentiation produces an elevation of EPSP amplitudes that lasts for minutes, while LTP produces an elevation that lasts for days. Kandel discovered that STP only requires increased glutamate release while LTP requires transcription and translation to induce synaptic growth (Asok et al., 2019). For STP, a short burst of serotonin (5-HT) release by a modulating neuron onto a sensory neuron increases post-synaptic concentration of cyclic adenosine monophosphate (cAMP). This intracellular second messenger binds to and activates protein kinase A (PKA), which transiently increases the release of glutamate into the sensory-motor synapse after the brief application of 5-HT. For LTP, when the sensory neuron axon receives prolonged 5-HT stimulation, PKA recruits p42 mitogen-activated protein kinase (MAPK). PKA activates cAMP

response element-binding protein 1 (CREB-1), which facilitates gene transcription, while MAPK suppresses CREB-2, which inhibits transcription. Activation of CREB-1 and inhibition of CREB-2 leads to the translation of somatostatin and other gene products that cause the growth of axonal branches following synaptic stimulation (Kandel, 2012).

The plasticity-related proteins generated from the LTP pathway can also be redirected to synapses that receive only a brief tetanization of 5-HT in a process called synaptic capture. This phenomenon was first proposed as synaptic tagging by Frey and Morris in early 1997 (Frey and Morris, 1997). Synaptic capture occurs when multiple synapses are stimulated within the same neuron, one of which receives prolonged tetanization that triggers LTP. The second synapse, which receives a brief tetanization, becomes marked and can capture LTP-inducing proteins such that both stimulated synapses undergo LTP (Redondo and Morris 2011).

With an understanding of the LTP pathway in hand, Kelsey Martin and colleagues (1997) asked why and how LTP-induced growth occurs at some synapses in a neuron, but not others. Additionally, they questioned where translation occurs within a neuron in order to evoke synaptic growth. The results of this study demonstrated the importance of local protein synthesis in synapse-specific LTP and provided evidence that synaptic capture did not require local protein synthesis. This study stands out from the body of work produced by the Kandel lab for its usefulness in teaching. It features meticulous research methods and diverse research techniques, and it provides students and educators an entry point into Kandel's research history and the field of learning and memory.

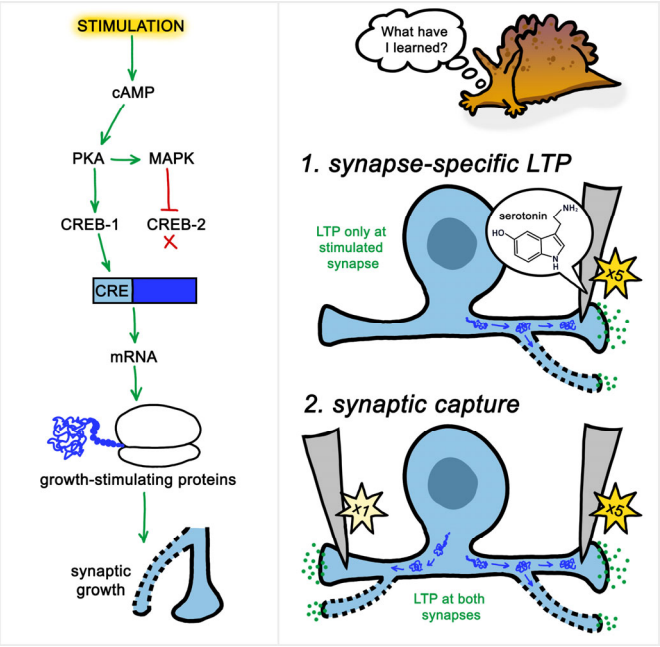

Figure 1. Schematic illustrating *(left)* the molecular pathway for LTP and *(right)* the differences between synapse-specific LTP and synaptic capture. *(Left)* Prolonged 5-HT stimulation at a synapse causes a cascade of cellular effects initiated by cAMP that eventually induces gene transcription and synaptic growth. *(Right)* For synapse-specific LTP, a single synapse is stimulated with prolonged 5-HT exposure (x5), which causes synaptic growth only at the stimulated synapse. For synaptic capture, two synapses on the same neuron are stimulated, one with brief 5-HT stimulation (x1) and the other with prolonged 5-HT stimulation, and both synapses experience LTP-induced synaptic growth.

RESEARCH SUMMARY

Martin and colleagues (1997) experimented on a cell culture model of a sensory-motor neuron synapse in the form of a bifurcated sensory neuron, a pseudounipolar cell that synapses onto two spatially-separated motor neurons. They had two primary goals: to demonstrate that the *in vitro* synapse would recapitulate its *in vivo* counterpart, and to target the pathway for synaptic capture. In pursuit of their first goal, Martin et al. provided evidence that their isolated sensory neuron and its synapses experienced localized STP and LTP, just like the *in vivo* neuron. Two motor neurons and one bifurcated sensory neuron were extracted from *Aplysia* and placed in culture. Using a microelectrode, the authors recorded EPSPs in the two motor neurons and found that the sensory-motor synapse was functional in culture. Bath-application of five brief 5-HT pulses to the entire sensory neuron resulted in elevated EPSPs in both motor neurons 24 hours after application, indicating that the synapses underwent LTP. Five pulses of 5-HT to an individual synapse induced LTP at the treated synapse and not the other. One pulse of 5-HT to an individual synapse produced elevated EPSPs in the postsynaptic motor neuron for just 10 minutes after treatment, indicating STP occurred at one synapse and not the other.

Second, Martin et al. (1997) demonstrated that the molecular mechanisms of LTP still functioned as expected in the *in vitro* model. Bath-application of actinomycin D – a transcription inhibitor – blocked LTP, as did the injection of anti-CREB antibodies into the cell body. The authors next injected sensory neurons with carboxyfluorescein to visualize synaptic growth by light microscopy. Five pulses of 5-HT resulting in LTP caused increased axonal branching onto the motor neuron, while one pulse did not, demonstrating the accompaniment of structural changes with the physiological changes of LTP *in vitro*.

Third, in pursuit of their second goal for the study, Martin et al. (1997) targeted the translation machinery at the synapse to test whether synapse-specific LTP depended on local protein synthesis. Local application of emetine – a protein synthesis inhibitor – at the synapse receiving five 5-HT pulses blocked synapse-specific LTP. The emetine applied at one synapse did not diffuse to the nucleus, nor did it block LTP at another synapse.

Finally, Martin et al. tested the requirement of local protein synthesis for synaptic capture. Interestingly, when one synapse received five 5-HT pulses, and another synapse immediately received one 5-HT pulse, both synapses experienced LTP. The single 5-HT pulse appeared to mark the second synapse so it could capture gene products necessary for LTP. Martin et al. recognized this as the synaptic capture phenomenon proposed by Frey and Morris (1997). Application of emetine on the second synapse receiving one 5-HT pulse did not block LTP at that

synapse. This demonstrates that synaptic capture does not rely on local protein synthesis. However, emetine application at the first branch receiving five 5-HT pulses effectively blocked LTP at the second synapse. This implied that a retrograde signal traveling from the initial synapse to the nucleus and second synapse may require protein synthesis.

The findings of this study demonstrated the importance of local protein synthesis for synapse-specific LTP and raised some additional questions about synaptic capture. The authors theorized that synapse-specific LTP relies on two separate events: a retrograde signal to the nucleus to induce transcription, and synaptic marking to direct the products of gene expression to the stimulated synapse. For synapse-specific LTP, the synapse appeared to rely more heavily on local protein synthesis than the retrograde signal to the nucleus. For synaptic capture, in contrast, tagged synapses relied more on retrograde signals than local protein synthesis. Although the exact mechanism for this was not fully understood in 1997, a later study found that PKA recruited by synaptic tagging plays a significant role in redirecting gene products to another synapse, further suggesting the importance of retrograde signals from the PKA-involved pathway at the first synapse (Kandel and Siegelbaum, 2013). The study by Martin and colleagues thus served as a fundamental steppingstone in our understanding of the molecular mechanisms of LTM, and it spurred additional research that furthered our understanding of these processes.

AN ALTERNATIVE THEORY

Researchers studying the molecular mechanisms for memory have generally accepted synaptic growth, as proposed by Kandel and his colleagues, as the primary mechanism for cellular-level LTM. However, there exists a growing body of evidence that changes in intrinsic excitability via differential expression of ion channels also play a significant role in cellular correlates of learning (Debanne et al., 2019). This hypothesis was promoted by Daniel Alkon, Kandel's contemporary, who similarly studied molecular mechanisms of LTM but with a focus on intrinsic cellular excitability. His research on the sea slug *Hermissenda crassicornis* demonstrated that changes in certain potassium currents left a lasting effect on intrinsic excitability that paralleled LTP in the *Aplysia* (Alkon, 1984).

Despite the relevance of Alkon's work, Kandel's research became more widely acknowledged and lauded. The reasons for this appear to be multifactorial. Kandel's greater renown, the earlier start to his research, and his relatively greater social profile within the scientific community compared with Alkon may have all contributed (Allport, 1986). As a result, the theory of synaptic growth and LTP is prevalent in undergraduate neuroscience teaching, while the equally viable and contributing theory of intrinsic excitability as a mechanism for memory is often neglected, despite the fact that Alkon's findings could supplement our understanding of LTM mechanisms. This represents a gap in students' understanding of learning and memory and therefore an opportunity for educators. Students can learn to study the history of science with a critical eye to identify hidden biases within a field of study, such as the omission of Alkon's work from summaries of cellular mechanisms of LTM.

TEACHING VALUE

The study conducted by Martin et al. (1997) stands out as a useful teaching tool for the quality and diversity of its research methods and the impact of its results on the field of learning and memory. Martin and colleagues use research methods that are detailed and exhaustive. In the first half of their study, they meticulously demonstrate the viability and generalizability of their *in vitro* model of the sensory-motor neuron synapse, setting an example for a carefully controlled experiment. The study also deploys a broad range of research techniques, including cell culture, electrophysiology, immunocytochemistry, and microscopy. Undergraduates can examine the methods used in this study as an example of the effective use of diverse methodologies. Furthermore, *Aplysia* is presented as a useful model organism for studying learning and memory. Studying the use of a sea slug expands students' understanding of the power of invertebrate animal models in Neuroscience.

Educators can present the results of the Martin et al. study with a narrow focus on the achievements within the study or from a broad perspective on the study's impact on subsequent research. With a narrow focus on the study itself, Martin and colleagues found important results, which they display in an easily understood format in their figures. Undergraduates will find the figures easy to comprehend in the context of the writing, which will help them to better understand the results. Additionally, undergraduates will find the writing style of this paper highly accessible, as it uses plain language to explain the research. In a broader sense, the results of this study catalyzed research on the molecular mechanisms of LTM, which makes it useful to educators to introduce the field of learning and memory to students. This article can ease students into understanding the large body of Kandel's research because of its numerous references to his previous work and its contributions to subsequent studies. For example, the findings of this study encouraged Kandel's lab to further investigate the involvement of proteins in synapse-specific LTP and discover the role of functional prions in maintaining LTP over time (Asok et al., 2019). Kandel's significant contributions to the field of learning and memory make him and his research ideal initiators for studying this extensive field of neuroscience research.

CLASSROOM AUDIENCE

Due to the complexity of the background information and techniques used throughout the study, this paper would be most useful in upper-level neuroscience courses. Students reading this paper would need a thorough understanding of foundational neuroscience topics such as the structural elements of a neuron, synaptic transmission, and second messenger pathways in order to understand the research and its implications. This paper would be suitable for a third- or fourth-year course focused specifically on the field of learning and memory or in a class that teaches different

research techniques. Students in these courses may have a more focused interest in pursuing a career in research, so an emphasis on research methods and strategies for developing research may benefit these audiences.

A discussion about differing scientific theories such as those from Kandel and Alkon may be instructive to students who read scientific papers on a regular basis. Teachers can encourage students to read articles or summaries of a researcher's work with a critical eye for how the data are interpreted, which may help them notice gaps in scientific theories. A single source of research (such as Kandel's) may not paint a complete picture, and another contributing body of research (Alkon's) may complement a student's understanding of a topic (such as LTM). Furthermore, Martin and colleagues' 1997 paper is an example of a meticulous study that will help students distinguish papers with strong experimental designs (e.g., well-controlled, use well-established methods to support or refute a hypothesis). It is important for students to recognize the strengths and weaknesses of published research to help them critically analyze and understand scientific findings.

ADDITIONAL RESOURCES

In a class focused on the field of learning and memory research, teachers using this paper could present the evidence for both synaptic growth and changes in intrinsic excitability as potential methods for molecular learning. Debanne, Inglebert, and Russier's review references historical and contemporary research studying intrinsic excitability as a mechanism for LTM (2019). In the case of Kandel and Alkon, this may present a unique learning opportunity for students to understand factors external to the results of a study that could contribute to societal acceptance of scientific theories. Teachers could pose discussion questions about the topic such as the following: To what degree do non-empirical factors influence the acceptance of a scientific theory? How do people without expert knowledge of a field decide which facts to accept as truth? How does one critically read a research article or learn about a field of research? Learning about the history of Kandel's and Alkon's controversy would encourage students to think critically about the information presented to them and stay vigilant for hidden biases toward one scientific idea versus another.

Students studying the Martin et al. (1997) paper could benefit from resources providing additional, detailed information about the molecular pathways for LTP. For example, Chapter 66 in *Principles of Neural Science* provides detailed explanations of the pathways Kandel studied (Kandel and Siegelbaum, 2013). Kandel himself has authored and co-authored several review papers which outline these pathways and expand upon the field of research, such as a review in 2012 and a review with Asok and colleagues in 2019. Chapter 20 in *From Molecules to Networks* provides a broader overview of learning and memory research and may be helpful to introduce the subject before discussing the details of LTP (Byrne et al. 2014). Supplementary to these readings, teachers could encourage students to visualize the molecular pathways, as exemplified in Figure 1, to solidify their understanding. For additional background on Kandel's history, students can read excerpts from his autobiography, *In Search of Memory* (Kandel, 2006). Overall, Martin and colleagues' 1997 study provides students with numerous avenues to learn about and understand the molecular mechanisms of learning and memory.

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