

## Memory and Synapses I: Aplysia

I want to spend the next few lectures on the subject of memory in which an understanding of synaptic transmission has contributed to a better understanding of the cellular and molecular changes that are responsible for changes in behavior in a number of organisms, including the marine mollusc, *Aplysia californica*, the fruit fly, *Drosophila melanogaster*, and mammals, mostly mice and rats. Over 50 years ago a psychologist named Donald Hebb proposed that learning was mediated by changes in synaptic strength or “efficacy”. What he meant was that as an animal learned to do something, some synapses became stronger; that is, that particular synapses in the neuronal pathways that were responsible for the learned behavior gave a larger postsynaptic response to the stimulation of the presynaptic neuron than they did before “learning”. He thought that what was required for this strengthening of particular synapses was coordinated activity of the pre- and postsynaptic neurons; if the activity was “uncoordinated”, then the synapse would lose efficacy. He didn’t specify whether this increased efficacy was caused presynaptically (such as by increased release of neurotransmitter) or postsynaptically (such as by increased number of receptors for transmitter). While this basic hypothesis was very appealing from the first, it is only within recent years that it has been possible to do experiments to test whether it is true. In the next couple of classes I want to discuss two heavily studied systems in which there is now good evidence that Hebb was essentially correct, and to talk about the details of how efficacy changes and what similarities and differences have been found in invertebrate and mammalian systems.

*Aplysia*, also called the sea slug or sea hare, has long been a favorite organism of neurobiologists because it has a relatively simple nervous system, containing about  $10^5$  neurons, about 1 millionth as many as humans, and it has “huge” (up to 1 mm diameter) neurons that are easy to put a microelectrode into and record from. Like most invertebrates, *Aplysia* doesn’t have a discrete brain, per se, but its nervous system consists of several groups of neuronal cell bodies, called ganglia, connected by bundles of axons called connectives. (See Fig. 24.1)

Each ganglion controls the sensory and motor behavior of a nearby region of the animal, and the connectives between the ganglia allow signals to be passed back and forth in order for the

different parts of the animal to coordinate their activities. The abdominal ganglion regulates several functions including digestion and reproduction, but the most heavily studied function of this ganglion is the movement of the gill and siphon, organs involved in respiration. On its dorsal surface, *Aplysia* has a flap of muscle called the mantle, which the gill and siphon normally stick out from under. If the animal is touched or threatened, the gill and siphon are withdrawn back under the mantle for protection--the "gill withdrawal reflex". Relatively few cells are involved in this simple, reproducible behavior, and it has been possible for scientists to identify them all (they think) because these cells are large, differently pigmented, and stereotyped in their location and connections. By stereotyped, I mean that the neurons that have the same function in different animals are in the same location in the ganglion and have the same pigmentation (black, orange, none, etc.; See Fig. 24.1B).

While it may be interesting that the *Aplysia* withdraws its gill when it is touched, what is more interesting is that it changes this response depending upon its previous experience. For example, Eric Kandel and his coworkers showed many years ago that if they gently squirted water from a Water Pik onto the mantle, the *Aplysia* would withdraw its gill and siphon. If they repeated this gentle squirt once or twice a minute, the withdrawal response would get weaker over time and eventually cease; the *Aplysia* learned to "ignore" the stimulus (squirt of water) because it was not harmful (Fig. 24.1C). Psychologists call this process habituation--a reduced response to a repeated benign stimulus. So *Aplysia* can habituate their gill withdrawal reflex. Further, Kandel showed that if he first got the *Aplysia* accustomed to the Water Pik, and so the withdrawal reflex had disappeared, and then shocked the tail of the *Aplysia* with a mild electrical jolt, the *Aplysia* instantly withdrew its gill the next time it was squirted with water (Fig 24.1C, Trial 14). In other words, the noxious shock on the tail, aroused the *Aplysia* so that now it responded to a stimulus--the Water Pik--that it had been ignoring. This process is called "sensitization" by psychologists. Showing that even a relatively simple beast like *Aplysia* was capable of a primitive form of learning allowed Kandel now to ask: what changes in the neurons and synapses of *Aplysia* when it changes its behavior?

The gill withdrawal reflex seems to depend on about 100 neurons in the abdominal ganglion. Roughly 50 are sensory neurons that respond to touch, whose nerve endings are located in the skin around the gill, siphon and mantle; about 40 are motor neurons that stimulate the muscles that retract the gill. The rest are interneurons--i.e., neurons that are contained entirely within the ganglion and don't make connections with the periphery. For example, interneurons might be innervated by sensory neurons and make synapses on motor neurons. A simplified "wiring diagram" of this neuronal circuit is shown in Fig. 24.2A.

You should read this diagram as follows. Stimulation of the skin excites the sensory neuron to fire an AP, which activates the motor neuron two ways--both directly, through a monosynaptic connection, and indirectly through a polysynaptic connection involving interneurons. If the motor neurons comes to threshold, it fires an AP, causing the gill muscle to contract and withdraw the gill. One can put electrodes into the sensory neurons (cell bodies), the interneurons, and the motor neurons either for the purpose of recording the Vm or in order to pass current into the neurons or to inject various biologically active compounds (see below).

What Kandel et al. first did was to show that during habituation the size of the epsp caused by the sensory neuron on the motor neuron decreased in amplitude. Conversely, they found that sensitization causes a big increase in the size of the sensory to motor neuron epsp (see Fig. 24.2B and C). What mechanisms could account for this? What experiments could you do to test those hypotheses?

- 1) Changes in the sensitivity of the postsynaptic cell (e.g., change in receptor number or function).
- 2.) More or less transmitter released/quantum
- 3.) More or fewer quanta released/stimulus.

How could you distinguish the possibilities? It turned out that they could actually get habituation to occur in a fragment of an animal consisting only of the mantle, gill, skin and ganglion with all the connections intact. This allowed Kandel et al. to record from and manipulate the neurons before and during the behavioral changes.

For Habituation, they showed that:

1). *Iontophoresis* of transmitter after habituation gave the same postsynaptic response as before habituation, implying that the sensitivity of the postsynaptic cell didn't change.

2). They conducted quantal analysis to analyze the size of the miniature excitatory postsynaptic potentials. The mepsp size didn't change during habituation.

But--the epsp size got smaller, implying that fewer quanta were released when the sensory neuron was stimulated.

Why? They believe, based on a number of experiments, that there is less  $\text{Ca}^{2+}$  entering the presynaptic nerve terminal (the sensory neuron) as a result of habituation. What is the evidence that a change in  $\text{Ca}^{2+}$  entry could be responsible for the strength of the synapse and how could the entry of  $\text{Ca}^{2+}$  into the nerve terminal be regulated? It turned out to be easier to study this question during sensitization, rather than during habituation.

In sensitization, when a tail shock increases the strength of the response they found that the epsp between the sensory and motor neurons suddenly got larger, because the number of quanta released per stimulus increased greatly (Fig. 24.2C). How does a tail shock affect what goes on in the abdominal ganglion? It turns out that the sensory neurons in the tail make synapses in other ganglia onto "facilitating (or modulatory) interneurons" that send their axons through the connectives up to the abdominal ganglion where they make synapses on the nerve endings of the sensory neurons coming from the gill (at both sensory to motor and sensory to excitatory interneuronal synapses) (refer to Fig. 24.2A). Shocking the head or tail activates the facilitating interneurons, which do not synapse directly onto the motor neurons or excitatory interneurons, and thus have no direct effect. Rather they somehow interact with the sensory nerve terminals and increase the amount of transmitter that the sensory nerve terminal releases/stimulus. This change can occur after only 1 shock.

Understanding of this phenomenon was greatly aided when Kandel et al identified 5-HT (serotonin) as the transmitter released by the facilitating interneuron. For example, "bath application" of 5-HT to the preparation enhanced the epsp in the motor neurons (in bath application, they basically just dumped serotonin into the dish of physiological saline, as we often do in lab). In addition they found that serotonin antagonists prevented sensitization, and radioactive

$^3\text{H}$  5-HT was taken up into vesicles in the nerve terminals of facilitating interneurons (FI). This pretty clearly shows that 5-HT is the transmitter from FI to sensory neurons in the abdominal ganglia. Knowing that 5-HT is the transmitter from FI to sensory nerve terminals, one could ask: how does the 5-HT affect transmitter release? They suspected that there was some biochemical change in the nerve terminal because the sensitization lasted a relatively long time--at least several minutes, and under some circumstances (such as when the animal was sensitized several times a day for several days, then tested to see how long sensitization remained in place), the effect could last for weeks. That implies, something more than the temporary opening of an ion channel was going on. Since 5-HT is known to activate several second messenger systems, Kandel et al. tested to see if any were affected at this synapse and found that 5-HT released by the facilitating interneuron increased cAMP levels in the sensory neurons, *and*, more important, they showed that the increased levels of cAMP were responsible for the increased release of transmitter.

Evidence: 1) Stimulation of FI increases cAMP levels in a single sensory neuron 3-4 times, as does bathing the sensory neuron in 5-HT.

2) Application of a membrane permeable analog of cAMP (dibutyryl cAMP) to sensory neurons increased the epsp.

3). Intracellular injection of cAMP into the sensory neurons increases the epsp.

4). Intracellular injection of the catalytic subunit of protein kinase A (PKA) increases the epsp.

These results all indicate that increased cAMP in the sensory neuron activates PKA and that somehow active PKA increases transmitter release.

The question is: How?

Although it was not possible to do voltage clamp experiments on sensory nerve terminals, it was possible to do that on the cell body (and to hope that the nerve terminal was also clamped).

The voltage clamp experiments indicated that sensitization increased an inward  $\text{Ca}^{2+}$  current into the sensory neurons. How? Maybe a  $\text{Ca}^{2+}$  channel is being phosphorylated. To test they blocked  $I_{\text{Na}}$  with TTX and  $I_{\text{K}}$  with TEA, so only  $I_{\text{Ca}}$  remained. When they did this, they saw no difference of  $I_{\text{Ca}}$  between normal and sensitized neurons. So then they blocked  $I_{\text{K}}$  with TEA and  $I_{\text{Ca}}$

with  $\text{Co}^{2+}$  and saw no change in  $I_{\text{Na}}$ . Finally, when they blocked  $I_{\text{Na}}$  with TTX and  $I_{\text{Ca}}$  with  $\text{Co}^{2+}$ , they discovered that  $I_{\text{K}}$  was reduced in sensitized neurons compared to normal neurons. Now what will be the effect on the AP if you reduce  $I_{\text{K}}$ ? It will be prolonged because the efflux of  $\text{K}^+$  is partly responsible (along with inactivation of  $\text{Na}^+$  channels) for repolarizing the membrane potential. And one effect of a prolonged AP will be increased entry of  $\text{Ca}^{2+}$  into the cells through the voltage dependent  $\text{Ca}^{2+}$  channels, which in turn should increase the release of transmitter by the sensory neuron.

Kandel et al. have found that there are at least 5 kinds of  $\text{K}^+$  channels in these sensory neurons. One is a  $\text{Ca}^{2+}$  modulated  $\text{K}^+$  channel, one is the standard Hodgkin-Huxley voltage gated  $\text{K}^+$  channel, a third is a ligand gated channel that's activated by ACh, and the other two have their conductance affected by serotonin. One of these two is called the  $\text{K}_s$ , serotonin-sensitive  $\text{K}^+$  channel and the other causes a transient  $\text{K}^+$  current and is called the  $\text{K}_v$  channel. Both seem involved in the normal repolarization of the action potential and both have their conductance reduced in the presence of 5-HT. The larger, and more important, is the  $\text{K}_s$  channel, which accounts for about 2/3 of the increase in  $\text{Ca}^{2+}$  entry into sensitized neurons.

The  $\text{K}_s$  channel is unique in several ways; one is that it is a voltage-gated channel that is modulated by a transmitter. It's actually modulated by two transmitters, because Kandel et al. also discovered inhibitory interneurons that affect the sensory to motor synaptic transmission (and which aren't shown in the schematic "wiring diagram" in Fig. 24.2A). Activation of these inhibitory interneurons once again decreases the size of the epsp on the motoneuron. Now they showed that the transmitter used by the inhibitory neurons is a peptide (FMRFamide; FMRF stands for the amino acid sequence of the peptide, and the carboxy terminal carboxy group has an amide group attached). They repeated the same kinds of expts with FMRFamide that they did with 5 HT and showed:

- 1). FMRFamide is present in inhibitory neurons.
- 2). FMRFamide applied directly to the sensory neurons reduces epsp, just like stimulating the inhibitory interneuron would do.

- 3). FMRFamide action on the sensory neuron causes a decrease in the entry of  $\text{Ca}^{2+}$  into the nerve terminal during an AP.
- 4) FMRFamide causes an increase in the  $\text{K}_s$  current (i.e., reactivates it).
- 5). FMRFamide does not affect cAMP levels in the sensory neuron. FMRFamide does increase the levels of arachidonic acid in the sensory neurons.

How can one make sense out of all these results? Kandel's working model is shown in Fig. 24.3. The facilitatory interneurons activate a G protein-coupled receptor for 5-HT, which in turn activates adenylyl cyclase. This causes an increase in cAMP, which activates PKA, which in turn phosphorylates the  $\text{K}_s$  channel, decreasing its conductance. This then prolongs the action potential in the nerve terminal, allowing greater influx of  $\text{Ca}^{2+}$  through voltage dependent  $\text{Ca}^{2+}$  channels, and because transmitter release is proportional to calcium entry, this will cause a greater amount of transmitter release. If the inhibitory interneurons are also activated, they release FMRFamide, which binds to different receptors that activate arachidonic acid as a second messenger. This probably turns on protein phosphatases that remove phosphate groups from the  $\text{K}_s$  and  $\text{K}_v$  channels, and restore the original condition, which reduces  $\text{Ca}^{2+}$  entry and transmitter release; in other words this has the opposite effect on the sensory nerve terminal as does the facilitatory interneuron.

More recently Kandel has shown that these alterations can cause long term as well as short term changes in these synapses. It's clear that long term changes require protein synthesis, because the learning that lasts more than a few hours depends on protein synthesis (it's blocked by inhibitors of protein synthesis.) It's known that in many systems, not only does cAMP activate PKA, but it can also bind to Cyclic AMP Response Element Binding proteins (CREB) proteins. CREB proteins are proteins that are transcription regulators in the nucleus (look back to Fig. 7.11). They bind cAMP in the cytoplasm, move into the nucleus, and bind to regions on DNA that control the expression of various genes. These cAMP stimulated genes all contain a common sequence of nucleotides in their transcriptional control region that is called the cAMP response element or CRE. So the idea is that stimulation of cAMP levels in the cell by 5-HT not only leads to phosphorylation and dephosphorylation in the synapse, but also to gene transcription and protein synthesis in the

cell body. James Schwartz et al. have proposed that one of the proteins that gets synthesized in response to high levels of cAMP is a molecule, ubiquitin hydroxylase, that promotes degradation of the regulatory subunit of PKA. If true, this would shift the balance toward continuous activity of PKA even in the absence of cAMP. Thus, the Ks channel and the Kv channel would be continuously phosphorylated and inhibited, resulting in a more or less permanent increase in Ca<sup>2+</sup> influx into the nerve terminal during APs.

Here, activation of a synapse causes long term changes in synaptic efficacy mediated through intracellular second messenger systems. In the case of *Aplysia*, however, the behavioral changes that the animal is going through can be studied and correlated to the changes in synaptic efficacy that are measured. Thus, in this system it's possible to show that a simple form of learning is dependent on alterations in synaptic "strength", which is one reason why the same is presumed to be true in vertebrates, like humans, though this has not yet been shown directly.

As discussed in Box A of Chapter 24, genetic studies in *Drosophila* have also implicated cyclic AMP as an important mediator of memory formation in that organism. Many mutants that interfere with learning in fruit flies have been shown to affect genes involved in cAMP synthesis or degradation. Thus, as in *Aplysia*, the cAMP/PKA/CREB system is likely to be responsible for long-term changes in behavior in *Drosophila*, suggesting that it may be generally involved in learning and memory in animals.

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