

A Presynaptic K⁺ Channel-Independent Neurotransmitter Release Mechanism Caused by Serotonin May Exist in Crayfish.

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ABSTRACT

Recent studies on the sea-slug *Aplysia* have shown that the neurotransmitter serotonin (5-HT) may trigger an additional ion-channel-independent mechanism that contributes to the presynaptic release of neurotransmitter thereby enhancing excitatory post-synaptic potential (EPSP) sizes and facilitation in chemical synapses beyond what is already known to be caused by 5-HT's inhibition of K⁺ channels. Though numerous studies of this mechanism have been performed on *Aplysia*, few or none have been done on crayfish. In order to test the universality of this additional K⁺ channel-independent mechanism in other organisms, we devised a system of experiments attempting to isolate the mechanism in the neuromuscular junction (NMJ) of the superficial crayfish extensor muscle. By blocking presynaptic K⁺ channels through application of the drug 3-4 diaminopyridine (3-4 DAP), we tried to account for how much the K⁺ channels contributed to overall EPSP enhancement and facilitation when exposed to 5-HT. Our results, however, contradicted our predictions; 3-4 DAP decreased both EPSP amplitude and facilitation in both test conditions. This, along with some apparently inconsistent behavior between experimentation days, prevents us from being able to support the mechanism's existence. Nevertheless, the broadened shapes of the experimental EPSPs hint towards presynaptic action potential lengthening under our test conditions, a sign that this K⁺ channel-independent process may in fact exist in crayfish.

INTRODUCTION

The neurotransmitter serotonin, or 5-HT, has long been known to be important in the fields of neurobiology and neuropharmacology. Its effects on the brain are the basis for many relatively common drugs and antidepressants. Surprisingly, relatively little is known about how 5-HT actually works. On the cellular level, 5-HT is known to cause short-term facilitation of synaptic transmission between neurons, making it important to the understanding of cellular-level memory, which can be seen through the basic learning behaviors of sensitization and dishabituation. Though the specific mechanisms behind 5-HT's effects are still partially unknown, early studies suggested that a secondary messenger pathway within the neuron leads to an enhanced EPSP response. Under this system, 5-HT receptors on a presynaptic sensory neuron would trigger cyclic AMP (cAMP) production, activating protein kinase A (PKA), which would then inhibit K⁺ channels, lengthening the duration of the presynaptic action potential (Hochner et al, 1992). The result if this process would be the release of more neurotransmitter and thus a greater EPSP response and facilitation in the postsynaptic motor neuron (Goldsmith et al, 1992). More recent studies by John H. Byrne and Eric R. Kandel, however, suggest these effects are also caused by an additional "spike duration independent" (or presynaptic action-potential independent) mechanism, which is shown in Figure 1 (Byrne et al, 1996).

The majority of these types of experiments have been conducted on *Aplysia*, whose sensory-motor neuron synapses can be tested in cell culture (Bao et al, 1997). Therefore, in order to investigate the universality of this proposed model, we chose to look for it in crayfish. The simple dissection and large tail extensor muscle cells of the crayfish make it an ideal preparation for this experiment.

We theorized that, assuming Byrne and Kandel's model were accurate, we should find enhanced EPSP amplitudes and facilitation with the addition of 3-4 DAP, and even more of these effects with the 5-HT/3-4 DAP combination. To support this theory, we needed to account for how much the K⁺ channels of the presynaptic neuron contributed to the postsynaptic EPSPs. The

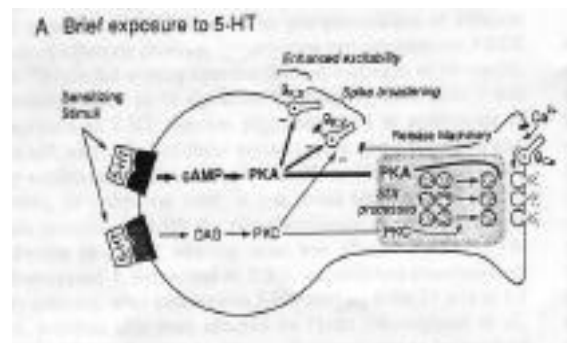


Figure 1. The Current Model for Presynaptic Facilitation John H. Byrne and Eric R. Kandel's model for presynaptic facilitation by serotonin (5-HT) from their 1996 journal report "Presynaptic Facilitation Revisited: State and Time Dependence" as published in *The Journal of Neuroscience* 16(2): 425-435

chemical compounds 3-4 diaminopyridine (3-4 DAP) and 4-aminopyridine are both known to block K^+ channels that affect presynaptic spike broadening (see $g_{k,v}$ in Figure 1), leaving K^+ channels that create the resting membrane potential (see $g_{k,s}$ in Figure 1) untouched (Clark Lindgren, Grinnell College, November 2002). By applying 3-4 DAP to our crayfish preparation, we hoped to prematurely “shut off” the K^+ channels. Then, by stimulating the nerve and recording its behavior, we could compare a 3-4 DAP-modified EPSP with a control EPSP, giving us an indication of how much the closed K^+ channels would contribute EPSPs enhancement and facilitation. By comparing the 3-4 DAP test results with the results gathered from the additional application of 5-HT, we could see if there was any additional EPSP enhancement or facilitation, implying that some other process—one independent of the closed K^+ channels—accounted for the increase. Our results, however, show the exact opposite of what we had expected, seemingly negating such a hypothesis.

MATERIALS AND METHODS

The Dissection

After anesthetizing the crayfish specimen in ice water for 15 minutes, our lab assistant removed the entire tail, which we placed in a glass dissection dish containing a physiological saline solution designed to imitate the chemical concentrations of a crayfish’s internal fluid. The specific composition of the solution is listed in Table 1. We made longitudinal cuts along both sides of the tail and carefully removed the flexor muscles and gut, leaving only the medial and superficial extensor muscles attached to the segmented exoskeleton. We pinned this preparation down to the Sylgard bottom of the dissection dish.

The Equipment

Using glass micropipettes filled with a 3M KCL solution, we made measurement electrodes and inserted them into the large cells of the crayfish superficial extensor tail muscle. The measurement and reference electrodes were connected to the Maclab recording peripheral, monitored on the computer by Scope v3.3 software. All tests were conducted using measurements from various cells within the same muscular bundle—the left lateral bundle of the superficial extensor in the second anterior segment—giving us a consistent and more accurate set of data points. An electric stimulator was placed just below this specific segment with each of the two tips straddling the corresponding nerve in order to assure proper triggering.

Equipment Settings and Recording Process

For our control experiment, the preparation was bathed only in standard crayfish saline, the specific composition of which is listed in Table 1. This solution was emptied and refreshed every 15 minutes to minimize physiological rundown caused by ionic changes within the saline. We used the paired-pulse option on the electric stimulator and shocked the neuron at a frequency of 0.5 Hz at an interval of 100 milliseconds between paired-pulses. The voltage and duration of the stimulation were set to the smallest values possible to create a noticeable EPSP, thereby triggering as few muscle fibers as possible. Typically, the stimulus had a voltage between 90 and 100 volts and durations of .05 or fewer milliseconds. We saved a computerized readout of each paired-pulse that yielded EPSPs, having a minimum of four readouts per test condition. Later, we analyzed the peak amplitudes and shapes of both EPSPs of each paired-pulse to determine EPSP enhancement, facilitation, and EPSP broadening. This procedure was repeated for each following test condition.

Agent	Concentration in mM
KCl	5.4
NaCl	205
MgCl ₂ *6H ₂ O	2.6
NaHCO ₃	2.3
Dextrose	2.0
CaCl ₂ *H ₂ O	13.5

Table 1. The chemical composition of the “control” crayfish solution.

Test Experiments and Test Solution Compositions

After digitally recording the preparation’s behavior under control conditions, we emptied the crayfish solution and replaced it with a new test solution containing 50 μ M 3-4 diaminopyridine (3-4 DAP) diluted in crayfish solution at a ratio of 1:1000. Then, we stimulated the neuron and collected our data as described in the above paragraph. Once the data was collected, we disposed the test solution and immediately applied a final solution containing both 50 μ M 3-4 DAP and 100 μ M 5-HT in crayfish solution at a ratio of 1:1000. For consistency, both test solutions were made from the same original batch of 50 μ M 3-4 DAP solution. Finally, to identify any possible spike-independent process, the results were compared with each other and in reference to the baseline conditions of a normal cell.

Data Extraction and Analysis

Using the Scope analysis software, we recorded the peak amplitudes of both the first and second EPSPs gathered from each paired-pulse readout. We calculated

facilitation percentages by dividing the difference of EPSP1 from EPSP2 by EPSP1. We also used the Scope software to average the readouts of each control and test experiment for each day, creating a graph of EPSP amplitude over time that could be analyzed for differences in shape and size, as seen in Figure 4.

RESULTS

Due to various problems such as muscle spikes and electromagnetic interference, we could not properly analyze some of our data, limiting us to two days worth of usable information. Within these two days of data, both similar and divergent trends were observed. Overall, we found three significant effects from our experimental manipulations.

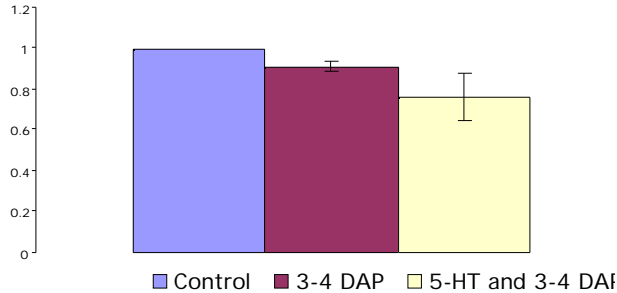


Figure 2. Average of Normalized EPSP Amplitudes This graph shows the average normalized amplitudes of the initial EPSPs from two different crayfish on two different days. Note the trend of reduced EPSP amplitude after the application of 3-4 DAP, and the further reduction with the addition of 5-HT. Though this data directly contradicts our expected results, the trend was observed during both days of experimentation. The error bars indicate +/- standard error.

First, we found that the addition of 3-4 diaminopyridine (3-4 DAP) caused a significant average decrease ($t \leq 9.06 \times 10^{-07}$) in the magnitude of the first EPSP. The addition of serotonin (5-HT) caused a trend of increased reduction of EPSP amplitude, however a t-test ($t \leq 0.84$) revealed that we could not safely assume a significant difference between the two conditions. The averages of these results, normalized between the two days of testing, can be seen in Figure 2.

Secondly, we found that 3-4 DAP caused a significant decrease in facilitation relative to the control ($t \leq 2.96 \times 10^{-10}$), as can be seen in Figure 3. This trend continued with the additional application of 5-HT, causing another ($t \leq 0.0028$) decrease. Also, our attempts to reverse this process and wash out the 5-HT and 3-4 DAP in our prep caused the percent of facilitation to increase significantly

($t \leq 1.89 \times 10^{-11}$), though not all the way back to the levels observed in the initial control conditions.

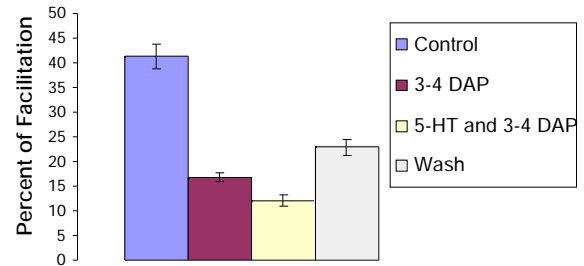


Figure 3. Average Percent of Facilitation This graph depicts the average percent of facilitation (difference in maximum EPSP amplitude between first and second EPSP divided by the amplitude of the initial EPSP) measured for each experimental condition. The addition of 3-4 DAP causes a decrease in facilitation, and the further addition of 5-HT causes an even lower decrease. The wash condition, in which we rinsed the 3-4 DAP and 5-HT from the prep with normal crayfish saline and allowed it to sit for several minutes, caused a fairly large return towards the initial facilitation values. This indicates that the effects observed in the experimental conditions were not primarily the result of some unidentified process of decay. The error bars indicate +/- standard error.

Finally, we found that the shape of the EPSPs varied with each test condition, as can be seen in Figure 4. The EPSPs observed with the presence of 3-4 DAP were broader than those observed under the control conditions—that is, they took longer to return to the resting membrane potential. The combination of 5-HT

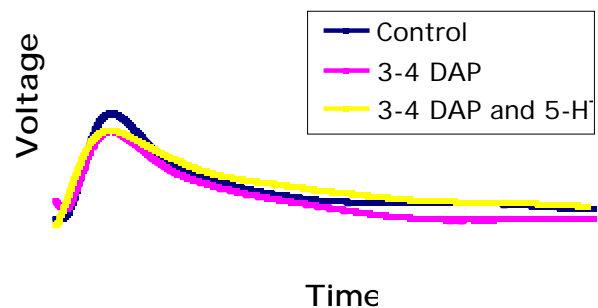


Figure 4. EPSP Shape This picture shows the averaged images of the initial EPSPs from the three primary experimental conditions taken within a single day. When compared to the control condition, the 3-4 DAP-modified EPSP has a broader shape, or a longer period of time before the cell returns to its resting membrane potential. The addition of 5-HT to the 3-4 DAP further enhances this effect. Because EPSPs are indicative of the amount of neurotransmitter release, which is a direct result of presynaptic action potential broadening, this data seems to support the existence of an additional K⁺ channel-independent release mechanism.

and 3-4 DAP caused the shape of the EPSP to be even broader than that of 3-4 DAP on its own.

DISCUSSION

Because our data was the exact opposite of what we expected, we are confronted with a number of possibilities. One possibility is that this discrepancy might be due to equipment failure or human mistakes. If we contaminated the solutions or damaged the sample in some way, it might yield unexpected results. For instance, damaged ion channels might have an effect of 3-4 DAP, or 3-4 DAP may have other side effects we are currently unaware of. Another possibility is that there is not in fact a K⁺ channel-independent process in the crayfish neuromuscular junction.

The latter conclusion, while it would certainly be more significant, seems unlikely given the traditional universality of cellular mechanisms within neurons. (For instance, an action potential is propagated using the same ionic mechanism despite the species of organism the neuron was taken from.)

Interestingly, the depressed conditions we went out of our way to prevent may have been the ideal conditions to isolate spike duration independent (SDI) facilitation. In their report, Byrne and Kandel mention that the SDI was first suggested based on experimental scenarios where 5-HT caused high demand on transmitter release (1996). They suggest that under these conditions (when the amount of available neurotransmitter is decreased due to repeated stimulation) 5-HT still enhanced neurotransmitter release. This idea was echoed in 1999 by L.L. Stark and T.J. Carew, who observed that 5-HT induced facilitation in “nondepressed synapses...is not reliable” (Stark and Carew, 1999). Had we tested our preparation under such conditions, we might have gotten more expected results.

Due to the questions surrounding our results, however, it would be advisable to recreate the experiment with higher frequency stimulation, deliberately making the cell depressed. By doing this, the independent mechanism might be more apparent.

Nevertheless, if Byrne and Kandel’s model is correct, neither of these states (depressed or non-depressed) should have demonstrated the trends we found in our data. By our reasoning, the facilitation ratios of the 3-4 DAP results should have been higher than those of our control. Spike broadening caused by the inactivation of the voltage-controlled K⁺ ion channels should have caused more neurotransmitter to release into the gap. With the addition of 5-HT to 3-4 DAP, the K⁺ channel independent mechanism (if it exists in crayfish) should have enhanced

neurotransmitter release, giving a noticeable effect on both the size of the EPSP and the cell’s facilitation. In fact, James Taggart and Evan Torner have recently confirmed our predictions on 5-HT’s behavior in Grinnell College’s Neurobiology Lab (Taggart and Torner, 2002). Yet according to our data, facilitation merely got smaller and smaller as we progressed through the experiment. Initially, the control conditions gave a 41 percent increase, followed by a 17 percent increase with the addition of 3-4 DAP, and a comparatively miniscule 12 percent increase with both 3-4 DAP and 5-HT. Though we initially supposed that this decrease might be caused by some kind of physiological rundown, the reversibility of the “wash” condition indicates these decreases were not due to some constant process of cellular degradation.

After some reconsideration of this dilemma, however, it seems as if our data gathered from the averaged shapes of the EPSPs might contain more information than we originally thought. Though these EPSP “traces,” when overlaid against one another, still demonstrate a reduction of EPSP amplitude, their shapes become progressively broader with each control condition. The area underneath these EPSP curves (if one were to integrate the function of the lines) is a closer representation of the amount of neurotransmitter binding to the postsynaptic receptors, which is an indication of the amount of neurotransmitter being released. Hypothetically, if the supply of neurotransmitter in the presynaptic cell were to become low and “max out” so that each action potential only released the same reduced amount of neurotransmitter despite the experimental conditions, then equal amounts of neurotransmitter per action potential would be delivered to the postsynaptic cell. The rates of neurotransmitter release, however, would still be affected by the various experimental conditions. Under this set of circumstances, a broadening of the EPSP length would be accompanied by a corresponding reduction of EPSP amplitude in order to preserve the same amount of area underneath the curve. In other words, the same amount of neurotransmitter would be more thinly spread across a longer period of time. This finding is significant because it provides scenario in which our data agrees with Byrne and Kandel’s model.

Therefore, we propose that future experiments on this topic focus on the integrals of the EPSP shapes as opposed to merely the peak amplitudes. By gathering more information in this manner, one could draw more specific conclusions about the existence of the K⁺ channel-independent neurotransmitter release mechanism.

Also, we should not lose sight of the significance of these experiments. In *Aplysia*, this variety of presynaptic facilitation occurs during sensitization and dishabituation, which many researchers believe are the keystones to understanding memory. By learning more about the specific ways experimental organisms “learn,”

we are enhancing our knowledge about the universality of neurons and how memories and other types of behavior are formed.

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