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H-89 Inhibits Serotonin Induced Short-Term Facilitation in the Crayfish Neuromuscular Junction.

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ABSTRACT

Eric R. Kandel and his colleagues' composite research on serotonin (5-HT) in *Aplysia* emphasizes serotonin's heterogeneous effects as a neurotransmitter on paired-pulse facilitation, since 5-HT not only opens up Ca^{2+} spike-broadening channels and Na-K channels to increase excitatory post-synaptic potentials (EPSPs), but also activates an independent protein kinase A (PKA) process of neurotransmitter regulation near the synaptic membrane. This PKA process increases the amount of neurotransmitter released (Byrne & Kandel, 1996). Since it had not yet been proven that this 5-HT-regulated process existed in more than a handful of other organisms outside of *Aplysia*, we set up an experiment to determine whether or not Kandel's PKA process is also present in the crayfish neuromuscular junction. We selectively inhibited the PKA process with N-[2-brommocinnamylamino] ethyl]-5 (H-89) and introduced 5-HT into the extracellular solution, measuring EPSPs to determine if the synaptic facilitation normally increased by 5-HT would be reduced. Our results show that the PKA process does in fact exist in the crayfish muscle neurons, since H-89 reduced the otherwise substantial 5-HT-triggered EPSP enhancement to insignificance. This suggests that Kandel's discoveries in 5-HT-regulated stimulus-response relationships apply to organisms other than *Aplysia* and suggests that further 5-HT studies on crayfish are relevant in the broader field of research on serotonin and its synaptic effects.

INTRODUCTION

Serotonin (5-HT) is one of the most prevalent neurotransmitters in the human brain, its regulation being of primary importance to sleep, mood, attention and learning (Campbell et al., 1997). Common anti-depressant pharmaceuticals like Prozac appear to produce their therapeutic benefit by altering the activity of serotonergic synapses. Thus, the study of serotonin has now become of substantial commercial/scientific importance. The mechanisms by which 5-HT affects the central nervous system at the cellular level, however, are not yet fully understood, making research of these processes governed by 5-HT vital to the further development of its usage.

For over thirty years, neurobiologist Eric R. Kandel and his colleagues have studied 5-HT, focusing on its effects on short-term presynaptic facilitation at the sensory-motor neuron synapse found in the sea slug *Aplysia.* Their research sought to uncover the chemical mysteries of how a behavior can be learned or unlearned. Kandel and others argue that the process of sensitization or dishabituation to stimuli is largely correspondent to the heterogeneous nature of 5-HT as a neurotransmitter: 5-HT generates an excitatory post-synaptic potential (EPSP) through the spike-broadening processes of opening Ca^{2+} channels and Na-K channels in the receiving neuron. Yet it also stimulates a process on the molecular level that uses protein kinase A (PKA) and protein kinase C (PKC) to regulate neurotransmitter release *independent* of the above-mentioned channel-opening and spike-broadening mechanisms (Dale et al., 1988). This PKA/PKC mechanism controls the ionic conductance leading to spike broadening, enhancement of Ca^{2+} influx and increased vesicle exocytosis (Byrne and Kandel, 1996). Together, these changes bring about increased synaptic facilitation given a paired-pulse stimulus.

While crayfish fast extensor muscles have been primarily used in research on glutamatergic synapses (Kawagoe et al., 1983.), they are also useful for our purposes for several reasons. As with *Aplysia* or *in vitro* giant squid axons, crayfish and their extensor muscles are useful because the cells are easily exposed and are, to a certain extent, large enough to see under a microscope when penetrating them with a micro-electrode. The superficial flexor muscles are inherently more excitable than most other muscle neurons and are specifically proven to be susceptible to 5-HT-induced EPSPs (Strawn et al., 2000). Nevertheless, serotonin research on the crayfish has been relatively sparse because of the inherent appeal of the *Aplysia* as a test subject due to its explicit demonstration of stimulus-response patterns controlled by serotonin's synaptic effects. Therefore, nothing involving H-89 PKA/PKC inhibition and the crayfish fast extensor muscles has appeared in the scientific literature. We aimed to provide further knowledge to the field of

crayfish plasticity and serotonin heterogeneity in multiple organisms.

The immediate goal of our research was to identify the existence of the PKA process in the crayfish neuromuscular junction. Our hypothesis was that if the PKA process of neuromodulation is present in the crayfish neuromuscular junction then 5-HT enhanced neurotransmitter release by the presynaptic neuron during short-term facilitation would be reduced by the presence of H-89. Before our experiment, PKA-mediated short-term synaptic enhancement has only been found in the *Aplysia*. Our evidence shows that this process exists in the crayfish neuromuscular junction as well, thus providing proof that the process is present throughout many different organisms. Finding the PKA process in at least several other species enhances the importance of Kandel, et al's research and removes doubt that the process exists in only one type of neuron in one specific animal.

MATERIALS AND METHODS

Preparation

To dissect the crayfish (of the *Procambaris clarkii* species), we covered it with ice for about fifteen minutes so as to anaesthetize the animal and then cut the tail where the abdomen of the crayfish meets the thorax. Immediately after the tail was cut away from the thorax, we submerged the tail in crayfish physiological saline solution for 20 seconds prior to the dissection. The contents of the physiological saline are the following: KCl (5.4 mM), NaCl (205 mM), $MgCl_2 \bullet 6H_20$ (2.6 mM), $NaHCO_3$ (2.3 mM), Dextrose (2.0 mM) and $CaCl_2 \bullet 2H_20$ (13.5 mM). Our dissection exposed the exoskeleton along the longitudinal indentations on both sides of the tail. In our removal of the dorsal portion of the tail shell from the ventral abdominal flexor muscle, we were careful not to damage the fast extensor muscles with our scissors. The ventral portion was discarded, leaving us with the fast extensor muscles of the crayfish tail. We used pins to fasten the crayfish tail, ventral side up, to the bottom of the Petri dish coated with Sylgardä . The saline inside the dish was changed at least every 30 minutes so as to prevent physiological run down of the fast extensor muscle.

Recordings

A Pul-1 by World Precision Instruments was utilized to make the glass micropipettes. We filled these microelectrode with 3 M KCl solution and placed them into a microelectrode holder also filled with 3 M KCl solution. The resistances of these microelectrodes were between 5 MΩ and 10 MΩ. We mounted the microelectrode and stimulating electrode on micromanipulators to precisely control their movements. To obtain excitatory post-synaptic potential (EPSP) recordings, we positioned the stimulating electrode across the crayfish motor nerve and inserted the microelectrode into the anterior side of the corresponding segment of the medial fast extensor muscle. We determined that we had penetrated the cell when we detected a rapid drop in the voltage with the bridge amplitude. We placed an indifferent electrode into the crayfish saline to complete the circuit. To shock the nerve with paired pulses we used a Grass SD9 electronic stimulator. The stimulator was set to deliver a single paired pulse stimulation at a delay of 100 milliseconds using the minimum voltage, so as to allow our observation of short-term synaptic facilitation. The EPSPs were recorded using Scope v3.6.3 software for Macintosh.

Experimental design.

For each solution we took EPSP recordings every minute over a 10-15 minute period of time. We recorded one paired pulse facilitation every minute so as not to depress the synapse. For our experiment, we used four different extracellular solutions consisting of two control solutions and two experimental solutions. We divided the experiment into an A-B pattern: Part A consisted of a control test with normal saline, and Part B consisted of an experimental test with diffused N-[2-brommocinnamylamino] ethyl]-5 (H-89) saline. One cell was penetrated for each trial A and B; both A and B were done on separate preparations. Immediately after EPSPs were recorded in normal saline, we added 25 µL of 10 mM serotonin (5-HT) saline to the 25 mL of normal crayfish solution in the petri dish using an automatic 20 µL pipetter, creating a modified 10 µM 5-HT crayfish saline solution. By adding the 25 µL of 10 mM 5-HT saline to the 25 mL of normal saline in the petri dish, we created a 1:1000 dilution of 5-HT. For the Part B experimental solutions, we used a 10 µM concentration of the protein kinase A (PKA) inhibitor H-89 crayfish saline solution and then added a10 µM concentration of 5-HT using the same techniques described in Part A. The dilution of H-89 and 5-HT was 1:1000.

Data analysis

To analyze our data, we measured the percent change in the amplitude of the second pulse when compared to the first pulse of paired pulse facilitation for all data sets. We used a bar graph to show the percent change of short-term facilitation between normal crayfish saline compared to modified 10µM 5-HT crayfish saline and modified 10 µM 5-HT crayfish saline compared to modified 10 µM 5-HT/10 µM H-89 crayfish saline. To find the statistical significance of our data, we ran unequal variance paired t-tests to compare each data set. Error

bars showing standard error were also calculated for each bar of the graph.

RESULTS

In our attempt to establish the existence of the protein kinase A (PKA) process, one of our primary concerns was how long serotonin (5-HT) took to affect the synapse. Since we were uncertain as to how long it would take for 5-HT to diffuse in either the control saline or the H-89 saline, we conducted a 1 mM cadmium ($CdCl_2$) timing control test to ascertain how long it took for a 25 µL solution to diffuse throughout the petri dish and take its effect on crayfish EPSPs. $CdCl_2$ is a powerful Ca^{2+} channel blocker that produces an obvious reduction in excitatory post-synaptic potentials (EPSPs) once it has fully diffused to the syanpse (C. Lindgren, Grinnell College, November 8, 2002). After introducing 25 µL of 1 M $CdCl_2$ saline by way of the method used to put the same quantity of 10 mM 5-HT solution into the saline during our other tests, we pulsed the nerve every minute and timed how long it took for the EPSP percent difference to go to zero, indicating the full effects of the channel-blocker. We determined that it took approximately seven minutes for $CdCl_2$ to have the desired effect, thereby setting the time limit for the diffusion of a similar quantity of 5-HT. This narrowed our usable data to all 5-HT control/experimental data taken after seven minutes following the introduction of 5-HT into the saline, giving us *n* values between three and five. Timing was critical in our experiment because the PKA process is only prominent in non-depressed synapses up to 5 minutes after 5-HT exposure (Byrne and Kandel, 1996). This meant in practical terms that we pulsed the crayfish only once a minute so as to not depress the synapse and could only accept data that was up to five minutes later than seven minutes after 5-HT was introduced (i.e. between 7 and 12 minutes). These considerations also account for our relatively low number of n values in each data set.

We determined through t-tests that the difference between facilitation in normal saline and 5-HT saline is statistically significant ($p<.05$) and the difference between facilitation recordings taken in the PKA inhibitor N-[2-brommocinnamylamino] ethyl]-5 (H-89) saline and the H-89 saline with 5-HT to be statistically insignificant ($p>.05$). The percent increase in the amplitude of the EPSPs taken in the 5-HT saline when compared to normal saline was due to 5-HT's role in activating the PKA process of increased vesicle exocytosis. H-89 nullifies the PKA process by inhibiting cyclic AMP (cAMP), and thus reduces the enhanced EPSPs generated by 5-HT induced short-term facilitation.

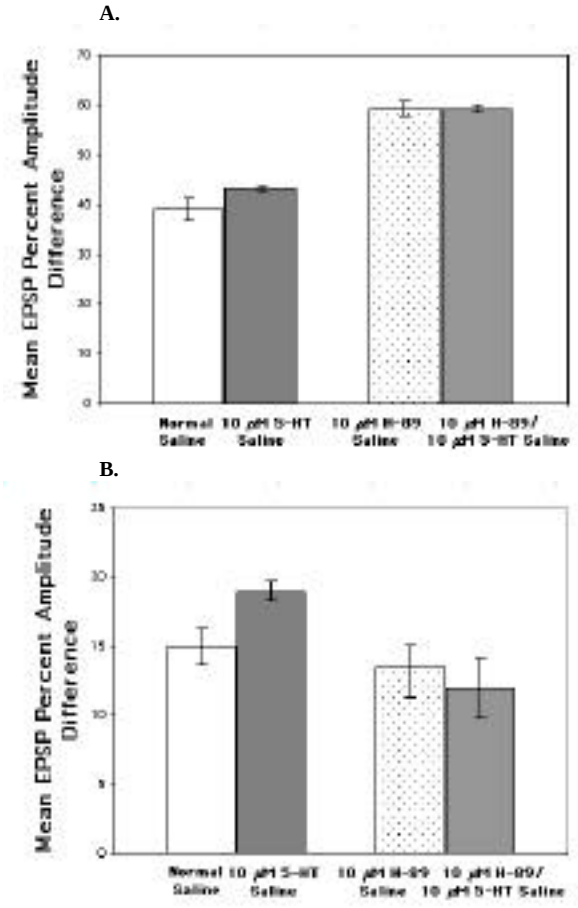

Figure 1. Mean percent difference in EPSPs between pulses in paired-pulsed facilitation for each experimental condition. Each bar is the average of the EPSP percent difference, with each pair of bars representing one crayfish. The two graphs are identical in every way except that the percent differences in Figure 1A are on a larger y-axis scale than Figure 1B because the crayfish involved in those experiments produced greater short-term facilitation. The numerical differences between the bars in each pair remained relatively similar between the graphs. The primary idea that these graphs demonstrate is that H-89 inhibits 5-HT facilitation; we proved that the difference (~4%) between the normal saline facilitation and the 5-HT facilitation is statistically significant, with $p<0.05$ in a single-variable t-test assuming unequal variance, whereas the difference between the H-89 bars in all graphs was found to be statistically insignificant ($p>0.05$). The error bars show the amount of standard error found after calculating each mean percent difference and illustrate the range of percent differences that each bar actually represents.

As seen in Figure 1, we determined the PKA process' existence by comparing the EPSP percent amplitude difference in our control group, in which 10 µM 5-HT saline proved to cause greater facilitation than normal saline, with that of our experimental group, in which 10 µM H-89/10 µM 5-HT saline proved to be statistically identical in facilitation with 10 µM H-89. The experimental variable was the presence or absence of

H-89 in the saline. We performed procedurally-identical control tests on two different crayfish tails, experimental tests on two other crayfish tails and our 1 mM $CdCl_2$ timing control test on an additional tail to determine how much data we had to discard. Figure 1 shows the results from the four primary crayfish in two bar graphs, since one control and one experimental group clearly exhibited greater EPSP amplitudes than the others. Judging from the actual differences in the facilitation percentages on both graphs, however, the amount that 5-HT and H-89 PKA inhibition affected EPSP differences in the respective amplitude ranges is almost identical. Regardless of the excitatory nature of the neurons in any given crayfish, the effects of 5-HT and H-89 on facilitation remained relatively constant. This provides yet further evidence that the PKA process is a consistent feature of all cells with $5\text{-}HT_3$ receptors and are susceptible to serotonin flux.

In order to obtain mean EPSP percent differences from the actual data that we kept, we calculated the difference in voltage amplitude between the peak and the baseline of a given EPSP produced by each pulse in paired-pulse facilitation, which shows the actual EPSP shifts regardless of how high or low the baseline is located in any given reading. The *peak* we defined as the most positive amplitude of voltage across the membrane that the muscle reached after the stimulus artifact, indicating the strength of the EPSP, and the *baseline* is the equivalent of resting membrane potential. After figuring out these differences for every reading in both pulses, we calculated by what percentage the second EPSP's amplitude increased or decreased from the EPSP produced by the first pulse, so as to measure short-term synaptic facilitation.

DISCUSSION

Our hypothesis was that if the protein kinase A (PKA) process exists in the crayfish neuromuscular junction then serotonin-enhanced neurotransmitter release by the presynaptic neuron during short-term facilitation would be reduced by the presence of N-[2-brommocinnamylamino] ethyl]-5 (H-89), a PKA inhibitor, in the saline. The data that we collected suggests that the serotonin (5-HT) and cyclic AMP-fueled PKA process that causes greater-than-normal excitatory post-synaptic potentials (EPSPs) exists in the crayfish neuromuscular junction, since the inhibition of this process reduces 5-HT EPSP growth to a statistically insignificant amount. Our data shows a 4 percent increase in EPSP amplitude short-term facilitation with the addition of 5-HT and no increase in EPSP amplitude with the addition of 5-HT while H-89 was present in the extracellular solution. Since H-89 is an inhibitor of the 5-HT-activated PKA process, the logical explanation for why increased facilitation didn't occur in neuromuscular junctions perfused with H-89 saline and an introduced 5-HT solution is that H-89 was blocking one of 5-HT's primary means of producing facilitation. Without the PKA process, 5-HT simply cannot induce substantial excitatory effects on short-term facilitation up to five minutes after it diffuses into the neurons.

5-HT is a water-soluble molecule that binds to the presynaptic $5\text{-}HT_3$ receptors which in turn activate a protein kinase process that brings about enhanced short-term facilitation. However, the EPSP generated by this process varies significantly depending on the duration of the stimulus and the state of the synapse (depressed/non-depressed) before the stimulus arrives. Eric R. Kandel's explorations reveal that this variance comes from the operation of twin PKA and PKC pathways that activate in addition to the channel-opening processes when 5-HT bonds with the $5\text{-}HT_3$ receptor protein. Specifically, once 5-HT molecules bind with the $5\text{-}HT_3$ receptors on the presynaptic terminal, secondary messenger molecules are activated that are then sent to produce the particular protein kinase corresponding with those molecules, which will then travel down a pathway to the neurotransmitter vesicles and regulate their release (Braha et al., 1993). The abundance of the regulatory molecules, cyclic AMP (cAMP) for PKA or diacylglycerol (DAG) for PKC, is dependent on state and time factors: if the synapse is non-depressed or experiences only a brief exposure to 5-HT ($\leq$5 minutes), then more cAMP is produced, stimulating more PKA production and making PKA more dominant in the process of neurotransmitter excitation. Similarly, PKC will take a more active role in a neuron on a depressed synapse or one that has experienced a long period (10-20 min.) of serotonin stimulation (Byrne and Kandel, 1996). Since we were examining only the PKA process we could only expose the crayfish tail to 5-HT for a maximum of five minutes. Otherwise, protein kinase C (PKC) would become dominant in the process of enhanced facilitation (Byrne and Kandel, 1996). PKA produces enhanced short-term facilitation by arousing vesicles near the presynaptic terminal so that they undergo exocytosis and eject a subsequently increased amount of neurotransmitter into the synaptic cleft than there would be if this process were non-existent. The percentage increase of EPSPs that we observed during our control experiment was created through the above-mentioned process.

The selective PKA/PKC inhibitor that we used is H-89 Dihydrochloride (H-89), also known as N-[2-brommocinnamylamino] ethyl]-5, with the chemical formula $C_{20}H_20BrN_30S.24Cl$. T. Chijiwa et al. (1990) were some of the first to use it as a cAMP inhibitor, and they discovered that PKA/PKC processes were also

inhibited by its addition into a solution. It is an isoquinolinesulfonamide permeable to the membrane, water soluble, potent at 10 µM and is used in several fields of cell biology other than neurology (McDonald et al., 1995). We inhibited the PKA/PKC mechanism with H-89 and detected no significant increase in the EPSPs generated with the addition of 5-HT. Our results showed that a significant part of 5-HT's role, that of a neurotransmitter regulator at the presynaptic terminal, was incapacitated with the inhibition of the PKA process. This proves the existence of such a regulation mechanism in crayfish and implies its existence in other non-*Aplysia* invertebrates.

In proving the existence of the PKA process in the crayfish, our research provided support to the applicability of Kandel's *Aplysia* studies to multiple organisms. Kandel's research focused on the cellular mechanisms behind dishabituation and sensitization that are fundamental to our understanding of how behaviors are learned and unlearned. His work with serotonin and the PKA/PKC processes provides a basis of knowledge in helping to understand phenomena such as muscle memory. In providing evidence to the universality of the PKA process, our research creates a stepping-stone to studying serotonin and its role in larger scale behaviors occurring in more complex organisms, such as the crayfish. To expand on our research, an experiment could be done to observe dishabituation and sensitization to stimuli in crayfish with and without the presence of serotonin. This investigation would require the development of new lab techniques to observe dishabituation and sensitization in the crayfish, but would contribute to limited knowledge of how serotonin affects synapses, and how its effects are expressed behaviorally. A more simple yet important future study is to replicate our research in this paper on another more complex organism.

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