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Carbon monoxide has varied effects on post-tetanic facilitation and depression in the crayfish neuromuscular junction.

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ABSTRACT

We varied the concentration of CO in the crayfish neuromuscular junction (NMJ) to observe the effects of CO on excitatory post-synaptic potentials (EPSPs) and post-tetanic facilitation and depression**.** The decrease of CO in the crayfish NMJ had significant effects on post-tetanic EPSP amplitudes. Additionally, decreased CO increased post-tetanic facilitation immediately following stimulation, while increased levels of CO did not significantly affect post-tetanic facilitation except when combined with an NOS inhibitor. While we did not observe PTP as expected, we observed post-tetanic facilitation and depression in the crayfish NMJ.

INTRODUCTION

In typical synaptic transmission, neurotransmitter released from the presynaptic nerve terminal binds to the receptors on the postsynaptic cell and opens the ion channels in the postsynaptic membrane. Nevertheless, several studies have demonstrated that synaptic transmission does not always follow this model and is instead modulated by diverse neuromodulators and secondary messenger systems.

Carbon monoxide (CO) is a gaseous atypical neurotransmitter which modulates transmission in the hippocampus by attaining the role of a second retrograde messenger (Zhuo et al., 1998). There have been many studies on CO in the hippocampus because of its importance in memory. Zhuo et al. (1998) found that increased concentrations of CO increase LTP in rat hippocampus synapses. However, to our knowledge, the effect that CO has on other forms of potentiation, such as post-tetanic potentiation (PTP), has not been studied.

PTP occurs after a rapid train of action potentials or tetanus. Immediately after the tetanus, the post synaptic response to a single action potential increases (Levitan & Kaczmarek, 1997). LTP occurs after PTP is observed for an extensive period of time, depending on the intensity and duration of PTP (Levitan & Kaczmarek, 1997). Since the mechanism for PTP is unknown, and CO has been found to have an effect on LTP, we pursued our study on the relationship between PTP and CO. Crayfish are suitable for this experiment due to their well-defined, easily accessible, single point of synaptic contact between a motor neuron and a muscle fiber (French, 2005). Crayfish also utilize glutamate as the major excitatory neurotransmitter and GABA as the major inhibitory neurotransmitter. These neurotransmitters are used in the majority of synapses in the human Central Nervous System (CNS). We therefore attempted to determine whether the gaseous neuromodulator CO has a direct effect on PTP in the crayfish neuromuscular junction (NMJ).

The long-term objective of the research is to understand how this gaseous secondary retrograde messenger modulates enzyme activity in the crayfish NMJ. Since CO has been shown to increase LTP, we hypothesized that it would also enhance PTP. Our study yielded inconclusive results about the relationship between CO and PTP, but provided information on post-tetanic facilitation in the crayfish NMJ. Post tetanic facilitation is the increase in the magnitude of EPSPs after a high-frequency tetanic stimulation.

MATERIALS AND METHODS

Crayfish dissection and intracellular recording

Crayfish (*Procambarus clarkii*) were obtained from Carolina Biological Supplies Co. The typical size of the animals was 5-7 cm head to tail. The crayfish were chilled in ice, their tails were cut off and dissected, and the fast extensor muscles of the tail were exposed. The crayfish were placed in Ringers solution (205.0 mol NaCl, 5.4 mol KCl,13.5 mol CaCl, 2.6 mol $MgCl_2$, 10.0 mol Tris [pH 7.4]), which mimics the physiological environment of the crayfish. We also changed the Ringers solution every 15 minutes in all our experiments to maintain longevity of the preparation.

Electrodes were prepared by pulling 1.2 mm glass capillaries to a fine point and were filled with 3M KCl (resistance 10-20 MΩ). A blunt tip electrode filled with Ringers solution was used as a suction electrode. We connected the suction electrode to a SD9 stimulator to stimulate the nerve. Microelectrodes were used to penetrate the muscle cells and obtain a steady membrane

potential. We then gradually increased the voltage to elicit a twitch in the lining of the muscle that was being stimulated, punctured it with a recording electrode, and measured the amplitude of its EPSPs using the Maclab recording instruments. EPSPs were recorded only from those muscle cells with membrane potentials below -40mV. Throughout the pre-tetanic portion of the experiment, we kept the stimulator frequency at .5 Hz, delay at 5 ms, and duration at .5 ms. We increased the frequency to 50 Hz to simulate tetanic stimulation. Voltage value was gradually increased.

We first measured the amplitude of EPSPs elicited in normal Ringers solution. Following this, we applied tetanus of 50 Hz for 5 seconds and measured the amplitude of EPSPs after the tetanus. The same protocol was followed in experimental solutions.

Drug preparation

CO was inhibited in the crayfish preparation by the application of Tin Protophyrin IX (Tocris, Ellisville, MO). Tin Protophyrin IX inhibits heme oxygenase (HO-2), the enzyme that produces CO in the postsynaptic cell. A stock solution of 5mM in DMSO was prepared. This stock solution was diluted to 12.5nM in the experimental solution.

Since Tin Protophyrin IX can possibly affect other enzymes including NO synthase (NOS) (Zhuo et al., 1998), as a control, we examined PTP upon a preparation in which NO production was inhibitied to ensure that NO had no effects at this synapse. NO production was inhibited by inhibiting NOS, the enzyme that produces NO, using a 0.3mM concentration of L-NAME (obtained from Tocris, Ellisville, MO) in the Ringers solution.

To increase the concentration of CO in solution, we bubbled the CO gas in 40mL of Ringers solution in a closed flask until saturation (for 5 minutes).

RESULTS

We found decreasing CO to have significant effects on baseline EPSP amplitudes. Decreasing CO by using Tin protoporphyrin (SnPP) to inactivate heme oxygenase-2 significantly decreased the mean amplitude of EPSPs to 46% (*SE* = 10.9%) of the height of control EPSPs, $t(11) = 2.74$, $p < .05$ (two-tailed). Increasing CO by bubbling it into the Ringer's solution and applying a NOS inhibitor alone or in combination with increased or decreased levels of CO did not significantly alter mean EPSP amplitudes. See Fig. 1 for the mean amplitudes of EPSPs in all experimental conditions relative to the control condition.

Additionally, we found effects of decreasing CO levels on the course of post-tetanic facilitation (see the normal course of EPSPs following tetanic stimulation in Fig. 2). To assess post-tetanic facilitation and depression, we averaged all readings following tetanic stimulation in each experimental condition for three ten-second periods: 0 to 10 seconds, 20 to 30 seconds, and 54 to 64 seconds. By doing this, we hoped to capture both initial facilitation effects and later depressive effects. Results are shown in Fig. 3. Although the effects of increasing CO were not statistically significant, decreasing CO significantly increased the amplitude of EPSPs between 0 and 10 seconds following post-tetanic stimulation, $t(7) = -2.51$, $p < .05$ (two-tailed). We also found that an NOS inhibitor alone significantly increased EPSPs between 54 and 64 seconds following post-tetanic stimulation, $t(16) = -4.58$, $p < .01$ (two-tailed). Additionally, we found that an NOS inhibitor plus increased levels of CO significantly increased EPSP amplitudes between 54 and 64 seconds following post-tetanic stimulation, $t(13) = -4.70$, $p < .01$ (two-tailed).

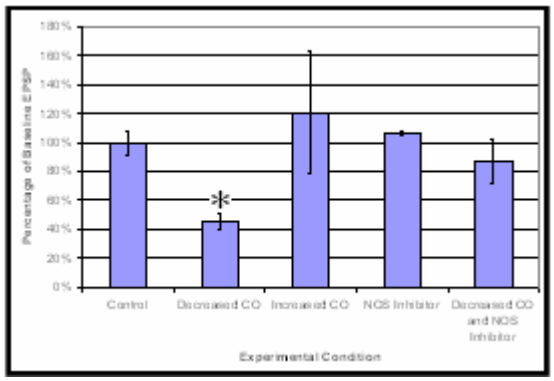

Figure 1. Mean baseline EPSP readings in experimental conditions relative to control EPSP readings. Each bar is based on mean readings from between two and eight crayfish ($M = 4.2$). * $p < .05$

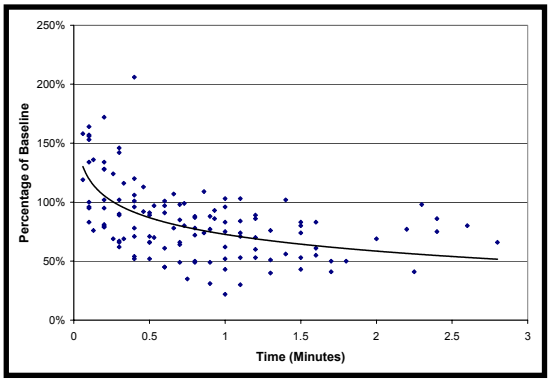

Figure 2. EPSP heights over time in a control condition following tetanic stimulation at 0 minutes. EPSP readings were taken in the neuromuscular junctions of eight crayfish.

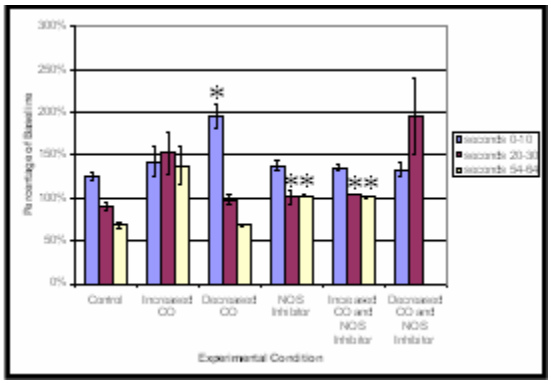

Figure 3. Mean post-tetanic EPSPs relative to baseline EPSPs in various experimental conditions. Readings occurring 0-10 seconds, 20-30 seconds, and 54-64 seconds following tetanic stimulation were averaged within each condition. Each bar represents an average of 6.1 readings. There were no readings taken between 54 and 64 seconds for the decreased CO and NOS inhibitor condition. * $p < .05$ ** $p < .01$

DISCUSSION

While we failed to observe LTP or PTP due to experimental method complications, we found that decreasing CO in the crayfish affects post-tetanic EPSP amplitudes and found that decreased CO increases post-tetanic facilitation immediately following stimulation; we observed post-tetanic facilitation and depression. These findings suggest the active role of CO in synaptic transmission in the crayfish NMJ. When a NOS inhibitor was combined with decreased levels of CO, however, mean EPSP levels were not significantly different from a control. This suggests that NO may interfere with the initial effect of decreased CO. Though we consider our observations on the effects of NO preliminary, they support our initial hypothesis that CO has an important role in synaptic transmission.

Increased levels of CO did not significantly affect post-tetanic facilitation except when combined with an NOS inhibitor. In this case, the post-tetanic depression occurring between 54 and 64 seconds following tetanic stimulation was significantly reduced—in fact, post-tetanic depression did not occur in this condition. One explanation is that NO has a phasic stimulation on post tetanic stimulation as Zhuo et al. (1998) suggests in their study. On the other hand, decreased levels of CO increased facilitation immediately following tetanic stimulation. This finding was contrary to our expectation that decreased levels of CO would inhibit post-tetanic facilitation.

In the future, more experimental trials should be conducted with increased CO to enhance the reliability of our data. Additionally, more experiments should be conducted on increased CO combined with NOS inhibitor to support our findings that suggest a role of NO in this synapse. Experiments in which NO is increased should be conducted to see if the same results occur as when CO is increased. This would give information on how NO affects post tetanic depression and facilitation in the crayfish NMJ.

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REFERENCES

Barañano, D. E., Ferris C. D., and Snyder, S. H. 2001. Atypical neural messengers. *TRENDS in Neuroscience* Vol. 24 No. 2: 99-106.

Boehning, D. and Snyder, S. H. 2003. Novel neural modulators. *Annual Review of Neuroscience* 26:105-31.

French, L. B. Lab 8- Crayfish Synaptic Connectivity. <http://webpub.allegheny.edu/employee/l/lfrench/WordforWeb/Lab_8_Qs.doc>

Levitan, B., Kaczmarek, K., 1997. The Neuron. Oxford University Press. New York, NY.

Zhuo, M., Laitinen J. T., Li, X-C., and Hawkings, R:D. 1998. On the respective roles of nitric oxide and carbon monoxide in long-term potentiation in the hippocampus. *Learning & Memory* November/December 5(6): 467-480.