Pioneering Neuroscience, 2011, **12**, 17-20.

Exogenous NAAG Decreases the Excitatory Post-Synaptic Response at the Crayfish Neuromuscular Junction

ABBY STEVENS, NATALIA NAKAIDZE, and MARTIN ESTRADA
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Glutamate is widely held to be one of the most abundant neurotransmitters in the nervous systems of many organisms. Another neuromodulator, N-Acetylaspartylglutamate (NAAG), has been shown to exhibit both agonistic and antagonistic properties at various glutamatergic receptors. Much current research seeks to determine its role at synapses. We sought to determine the role of NAAG at the neuromuscular junction of crayfish, and to compare its effects with those of glutamate. Using intracellular recording techniques, we measured the Excitatory Post-Synaptic Potentials (EPSP) of stimulated crayfish muscle cells in regular saline, in the presence of exogenous NAAG, and in the presence of exogenous glutamate. Our results support our hypothesis that the addition of NAAG results in a decreased amplitude of EPSPs in comparison to those of the baseline measurements. Our results also indicate that the addition of exogenous glutamate increases the amplitude of the EPSP, demonstrating the independence of NAAG as a neuromodulator. An analysis of the paired-pulse ratio indicates that NAAG may act post-synaptically at the crayfish neuromuscular junction.

INTRODUCTION

When neurons communicate via chemical synapses, the postsynaptic membrane may exhibit an excitatory post-synaptic response (EPSP) upon activation of its ionotropic channels. Research has shown that this depolarizing effect on the membrane can be elicited by the release and binding of neurotransmitters, such as glutamate, from pre-synaptic vesicles.

Studies have shown, however, that glutamate is not the only neuromodulator known to activate glutamatergic receptors. N-Acetylaspartylglutamate (NAAG), discovered in the mammalian nervous system in the mid-1960s, was found to activate Group II metabotropic glutamate (mGlu) receptors (Neale *et al* 2005). As an agonist at mGluR3, NAAG reduces voltage-sensitive calcium currents and suppresses glutamate release by acting on presynaptic $mGluR_3$ (Bacich *et al* 2002). Glutamate carboxypeptidase II (GCPII) is a known deactivator of NAAG, and hydrolyzes NAAG into glutamate and N-acetylaspartate. Studies have found that the inhibition of GCP II reduces the levels of extracellular glutamate (Bacich *et al* 2002). Some research indicates that the inhibition of GCPII does not decrease the amplitude of miniature EPSPs but rather their frequency, which suggests that NAAG in its non-hydrolyzed form works pre-synaptically (Sanabria 2003). However Bergeron found that NAAG acts as an antagonist of NMDA receptors on the post-synaptic membrane (2007). The differences in these findings suggest that the location of the mechanism of NAAG modulation is not yet clearly determined.

The above research was all conducted on mammals. Our lab is interested in the role of NAAG at glutamatergic receptors in non-mammalian crayfish cells. The only research currently available that investigates the role of NAAG in crayfish, conducted by Gafurov *et al* (2001), demonstrates that glial cell hyperpolarization, which previous reports indicated was induced by glutamate or high-frequency stimulation, could also be induced by NAAG in adult crayfish cells. In the experiments conducted by Gafurov's lab, 2-PMPA, a GCP II inhibitor, was added to rule out NAAG hydrolysis as the source of glutamate. The study found that NAAG in its non-hydrolized form could be the prominent agent in axon-to-glia signaling in crayfish.

Studies of NAAG have the potential to advance the "glutamate theory" of schizophrenia. There are many reasons to hypothesize that not only is the dopamine system abnormal in schizophrenia, but so is glutamatergic transmission. First, glutamate is the most abundant neurotransmitter in the cerebral cortex. It is widely found in brain structures that are commonly implicated in schizophrenic patients, such as the entorhinal region, the hippocampus, and the frontal cortex. Second, the drug phencyclidine, which is able to induce schizophrenia-like psychosis in healthy people, acts on glutamate receptors. Phencyclidine acts as an antagonist at NMDA receptors, which results in the hypofunction of the receptors, a phenomena proposed to play a role in the pathophysiology of schizophrenia (Bergeron 2007), resulting in an analgesic and anesthetic effect on the patient (Heinrichs 2002). Studies indicate that, since NAAG is a possible antagonist to NMDA receptors, an increase in extraceular NAAG may be one of the many causes of NMDA hypofunction

© 2011 Grinnell College

and thus schizophrenic-like symptoms (Bergeron 2007). Neale presents another interpretation of the role of NAAG in schizophrenia. Since NAAG acts as an agonist to Group II mGlu receptors, NAAG's presence may help mitigate the effect of glutamate hypofunction because it is activating glutamatergic receptors (2005). A better understanding of the role of NAAG will help differentiate between these competing theories and lead to a more precise glutamate theory of schizophrenia.

We are interested in the role of NAAG at the crayfish neuromuscular junction. The crayfish neuromuscular junction is an ideal test specimen because we can directly measure what is happening at individual synapses. We are exploring the possibility of the crayfish neuromuscular junction serving as a model synapse for studying NAAG.

We sought to reconcile the various research findings in order to determine how exogenous NAAG affects the EPSP elicited by low-frequency stimulation at the crayfish neuromuscular junction. We hypothesized that the presence of exogenous NAAG would decrease the amplitude of the EPSP relative to the baseline measurements, given its proposed antagonistic effects at NMDA receptors. We tested NAAG in its non-hydrolized form, and compared the results with those of exogenous glutamate to differentiate NAAG from its hydrolysis products as an independent neurotransmitter. We used paired-pulse stimulation to determine the location of the mechanism of NAAG modulation, and measured the EPSP induced in the presence of NAAG and Glutamate under the condition that GCPII was inhibited by ZJ43, which eliminated any effects of the hydrolysis of NAAG. Our results supported our hypothesis that NAAG decreases the amplitude of EPSPs and that it does not elicit the same response as glutamate. The paired-pulse ratio showed us that NAAG acts postsynaptically.

MATERIALS AND METHODS

Crayfish Preparation

Crayfish were obtained from Wards (Rochester, New York). We anesthetized the crayfish on ice prior to the experiment. We removed the tail from the rest of the crayfish and made two longitudinal cuts along the ventral surface, allowing the underside of the exoskeleton and extraneous muscle tissue to be removed. We used the exposed dorsal extensor muscles for experimentation. We pinned the dorsal exoskeleton with extensor muscles exposed in a silicon dissecting dish and covered with 100 mL of saline. We collected data from three different crayfish specimens.

Solutions

We added GCP inhibitor ZJ43 (Tocris) to each solution at a concentration of 1 μM. This blocked the hydrolysis of endogenous NAAG in order to control levels of glutamate. We took our initial baseline measurements in 100 mL of regular crayfish saline, which was composed of 5.4 mM KCl, 196 mM NaCl, 2.6 mM $MgCl_2$, 13.5 mM Ca Cl_2, 10 mM HEPES, H_20, and had a pH of 7.4. We then replaced 25 mL of regular saline with 25 mL of saline containing NAAG (Sigma) to create a final concentration of 10 μM. We washed the NAAG out by systematically removing the solution and replacing it with regular saline. We tested glutamate (Sigma) with the same procedure at the same concentration.

Electrophysiology

We recorded the resting membrane potential under each condition using intracellular recording techniques. We pulled the microelectrodes from 1.2 mm diameter borosilicate glass capillaries using a WPI Pul-1 (Sarasota, Florida) micropipette puller. We filled these with 3M KCl and kept the resistance between 4MΩ and 10MΩ. We used a suction electrode to suck up and stimulate crayfish nerves. We used low-frequency stimulation (0.4 pps) to generate the EPSPs at an average applied voltage of approximately 1V. Because EPSP amplitudes vary from cell to cell, we took baseline measurements, measurements with glutamate or NAAG, all in the same cell. We accomplished this by leaving the microelectrode in a single cell while changing the solutions.

Statistical Analysis

We performed a student's t-test using MiniTab to determine statistical significance at a $p<0.05$ level. We compared the EPSP measured under each treatment with that of its associated baseline measurement.

RESULTS

We studied the effect of NAAG on the EPSP at the crayfish neuromuscular junction. We hypothesized that the presence of exogenous NAAG would decrease the amplitude of the EPSP relative to the baseline measurements. We used ZJ43 in each solution throughout the experiment to inhibit GCP II and prevent the effects of NAAG's hydrolysis product, glutamate. We took baseline measurements in regular crayfish saline with ZJ43 and then replaced 25 mL of this saline with saline containing NAAG. Using low-frequency paired-pulse stimulation, we elicited EPSPs and using intracellular recording techniques we found that NAAG significantly lowered the amplitude of the EPSP relative to the baseline measurement (Figure 1).

© 2011 Grinnell College

To differentiate this result from glutamate, we performed the same experiment with glutamate and found that the EPSP significantly increased relative to the baseline measurement (Figure 1). This indicates that non-hydrolized NAAG and glutamate perform different roles at the crayfish neuromuscular junction.

We measured the paired-pulse ratio by taking the ratio of the EPSP generated by the second pulse to that generated by the first. We found that the paired-pulse ratio did not change upon the addition of NAAG (Figure 2). We also found that the paired-pulse ratio significantly increased upon the addition of glutamate (Figure 2), though with only one trial we form no conclusions.

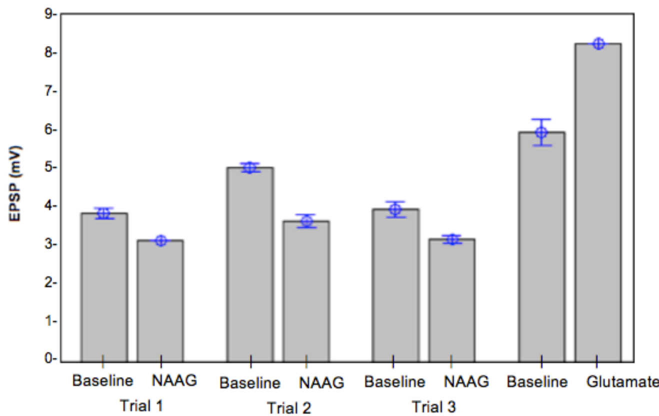

Figure 1. Average EPSP as a result of various chemical treatments. NAAG decreases the amplitude of the EPSP in relationship to the baseline measurements (p=0.000, p=0.001; p=0.000; n=20 for each Baseline average, n=50 for each NAAG average) while Glutamate increases the amplitude of the EPSP ($p<0.05$; n=20 for the Baseline, n=50 for glutamate). Each trial represents data taken from a single cell. Error bars represent standard error of the mean at a 95% confidence level. Student's t-test was used to determine statistical significance.

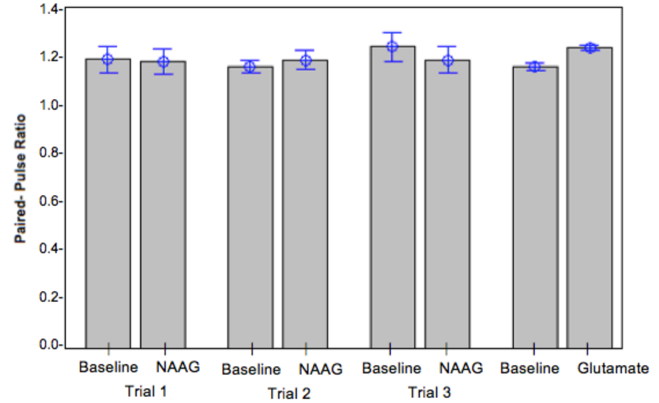

Figure 2. Average ratio of the EPSP induced by the second pulse to the EPSP induced by the first pulse. NAAG induced no change in the paired-pulse ratio (p=0.987; p=0.214; p=0.175; n=20 for each Baseline average, n=50 for each NAAG average) while Glutamate increased the paired-pulse ratio (p=0.000; n=20 for baseline, n=50 for NAAG). Error bars represent standard error of the mean at a 95% confidence level. Student's t-test was used to determine statistical significance.

DISCUSSION

We conclude that the data support our hypothesis: the application of NAAG caused the amplitude of the EPSP to significantly decrease, a result that opposed the action of glutamate. An analysis of the paired-pulse ratio of the EPSP generated by the second pulse to the EPSP generated by the first shows no change upon addition of NAAG, and a slight (but significant) increase upon addition of glutamate. The first result indicates that the pre-synaptic cell is not being influenced by NAAG and thus suggests that NAAG acts post-synaptically. The change in paired-pulse ratio found upon the addition of glutamate indicates that glutamate has a pre-synaptic effect, though with only one set of experimental results we draw no conclusion regarding the location of glutamate's action at the crayfish neuromuscular junction.

The increase in the EPSP upon the addition of glutamate suggests that there may exist metabotropic glutamatergic receptors at the neuromuscular junction of crayfish, and that NAAG may act as an antagonist to some of them. While this data provides no indication of whether NAAG acts as an agonist at mGlu receptors, as it does in mammals (Neale *et al* 2005), it provides evidence that supports the conclusion that there may exist NMDA receptors at the neuromuscular junction of crayfish, since NAAG is known to inhibit these receptors in mammals (Bergeron *et al,* 2007). In order to determine the location of NAAG's role at the neuromuscular junction of crayfish, future research may want to pharmacologically isolate certain receptors and repeat the experiment.

© 2011 Grinnell College

Since non-hydrolyzed NAAG does not elicit the same response as glutamate, we suggest that NAAG acts as an independent neuromodulator at the crayfish neuromuscular junction; that is, its effects are not restricted to those of its hydrolysis products.

Because our results indicate that NAAG may inhibit post-synaptic glutamatergic receptors, our results may support the glutamate theory of schizophrenia in terms of NAAG causing glutamate hypofunction in NMDA receptors (Bergeron 2007). Further research may seek to better isolate the location of glutamate hypofunction in schizophrenic patients in order to determine if NAAG's role is more prominent at mGlu or NMDA receptors and thus if it therapeutic or symptom-causing itself.

This experiment had many limitations. Given the time-frame under which this experiment had to be conducted and the limited number of specimens upon which we were able to perform our experiment, we were unable to reproduce the results as many times as we hoped in order to provide more conclusive evidence. Furthermore, we were unable to determine the specific location of NAAG's effects, and could only use our paired-pulse ratio analysis to draw broad conclusions. Future research should include many more trials and should focus on understanding the location at which NAAG acts. Also, it would be insightful to test different concentrations of NAAG and see whether the amplitude of EPSP depends on the concentration. Another variable that might be important is the duration under which crayfish is exposed to specific treatment.

ACKNOWLEDGEMENTS

We would like to thank our professor Clark Lindgren, our lab instructors Sue Kolbe and Ashley Milleti, and our Teaching Assistant Chris Kaiser-Nyman for their patience and willingness to make this investigation as seamless and educational as possible.

REFERENCES

Bacich, D.J. *et al.* 2002. Deletion of the glutamate carboxypeptidase II gene in mice reveals a second enzyme activity that hydrolyzes *N*-acetylaspartylglutamate. *Journal or Neurochemistry* 83: 20-29.

Bergeron, R *et al.* 2007. Endogenous N-acetylaspartylglutamate reduced NMDA receptor-dependent current neurotransmission in the CA1 area of the hippocampus. *Journal of Neurochemistry* 100: 346-357.

Heinrechs, W.R. 2001. In search of madness: schizophrenia and neuroscience. Oxford University Press, New York.

Gafurov, B. *et al.* 2001. N acetylaspartylglu tamate (NAAG) is the probable mediator of axon-to-glia signaling in the crayfish medial giant nerve fiber. *Neuroscience* 106(1): 227-235.

Neale, J. *et al.* 2005. The neurotransmitter N-acetylaspartylglutamate in models of pain, ALS, diabetic neuropathy, CNS injury, and schizophrenia. *TRENDS in Pharmacological Sciences* 26(5): 477-484.

Sanabria, E. Krystyna M. Wozniak. Barbara S. Slusher and Asaf Keller. 2003. GCP II(NAALADase) Inhibition Supresses Mossy Fiber-CA3 Synaptic Neurotransmission by a presynaptic Mechanism. *Journal of Neurophysiology* 91:182-193.

© 2011 Grinnell College