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The Role of NAAG as a Possible Postsynaptic Modulator at the Crayfish Neuromuscular Junction

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ABSTRACT

We investigated the effects of NAAG and β-NAAG on synaptic transmission in the crayfish neuromuscular junction (NMJ). Previous research has shown that exogenous NAAG regulates EPSPs in mammals both presynaptically and postsynaptically. To determine whether NAAG modulates synaptic transmission at the crayfish NMJ (and whether the modulation is pre- or postsynaptic), we measured paired-pulse ratios (PPRs) and excitatory post-synaptic potential (EPSP) magnitudes before and after adding NAAG or β-NAAG. We observed no change in PPR for any experiment, and we found that β-NAAG had no effect on EPSP magnitude. However in one experiment, we observed a decrease in EPSP magnitude after applying NAAG.

INTRODUCTION

N-acetylaspartylglutamate (NAAG) is a widely distributed neurotransmitter in the mammalian nervous system (Neale *et. al*, 2005). NAAG binds to both pre- and postsynaptic Group II metabotropic glutamate receptors (mGluR II) (Neale *et al*., 2005). Other research has also found that endogenous NAAG binds to postsynaptic NMDA receptors as an antagonist (Bergeron *et al*., 2007). NAAG is hydrolyzed by the peptidase glutamate carboxypetidase II (GCP II) into N-acetylaspartic acid and glutamate (Gafurov et al., 2001). However, β-NAAG, a synthetically produced analogue of NAAG, cannot be hydrolyzed by GCP II.

Previous research indicates that NAAG modulates synaptic transmission both presynaptically and postsynaptically. In Pöeschel *et. al.*'s (2005) research, application of NAAG reduced paired-pulse depression, which supports the theory that NAAG has a role in the presynaptic terminal; application of β-NAAG produced no change in paired-pulse ratios, suggesting that β-NAAG acts postsynaptically as an antagonist (Pöeschel, 2005).

As for the postsynaptic effects of NAAG, researchers have investigated NAAG's effects on mini-excitatory postsynaptic currents and long-term potentiation. Bergeron *et. al* (2007) found that application of NAAG led to a significantly reduced amplitude of the NMDAR component of the mini-EPSC, indicating that NAAG could change the sensitivity of the NMDA receptor to glutamate.

Although most of the research concerning NAAG and its effects has taken place in mammalian hippocampi, previous studies have shown that NAAG hydrolysis produces glutamate in the crayfish medial giant nerve fiber (Gafurov et al., 2001). Because little is known about NAAG in invertebrate species, we tested the effects of NAAG and β-NAAG in the crayfish neuromuscular junction. The crayfish is a model organism because it is relatively easy to observe glutamatergic synapses in crayfish muscle cells. We hypothesized that NAAG would have an effect on the magnitude of EPSPs and the paired-pulse ratio (PPR), while β-NAAG would have no effect. A change in the PPR would indicate that at least some of the change is presynaptic, while no change in PPR suggests that the change is exclusively postsynaptic.

We could not detect any significant change in the PPRs for either NAAG or β-NAAG. β-NAAG also had no substantial effect on the average amplitude of the first EPSP of the pair (E1). In our first NAAG experiment, we observed a change in the average amplitude of E1, and no change in the paired-pulse ratio. However, the second NAAG experiment showed no real change in either PPR or the amplitude of E1.

MATERIALS AND METHODS

Electrophysiology.

We used intracellular recording to measure the resting membrane potentials and EPSPs of the crayfish cells. To make microelectrodes, we placed small glass tubes (diameter 1.2 mm) into a horizontal electrode puller (PUL-1, World Precision Instruments). The puller heated the midsections of the tubes while pulling the two ends apart in order to create two micropipettes with sharp tips. We filled the micropipettes with 3 M KCL, then, using a micro-manipulator, inserted them into the crayfish muscle cells to record any changes in membrane potential or EPSPs. The resistance of each microelectrode was

between 4-10 MΩ. To stimulate EPSPs in the muscle cells, we sucked up nerves into a suction electrode that was attached to a Grass SD9 Stimulator and sent a current through the electrode and across the nerve. The nerves were stimulated with paired stimuli at a low frequency (.4 pps), a voltage of approximately 40 volts (range of 40 - 42 V), and a duration of .3 ms, with a delay of 40 ms between each stimulus. Recordings of the EPSPs were collected by using the computer program SCOPE.

Crayfish.

We dissected crayfish (Wards) by separating the crayfish's abdomen from the thorax. To minimize suffering of the crayfish, we froze the crayfish to slow the crustacean's bodily functions, which had an effect equivalent to anesthesia. We cut along the lateral ridges towards the posterior of the crayfish abdomen in order to separate the ventral side from the dorsal side of the crayfish. To expose the extensor muscles, we pushed all excess tissue out. With two pins, we secured the dorsal exoskeleton and superficial extensor muscle to the silicon elastomer at the bottom of a bowl filled with Ringer's solution.

Solutions and treatments.

The physiological saline solution used when establishing the baseline EPSPs, known as Ringer's solution, contained 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl2, 13.5 mM CaCl2, 10 mM HEPES and had a pH of 7.4. Both NAAG (Sigma-Aldrich) and β-NAAG (Sigma-Aldrich) were diluted from a 10 mM stock solution to a concentration of 40 μM by dissolving 400 μL of stock solution in 100 mL of Ringer's solution. We added the solutions and treatments to the observation dish via a syringe. To establish the baseline measurements, we added 100 mL of Ringer's solution to the observation dish and took measurements for 5 minutes. In experiments with NAAG and β-NAAG, we removed 25 mL of Ringer's solution from the observation dish and replaced it with 25 mL of treated solution applied via a syringe with measurements taken for 15 minutes afterwards. After the experiments with NAAG and β-NAAG, we washed the treatment from the solution by removing 50 mL of the treated solution from the observation dish and adding 50 mL of Ringer's solution. One wash repeated this process three times to ensure that the concentration of NAAG or β-NAAG was an eighth of its original concentration. For each experiment, we performed the wash twice to ensure the concentration went down to a sixty-fourth of its original concentration, to a negligible amount.

Analysis.

To analyze the data, we compared the average amplitudes of the 1st EPSP of the pair (E1) during the treatment to those during the washout and baseline periods. We also compared the paired-pulse ratios to determine whether changes are taking place presynaptically or postsynaptically. We calculated paired-pulse ratios by dividing the amplitude of the second EPSP by the amplitude of the first EPSP (E2/E1).

RESULTS

We observed the effect of NAAG and β-NAAG on EPSPs in the crayfish neuromuscular junction by measuring the amplitude of the EPSPs elicited by paired-pulse stimuli and the resulting paired-pulse ratios. Our paired-pulse stimulation involved sending a pulse into the crayfish nerve, followed 40 ms later by a second pulse (also known as 'twin pulses'). We used paired-pulse facilitation because PPF is a presynaptic phenomenon, and changes in PPF indicate whether modulation is pre- or post-synaptic. As seen in Fig. 1, in four separate experiments (two experiments for each treatment), we found no significant difference in the average amplitude of E1 when NAAG or β-NAAG was applied to the crayfish neuromuscular junction; however, we did observe a decrease in EPSP magnitude after adding NAAG to our solution in the first experiment (Fig.1). Our study also found no significant difference in average PPRs when NAAG or β-NAAG was applied (Fig.2).

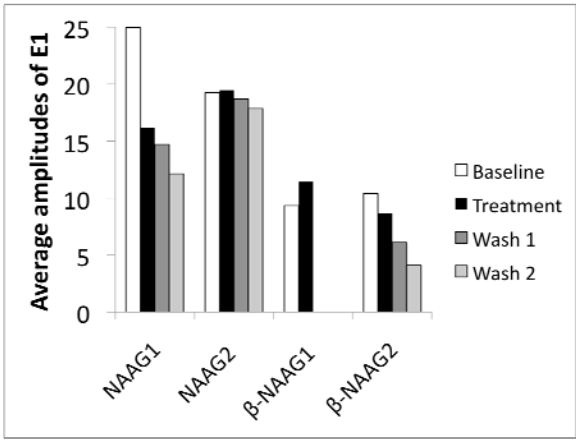

FIG. 1. Average amplitudes of E1(all data measured in millivolts (mV)) at baseline, treatment, first wash, and second wash. Each group of bars is labeled with the treatment performed. Each bar represents a different part of the experiment. Data was obtained by measuring the amplitude of E1 (time between recordings: <0.00 seconds) and calculating the average of these amplitudes for each part of the experiment. We did not perform the washes on the first experiment (β-NAAG1).

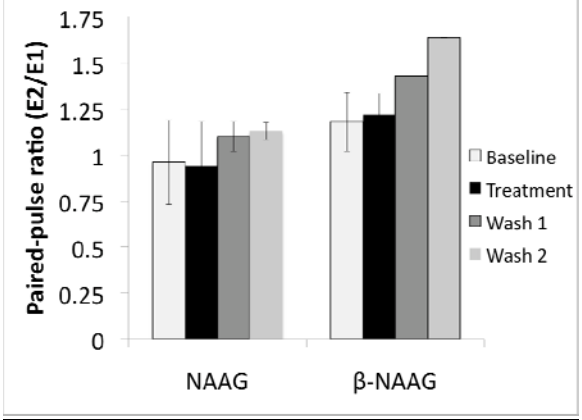

FIG. 2. Average paired-pulse ratios (E2/E1) at baseline, treatment, wash 1, and wash 2. Errors bars represent the difference of the two averages found. The data calculated determined that there were no significant differences to be found in the average paired-pulse ratios when either NAAG or B-NAAG (10 μM) was administered in the crayfish neuromuscular junction.

DISCUSSION

We used EPSP magnitude and the paired-pulse ratio to determine whether there were any changes in synaptic transmission during treatments and, if so, whether the changes were due to post- or presynaptic regulation. Paired pulse ratios (PPRs) would change if there were presynaptic regulation. However, if NAAG had a postsynaptic effect, we would not observe a change in PPR. We expected to observe a change in PPR after we added NAAG to the crayfish solution. Contrary to our hypothesis, our results show that while there were very minor differences between the magnitudes of the baseline and treatment PPRs for both NAAG and β-NAAG, there was some overlap between the margins of error. We therefore conclude that neither NAAG nor β-NAAG affected PPR. This result indicates that any change in amplitude of EPSPs between treatment and the baseline should be credited exclusively to postsynaptic regulation.

In comparing the change between the 1st EPSP elicited of the pair (E1) baseline and treatment amplitudes, we found mixed results. In the first trial of NAAG, the amplitudes of the EPSPs decreased during treatment. The change between baseline and treatment E1 amplitude was substantial enough to indicate that NAAG may have had some effect. This would suggest that NAAG postsynaptically increased the effect of the neurotransmitters or affected the sensitivity of the receptors on the postsynaptic cell. However, in our second trial of NAAG, the difference between the average baseline and E1 amplitudes is too small to be consequential. We observed very small changes in the average amplitudes of the baseline and treatment E1 for the β-NAAG experiments; however, we believe the differences are negligible. This may be due to the fact that β-NAAG cannot be hydrolyzed into glutamate and N-acetylaspartic acid by GCP II.

A few factors may have contributed to the difference between our results from the first and second NAAG experiment. At the beginning of our second NAAG trial, we observed several large Ca^{2+} residuals, so we switched mid-experiment to a solution composed of Ringers solution with half the concentration of Ca^{2+}. NAAG may require a normal Ca^{2+} concentration to be effective. Further, we may attribute this disparity in our results to the degree of variability that is present in all experimentation.

We also wish to point to a possible reason that the EPSP amplitudes during the wash never returned to the pre-treatment levels in our first and third experiment. This may be due to the weakening of synapses over time independent of the treatment, or because of a longer lasting effect of the treatment on the neuromuscular junction after the drug is washed out of the saline solution.

There has been an increased interest in studying NAAG's effects on synaptic junctions because of the theory that low levels of NAAG have a role in the development of schizophrenia (Neale *et al.* 2005). Glutamate hypoactivity may be a factor behind schizophrenia; when NAAG regulates postsynaptically, it has been shown to act as an antagonist to glutamate receptors, which would create hypoactivity.

Prior research has indicated that NAAG may affect EPSPs and the paired-pulse ratios. Pöeschel *et. al.* (2005) showed that exogenous NAAG has a diminishing effect on paired-pulse depression in rat hippocampi (suggesting that NAAG affects a presynaptic mechanism), while Bergeron *et. al* (2007) found that NAAG might reduce the postsynaptic cell's permeability to glutamate. The main questions we were concerned with were, "Will NAAG have effects similar to these in the neuromuscular junction of a crayfish?" and "How will β-NAAG affect EPSP amplitude and PPR?" Our experimental results agree with Poeschel *et. al.*'s (2005) study that found that β-NAAG does not affect either EPSP amplitude or the PPR. However, our two experiments with NAAG provided equivocal results. We would suggest that in any future experiments of this nature, performing more than two trials using NAAG would help clarify its effects at the crayfish neuromuscular junction. Such experimentation would contribute greatly to the growing body of research concerning the effects of NAAG in non-mammalian species.

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REFERENCES

Bergeron R., Imamura Y., Frangioni J.V., Greene R.W., & Coyle J.T. 2007. Endogenous N-acetylaspartylglutamate reduced NMDA receptor-dependent current neurotransmission in the CA1 area of the hippocampus. *Journal of Neurochemistry* 100(2), 346-57.

Gafurov B., Urazaev A.K., Grossfeld R.M., & Lieberman E.M. 2001. N-acetylaspartylglutamate (NAAG) is the probable mediator of axon-to-glia signaling in the crayfish medial giant nerve fiber. *Neuroscience* 106(1): 227-35.

Lea P.M., Wroblewska B., Sarvey J.M., & Neale J.H. 2001. Beta-NAAG rescues LTP from blockade by NAAG in rat dentate gyrus via the type 3 metabotropic glutamate receptor. *Journal of Neurophysiology* 85(3): 1097-106.

Neal, J., Olszewski, R., Gehl, L., Wroblewska, B., & Bzdega, T. 2005. The neurotransmitter N-acytylaspartylglutamate in models of pain, ALS, diabetic neuropathy, CNS injury and schizophrenia. *Pharmacological Sciences* 26: 477-484.

Pöschel, B., Wroblewska, B., Heinemann, U., & Manahan-Vaughan, D. 2005. The Metabotropic Glutamate Receptor mGluR3 is Critically Required for Hippocampal Long-term Depression and Modulates Long-term Potentiation in the Dentate Gyrus of Freely Moving Rats. *Cerebral Cortex* 15: 1414-1423.