Pioneering Neuroscience, 2010, **10**, 27-31

NAAG as a Potential Neurotransmitter at the Crayfish Neuromuscular Junction

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ABSTRACT

N-acetylaspartylglutamate (NAAG) is the most abundant peptide neurotransmitter in the mammalian central nervous system. It is composed of aspartate and glutamate, two fundamental amino acids, and modulates synaptic transmission via the activation of group II metabotropic glutamate receptors (mGluR II). In the current study, we asked whether NAAG has a similar role in non-mammals, specifically invertebrates, such as the crayfish. Our work reported here suggests that NAAG does indeed modulate synaptic transmission at the neuromuscular junction of crayfish. Metabotropic glutamate receptors, and more specifically mGluR II, facilitate the modulation. Through the application of NAAG and LY341495 (a mGluR II Antagonist), membrane depolarization was modulated after high frequency stimulation, indicating a change in synaptic transmission. Application of NAAG increased EPSP amplitude in a dose-dependent manner. Application of LY341495 decreased EPSP amplitude. Through this increased understanding of NAAG's role in the neuromuscular junction, we will enhance our knowledge of the phylogeny of NAAG and the pathways necessary for plasticity in crayfish synapses.

INTRODUCTION

N-acetylaspartylglutamate (NAAG) is distributed in millimolar concentrations throughout the mammalian brain. NAAG has been identified as a classical neurotransmitter concentrated in synaptic vesicles and co-localized with several other neurotransmitters such as GABA, glutamate, acetylcholine (ACh), and dopamine (Keller et al., 2004). Although this research does not specifically address NAAG's catabolic enzyme, GCP II, that is unique to astrocytes, NAAG is capable of playing an important role in cell-specific glial signaling. The role of NAAG in the vertebrate brain is central to the regulation of neurons, as well as the function and maintenance of the nervous system and can be considered as an important direction for future research (Baslow, 2000).

Being a classical neurotransmitter, NAAG is released in a Ca^{2+} dependent manner from synaptic vesicles and has been shown to bind to group II metabotropic glutamate receptors (mGluR II) with high affinity (Keller et al., 2004). Once NAAG activates mGluR II, the result is a presynaptic inhibition of transmitter release, which implies that it may serve as a potent presynaptic inhibitor of EPSPs (Keller et al., 2004). Little is known about the role of NAAG in non-mammalian organisms such as invertebrates. Since NAAG is a prevalent neurotransmitter in animals higher on the phylogenic tree, there is reason to believe that its effects may be seen in more simple organisms such as the crayfish.

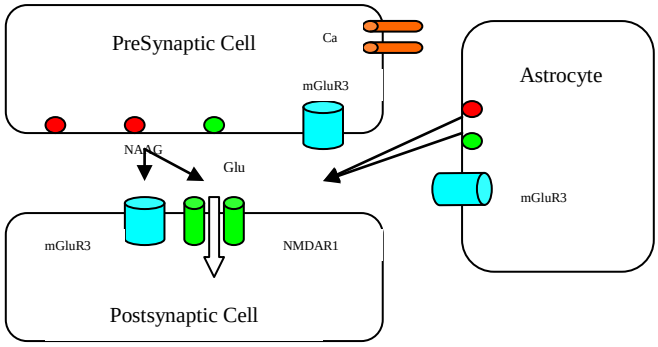

Figure 1. . Cell Diagram of the current idea of NAAG function. Modified from Neale *et al* 2000.

mGluRs are a family of eight receptors divided into three groups. The individual groups are based upon pathway similarity, pharmacological responses, and relationships to second messengers, with the principal of altering neuronal excitability (Neale and Salt, 2006; Balschun et al., 1999). Excitability is affected through the mGluRs presynaptic effects on neurotransmitter release and postsynaptic modulation of ionic conductance. The activation of mGluRs influences neuronal activity from a few seconds to making permanent changes in synaptic efficacy (Neale and Salt, 2006).

Using standard intracellular recording, NAAG was found to possess the ability to increase synaptic transmission at the neuromuscular junction of the crayfish. The mGluR2 antagonist LY341495 was also shown to effectively decrease EPSP output when present in the extracellular solution. These findings further indicate NAAG's importance as a neurotransmitter by

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extending its prevalence to animals such as the crayfish. The mGluR2 target is also imperative to signaling in this neural circuit.

MATERIALS AND METHODS

Materials
Crayfish ringer solution (Grinnell College) 5.4mM KCl, 1.96mM NaCl, 13.5mM $CaCl_2 \cdot H_2O$, 2.6 mM $MgCl_2 \cdot 6H_2O$, and 10mM HEPES was made weekly and refrigerated. Synthetic NAAG (Tocris) was diluted in crayfish ringer solution and stored at 0ºC. Synthetic LY341495 (Tocris) was diluted in DMSO and stored at 0ºC.

Animals
Crayfish were obtained through Grinnell College and kept according to housing standards. Animals were placed on ice for a minimum of 15 minutes before any experimentation was conducted

Application of NAAG
EPSP amplitude was measured in response to various doses of NAAG (0 (control), 10µM, 20µM, 100µM) applied for 25 minutes prior to recording in an *in vivo* preparation of the crayfish tail extensor muscle. Crayfish tail extensor muscle was exposed by a complete severing of the tail followed by removal of the ventral exoskeleton. The flexor muscle and neuronal bundles were removed by rough hand dissection from anterior to posterior end. Nerve endings were isolated and suctioned into a glass electrode across which a stimulus was applied (4-100V, duration 0.9 ms) to evoke EPSPs. Glass micropipette electrodes filled with 3M KCl were placed in the muscle that is innervated by the isolated nerve (Resistance: 4-12 MΩ). Nerves were stimulated by high frequency stimulation to induce synaptic modulation by 5 Hz stimulation 3 times for 5sec with 1sec between each train. Recording of the voltage EPSPs are collected by SCOPE computer program.

Application of mGluR II Antagonist +/- NAAG
The same recording and dissection described above was used. LY341495 was applied (0 (control), 20nM) in the absence of synthetic NAAG and EPSPs were measured. LY341495 and 20µM NAAG were added to the bathing solution at the same time EPSPs were measured. An average of 10 recordings from 3 different preparations were quantified.

Statistical Analysis
HFSR (High Frequency Stimulation Ratio)= post high frequency stimulation EPSP amplitude/ pre high frequency stimulation EPSP amplitude.
Repeated measures ANOVA was used to analyze SCOPE data for changes in amplitude of EPSPs and duration or increased voltage.

RESULTS

We used electrophysiological techniques to record EPSPs before and after high frequency stimulation in the presence of NAAG and an antagonist for one of its receptors, mGluR group II. Using both a normal stimulus to evoke action potentials and high frequency stimulation, the application of NAAG effectively increased both amplitude and occurrence of EPSPs. The mGluR II antagonist LY341495 decreased EPSPs after the normal and high-frequency stimulations. Figures 2, 3, and 4 depict the voltage recordings under the various experimental conditions.

Figure 2 characterizes the EPSP outputs at the various NAAG dosage levels. As more NAAG was added to the bathing solution larger EPSP amplitudes were evoked. Figure 3 shows the EPSP response when LY341495 was added to the bathing solution. With only the antagonist present, EPSPs decreased as the concentration of LY341495 increased (Figure 3A, 3B). The addition of both NAAG and the LY341495 had EPSP amplitudes that increased by 2mV.

HSFR were maximized in the NAAG preparation at 10 µM. This was due to the continual rise of prestimulation EPSPs at high doses of NAAG.

The last aspect that was addressed was the tendency of EPSPs to decrease over a period of time after high stimulation, which indicates short term facilitation (Figure 4).

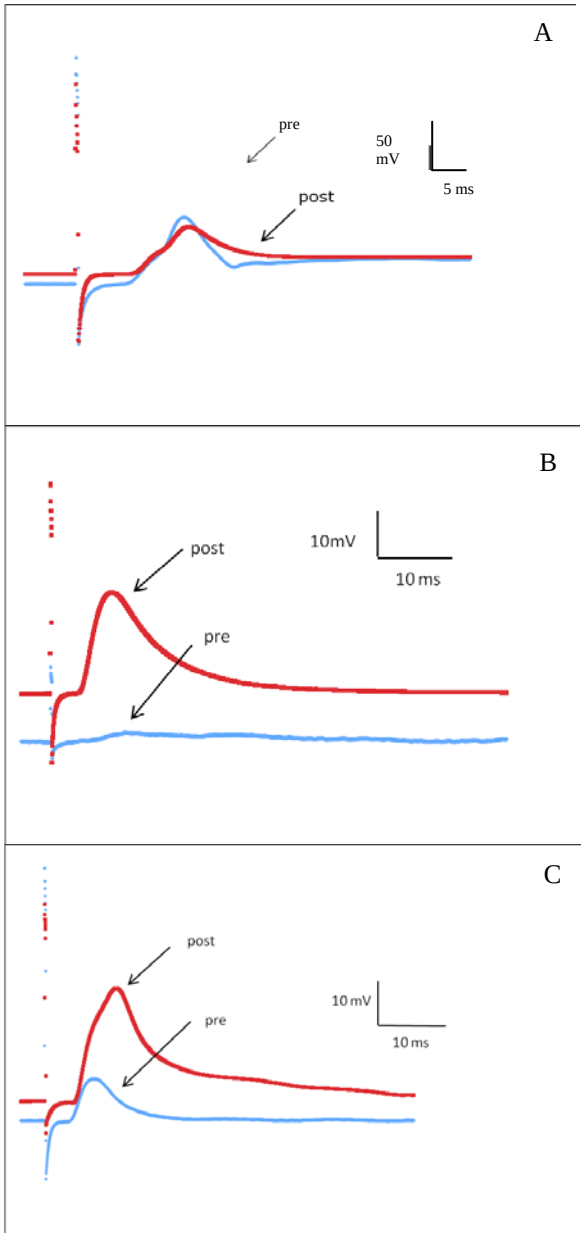

Figure 2. EPSPs at the crayfish neuromuscular junction with dose response to NAAG. A. 0 µM of NAAG was applied. Amplitudes were comparable. B. 10µM of NAAG was applied. Amplitudes are 20mV greater after high frequency stimulation (+/- 4). C. 20µM of NAAG was applied. Amplitude is 15mV greater after high frequency stimulation (+/- 2). (n=3).

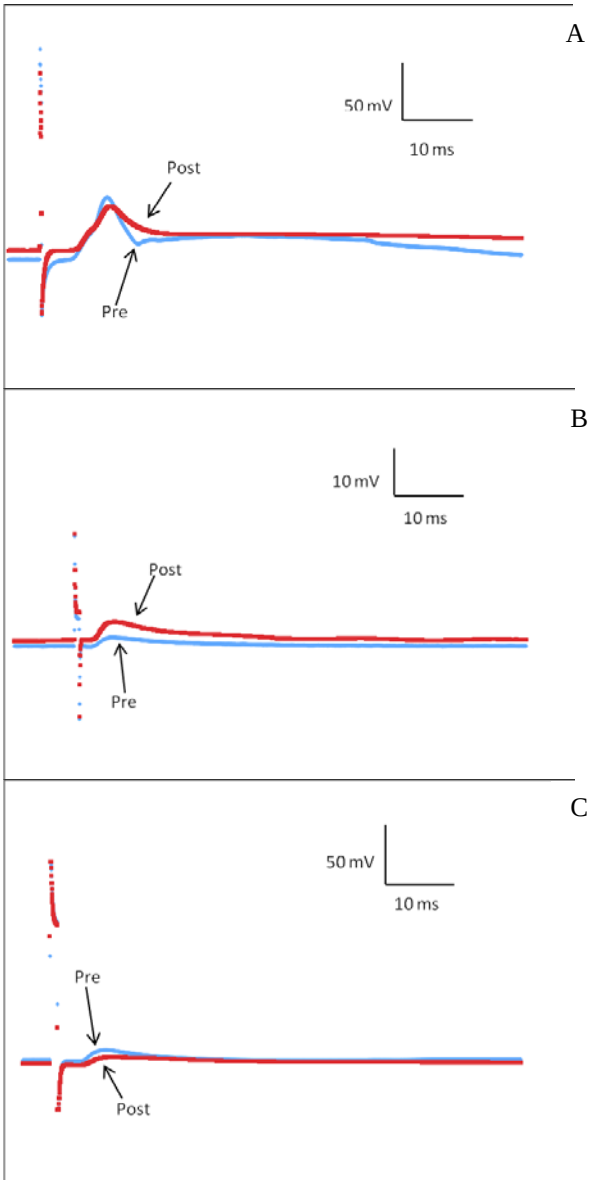

Figure 3. EPSPs of the crayfish neuromuscular junction with the application of synthetic mGluR II Antagonist +/- NAAG. A. 0nM mGluR II Antagonist and 0µM NAAG was applied. Amplitudes were comparable. B. 20nM mGluR II Antagonist and 0µM NAAG was applied. Amplitude is 3mV greater before high frequency stimulation. C. 20nM mGluR II Antagonist and 20µM NAAG was applied. Amplitude is 2mV greater post high frequency stimulation. (n=3).

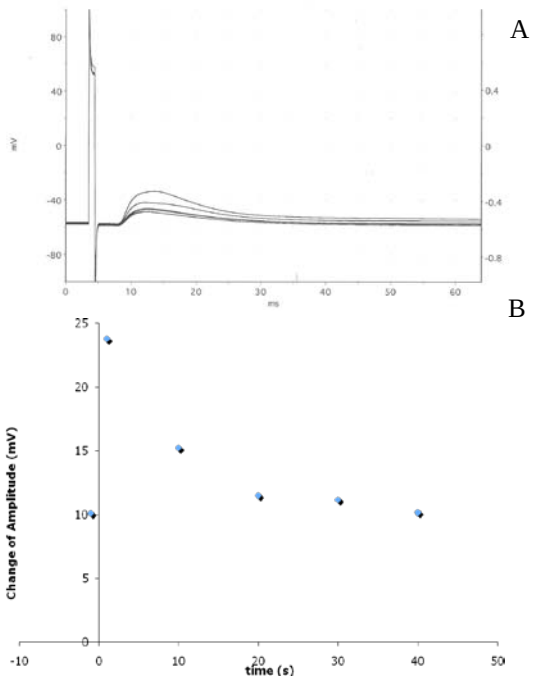

Figure 4. Time sequence of decrease in EPSP over time after high frequency stimulation. Poststimulation traces were collected immediately after high frequency stimulation as an average of 10 scans (each scan every 0.5 sec) with 5 sec between traces (total of 5 poststimulation traces). A. Scope data from NAAG 20 µM application. Bottom trace from prestimulation and poststimulation trace 5. B.ΔAmplitude of EPSP over time with application of 20 µM NAAG. Time spans for mGluR EPSP short term potentiation were comparable (Data not shown). (n=4).

DISCUSSION

NAAG does affect the amplitude of EPSPs indicating that NAAG is a neurotransmitter at the crayfish neuromuscular junction. We applied NAAG and a pharmacological block of mGluR group II (LY341495) and measured the pre and post high frequency EPSP. NAAG and its receptor play a distinctive role in modulation of EPSPs at the neuromuscular junction. When NAAG was applied to the prep, EPSP amplitude increased and HFSR increased, and when the mGluR group II antagonist was applied the EPSP amplitude and HFSR decreased. However when both NAAG and mGluR group II antagonist were applied, effects were reduced compared to just NAAG, but still had positive increases in HFSR.

Previous experimentation on synaptic modulation at the neuromuscular junction of crayfish have found post stimulated EPSPs to remain elevated for hours (Krasne and Edwards, 2002). However, Figure 4 indicates that the EPSP stimulated in these experiments only lasted for minutes. This indicates that the results that are present in Figure 2 and 3 are the result of short-term potentiation instead of long term.

NAAG dose response continued to increase as the dose of NAAG increased (Figure 1). Previous data has shown that NAAG has a plateaued response after 100µM (Westbrook, 1986). We were not able to use high frequency stimulation with such high concentrations of NAAG. However, it would be useful to have a more complete dose response curve at the crayfish neuromuscular junction. This continual increase in EPSP could be an effect of the synthetic application. This technique introduces unnatural amounts of NAAG to the neuromuscular junction. However, because of the continued increase in EPSPs even up through 100µM, the receptors for the NAAG must still be present, but unused in the natural crayfish. Excess amounts of receptors could be present at this location for non-normal usage (i.e. cases of extreme conditions where the crayfish must modulate its behavior). This supports the notion of NAAG being involved in short-term potentiation (Figure 4), because this response is only necessary for small amounts of time in order to change synapses for short term changes in the environment.

NAAG has been associated with mGluR group II and group III (Neale *et al.*, 2000). The group II antagonist alone decreases the effects of some but not all effects of synthetically applied NAAG. In Figure 3B, there is a decrease in EPSP after high frequency stimulation, which is the opposite result from the application of NAAG. Thus NAAG's effects could be mediated through mGluR group II. However, when NAAG was added with the group II antagonist, there is still an increase in amplitude after the high frequency stimulation (Figure 3C). If NAAG was only binding to group II, the amplitudes should be equivalent. Since they are not equivalent, NAAG must still be affecting the neuromuscular junction. This effect is most likely to be mediated by mGluR group III.

Thus NAAG, when applied to the crayfish neuromuscular junction, increases EPSPs. NAAG is a neurotransmitter in this system, which binds to mGluR group II, and most likely group III as well. It is still unclear if NAAG is endogenous to the crayfish neuromuscular junction. However, due to the quantity of receptors, NAAG could be present in very high amounts during certain situations that could indicate that it would need a feedback mechanism that was highly regulated, which would most likely occur via the astrocyte neighbor cell. However, there are several other agonists for mGluR group II that could also be binding to the receptors instead of NAAG. Further investigation should block additional potential NAAG receptors to isolate the effects of mGluR group II.

ACKNOWLEDGEMENTS

We thank Clark Lindgren, without whom we would not have discovered the importance of NAAG and for all of the preparations he did for obtaining chemicals, crayfish and other lab necessities.

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