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N-Acetylaspartylglutamate and Glutamate Carboxypetidase II are Present at the Crayfish Neuromuscular Junction

COLIN FRY, AARTI KOLLURI, and NIKKI SCHERRER
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

N-acetylaspartylglutamate (NAAG) is the most abundant neuropeptide in the mammalian central nervous system and has been shown to be involved in axon-glia communication in crayfish. Studies have established that NAAG is hydrolyzed by glutamate carboxypetidase II (GCP II), resulting in the breakdown of NAAG into NAA and glutamate. Additionally, NAAG is an agonist of the metabotropic glutamate receptor, type 3 ($mGluR_3$). In our study, we explored the existence of GCP II at the crayfish neuromuscular junction by inhibiting GCP II and $mGluR_3$. We measured excitatory post synaptic potentials (EPSPs) in control settings, in the presence of GCP II inhibitors and in the presence of $mGluR_3$ antagonists with GCP II inhibitors to determine if GCP II affected the strength of the potentials generated at the neuromuscular junction. Reductions in EPSPs in experimental groups compared to the control suggest that GCP II plays a role at the crayfish neuromuscular junction.

INTRODUCTION

N-acetylaspartylglutamate (NAAG) is an important neuromodulator in the nervous system of various organisms. Research has determined that NAAG plays a role in motor functions of mammals, specifically at the neuromuscular junction (Malomouzh et al. 2005). The neurotransmitter is also involved in the formation of glutamate. It has long been accepted that glutamate is an abundant neurotransmitter and that a portion of the glutamate found in an organism is produced as a result of the hydrolysis of NAAG, as facilitated by the enzyme GCP II (Malomouzh et al. 2005). Excess levels of glutamate have been linked with various neurological conditions and GCP II inhibition has been suggested as a viable treatment for these disorders.

A study conducted by Berent-Spillson et al. (2004) discovered that increased NAAG concentrations may protect against cell degeneration in high glucose environments. This was found to be due to the neuronal effect NAAG has on the group II metabotropic glutamate receptor, $mGluR_3$. It was predicted that GCP II inhibition or $mGluR_3$ inhibition could be used as a therapy for diabetes related neuropathies (Berent-Spillson et al. 2004). Similarly, another study published by Ajit et al. (2006) linked excessive glutamate to neurodegenerative disorders, including stroke. They hypothesized that GCP II inhibition would suppress hydrolysis of NAAG and therefore lower concentrations of glutamate, which would protect neurons from damage sustained by a stroke. They tested this by inhibiting GCP II and inducing a stroke in live rats. The rats were then studied and the findings were compared to data from rats who were subjected to a stroke without the GCP II inhibition.

It was found that the GCP II inhibition decreased the amount of neuronal damage and the effects of symptoms such as neuropathic pain. The experimental condition also increased the survival rate of the rats post-stroke, verifying that GCP II inhibition was an effective means of protecting from neuronal damage caused by excess glutamate (Thomas et al. 2006).

Previous research has established that both NAAG and GCP II are present in crayfish, specifically in axon-glia communication (Urazaev 2001). However, questions still remain regarding the specific function and role of NAAG and GCP II in the crayfish neuromuscular junction. Crayfish were used as a model organism to further our knowledge of synaptic transmission and NAAG signaling. We hoped to identify the presence of GCP II at the neuromuscular junction. More specifically, we wished to determine how the inhibition of GCP II hydrolysis of NAAG affects EPSP amplitude. To verify if NAAG activity is affected by GCP II in crayfish, we suppressed GCP II activity and monitored the change in EPSP measurements. In addition to GCP II inhibition, we also blocked $mGluR_3$, as NAAG also functions as an agonist at these receptors (Bergeron 2007) and it is necessary to eliminate any unanticipated interaction it may have, which might alter our results.

Following the inhibition of GCPII with ZJ-43 or the antagonism of mGluR3 with LY341495, we observed decreases in EPSPs in our experimental groups compared to our control group. This result suggests that GCP II is present at the crayfish neuromuscular junction.

MATERIALS AND METHODS

Crayfish Tissue Preparation

Procambarus clarkii were submerged in ice for 15 minutes or until rendered unresponsive. The tail was separated from the crayfish body. Then, the ventral half of the crayfish exoskeleton tail was removed, including the internal tissue and muscles leaving four superficial extensor muscle fibers intact on the dorsal side of the tail (Fig. 1). The dissected tail was then placed in a bowl, pinned at the base and head of the tail to silicone elastomer and submerged in 100ml of Ringer's solution. The solution was replaced every 30 minutes to refresh cells and discard cellular waste.

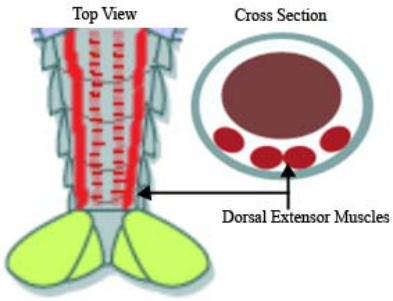

Figure 1. Dissected Crayfish Tail Following proper dissection, the crayfish tail resembles the figure above. Four strands of superficial extensor muscles are visible under the dorsal side. The large mass of muscles seen in the side view are removed leaving behind only the four strands of superficial extensor muscles.

Intercellular recording

Using a World Precision Instruments microelectrode puller, we made microelectrodes from 1.2mm borosilicate glass capillary tubes. The electrodes were filled with 3 M KCl and placed into an electrode holder also filled with 3 M KCl. Electrode resistance values ranged from 5-10MΩ. With a micromanipulator, the electrodes were maneuvered into the muscle cells allowing the membrane potential to be recorded using the computer program, SCOPE. The nerves of the crayfish were pulled into a suction electrode and stimulated at high (2-4Hz) and low (0.2-0.4 Hz) frequency. Each treatment, control, ZJ-43 application and LY341495 application, was applied for 5 minutes.

Solutions

Crayfish Ringer solution consisted of KCl 5.4mM, NaCl 196mM, MgCl2 2.6 mM, CaCl2 13.5mM, and HEPES Buffer 10mM. Crayfish Ringer solution was made weekly. The pH was adjusted to 7.4. The initial concentrations of ZJ-43 and LY341495 in stock solution were 10mM and 5mM, respectively. The final concentrations of the drugs in the Ringer's solution were 0.1μM ZJ-43(Tocris) and 1μM LY341495 (Tocris). The stock solutions of both drugs were stored at 0 .

Statistical Analysis

To analyze SCOPE data, we averaged five frames prior to high frequency stimulation and took the difference of the pre-high frequency average and high frequency average. The high frequency average was calculated by taking the mean of five values at the EPSP maximum. We divided the difference of the pre-high frequency average and high frequency average by the pre-high frequency average. This procedure was repeated for data under all conditions. The experimental data, specifically LY341495 and ZJ-43 treatments, were then compared to the control data to find the percent difference.

RESULTS

We measured EPSPs using intracellular recording techniques. We exposed control treatments, and experimental treatments to high and low frequency stimulation for durations of 5 minutes in the presence of ZJ-43, LY341495 and both chemicals toghether. Following the addition of GCP II inhibitor, ZJ-43, the high-frequency induced increase in EPSP amplitude was reduced (Figure 2B). Similarly, following the addition of $mGluR_3$ antagonist, LY341495, the high-frequency induced increase in EPSP amplitude decreased (Figure 2A). The addition of both drugs, regardless of order of addition, also resulted in a decrease in the high-frequency induced increase in EPSPs compared to control conditions (Figs 2 and 3).

Our results demonstrate that GCP II and $mGluR_3$ inhibitors also affect EPSP amplitudes during constant low-frequency stimulation. Therefore, GCP II like substances or GCP II itself may contribute to the activity of NAAG at the crayfish neuromuscular junction, as ZJ-43 is known to inhibit the hydrolysis of NAAG by inhibiting the actions of GCP II. A similar effect is seen when $mGluR_3$ are blocked by LY341495. The data suggests $mGluR_3$ may contribute to post synaptic neuromuscular junction because of the decrease in EPSP amplitude (Fig. 3).

Although we found noticeable decreases in EPSPs under experimental conditions, the decreases observed in the presence of only ZJ-43 were not equivalent to the decreases in the presence of LY341495 (Figures 2 and 3). This demonstrates that the inhibition of GCP II and the inhibition of $mGluR_3$ receptors do not have identical affects on synaptic plasticity at the crayfish NMJ. Further, the changes in EPSPs observed with the combination of both drugs differed depending on the order in which the drugs

were applied (Figures 2 and 3). This implies that the sequence of drug application may influence the extent of plasticity.

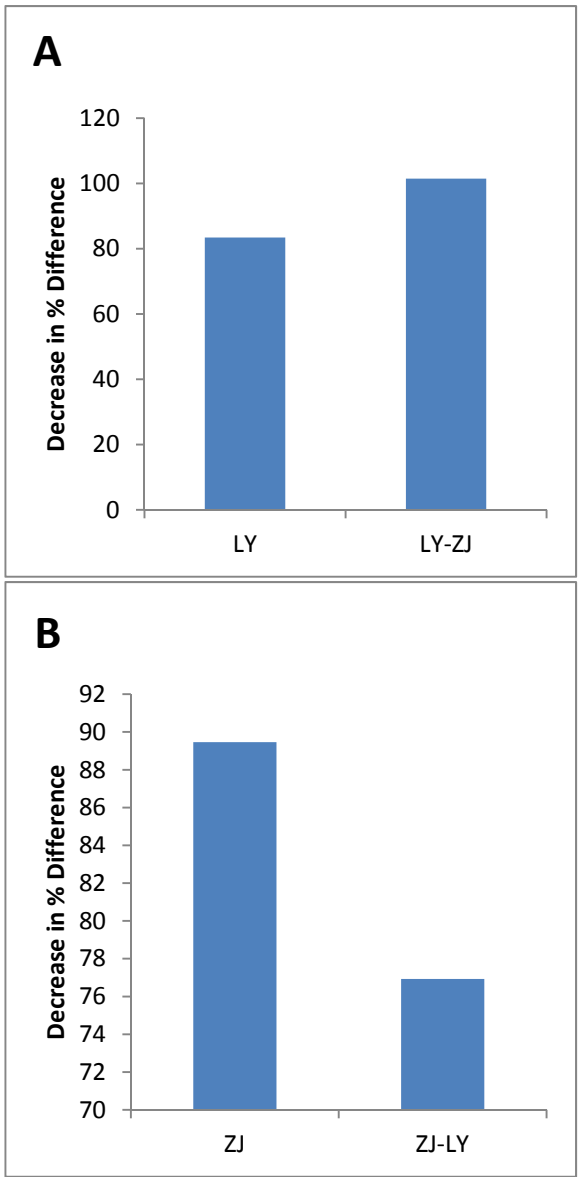

Figure 2. "% Difference of 3Hz Potentiation" – A) This graph depicts the negative difference between the average of the last 10 low frequency frames and the average of frames 170-180 for the high frequency stimulation. Each bar represents the negative % difference of 3Hz Potentiation in the presence of the indicted drug relative to baseline conditions. This depicts the data for the muscle cell that was subjected to LY341495 first and then both drugs. B) This graph depicts the same calculation method as figure 2a but using the average of the last 5 frames of the low frequency and the average of frames 19-23 of the high frequency. This depicts the data obtained from the muscle cell that was subjected to ZJ 43 first and then both drugs.

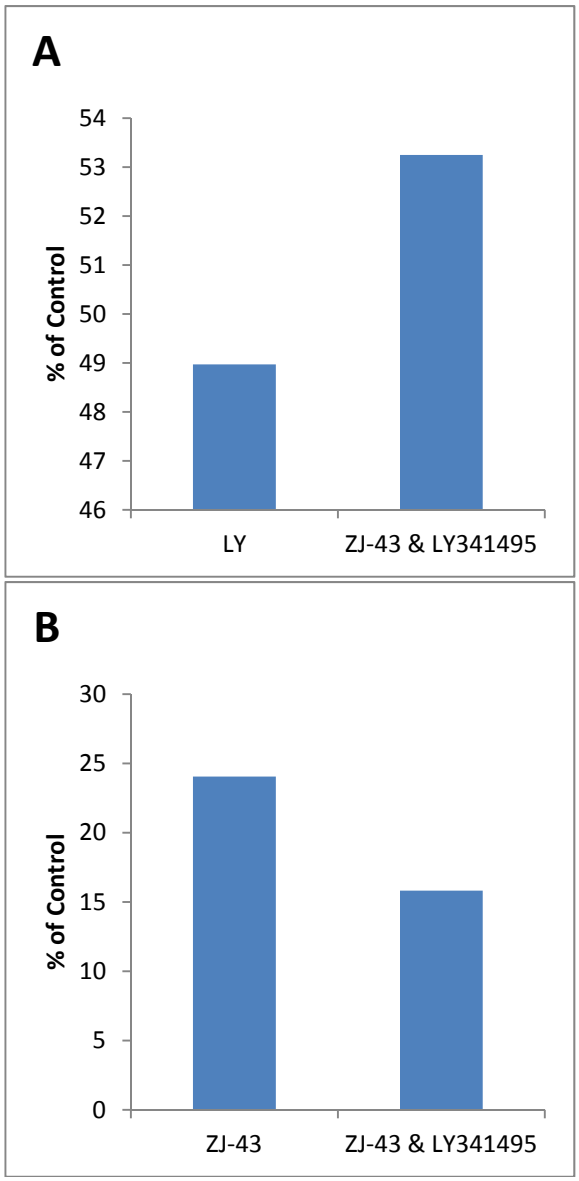

Figure 3. EPSP Strength as % of Control – A) This depicts the average EPSP strength during low frequency for each condition normalized as a percent of the control data at low frequency. This depicts the data for the muscle cell that was subjected to LY341495 first and then both drugs. B) This depicts the same data as figure 3a but for the muscle cell subjected to ZJ-43 first and then both drugs.

DISCUSSION

We hypothesized that the EPSP amplitude and the extent of high-frequency induced enhancement of EPSP amplitude would decrease following the addition of ZJ-43 and LY341495. As seen in Figures 2 and 3, there is a noticeable decrease in EPSP amplitude following the addition of ZJ-43, LY341495 and both drugs in combination compared to control conditions. The decrease in EPSP amplitude in the presence of ZJ-43 indicates that glutamate, possibly produced by NAAG hydrolysis, may play a role as a neurotransmitter.

The addition of ZJ-43 decreased the amplitude of EPSPs. This result points to not only the presence of GCP II but also the presence of NAAG at the neuromuscular junction of crayfish. This must be true because in order for a change in EPSPs to occur in the presence of an enzyme inhibitor, both the enzyme (GCP II) and the compound it acts on (NAAG) must exist simultaneously. Though NAAG has only been shown to be involved in axon-glia communication in crayfish (Urazaev et. al., 2001), both NAAG and GCP II have been shown to exist in in the neuromuscular junction of other species such as rats (Malomouzh et. al., 2005).

The addition of the $mGluR_3$ antagonist, LY341495 also lowered EPSPs, which suggests that $mGluR_3$ play a role in synaptic transmission at the crayfish neuromuscular junction. The addition of the $mGluR_3$ antagonist LY341495 in combination with the GCP II inhibitor ZJ-43 also lowered the EPSP amplitude, implying that un-hydrolyzed NAAG activates $mGluR_3$ when they are available. Our findings regarding the effect of $mGluR_3$ activation and potentiation, as well as the effect NAAG has on $mGluR_3$ are consistent with a previous study (Lea et al. 2005). As Lea et al. (2001) established through experimentation on the rat dentate gyrus, group II metabotropic glutamate receptors, specifically, $mGlurR_3$ are activated by unhydrolyzed NAAG.

We observed that the sequence of drug applications affects the amplitude of the EPSPs measure. The addition of ZJ-43 before LY341495 resulted in a reduction in the percent difference, while the addition of LY341495 before ZJ-43 produced an increase in the percent difference. The reason for this is unclear as the same concentrations of drugs were present in both conditions. Neale et al. (2005) noted that LY341495 counteracts the effects of NAAG peptidase inhibitors such as ZJ-43. This is consistent with our findings in which the addition of ZJ-43 preceded the addition of LY341495(Fig. 2B). Further research is needed to clarify these results.

The suggestions made regarding the presence of NAAG and GCP II in the crayfish neuromuscular junction are significant, because the crayfish neuromuscular junction can serve as a model for synaptic transmission. Inferences regarding NAAG and GCP II gleaned from experiments carried out on the crayfish neuromuscular junction can be applied to other glutamtergic synapses. Should further studies establish the presence of NAAG and GCP II in the crayfish neuromuscular junction, studies such as this could have implications regarding human medicine as well as further understanding of the mammalian synapse.

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