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Group II and III mGluR-linked paired-pulse facilitation is unaffected by agonist NAAG but possibly increased by LY-341,495 block

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ABSTRACT

We investigated the role of group II and III metabotropic glutamate receptors (mGluRs) in short-term synaptic plasticity at the crayfish neuromuscular junction by applying the agonist NAAG (*N*-acetylaspartylglutamate) and antagonist LY-341,495. NAAG is an endogenous neurotransmitter derived from glutamate and an agonist of group II and III (mainly group II) mGluRs. Past studies have investigated the role of NAAG and mGluRs in long-term synaptic plasticity and neurotransmitter release. Group II and III mGluRs are known to decrease synaptic plasticity when stimulated. The goal of this study was to extend that research to short-term synaptic plasticity, particularly paired-pulse facilitation (PPF). To determine whether LY-341,495 and NAAG affected PPF, we induced PPF in crayfish (*Orconectes* sp.) tails while soaked in a solution of control, LY-341,495, NAAG, or LY-341,495 + NAAG saline. We measured PPF with a ratio comparing the amplitude of the second EPSP to that of the first. While the LY-341,495 + NAAG solution significantly increased PPF and the LY-341,495 solution marginally increased PPF, NAAG alone did not affect PPF.

INTRODUCTION

Ever since their discovery in the middle to late 1980s, metabotropic glutamate receptors (mGluRs) have been of growing interest to researchers due to their role in long-term synaptic plasticity and their potential medical applications (Adedoyin et al. 2010; Conn and Pin 1997; Wierońska et al. 2015). The group II and III mGluRs in particular are known to inhibit neurotransmitter release at glutaminergic synapses. Both are antagonized by the drug LY-341,495, but they (the group II mGluRs in particular) are also activated by NAAG (Begeron and Coyle 2012; Kingston et al. 1998).

NAAG (*N*-acetylaspartylglutamate) is an endogenous neurotransmitter at crayfish glutaminergic synapses (Buttram et al. 2002). It is synthesized from glutamate (which can be derived from glutamine) and spartate (or its derivative NAA, *N*-acetylaspartate) (Lieberman et al. 2006). Once in the synaptic gap, NAAG can be hydrolyzed back to glutamate, and is nearly as potent as glutamate itself as an activator of group II mGluRs (Buttram et al. 2002).

LY-341,495, on the other hand, is a synthetic drug and highly potent antagonist of group II and III metabotropic glutamate receptors (Kingston et al. 1998). It is known to display very rapid kinetics for binding to these receptors (Johnson et al. 1999; Wright et al. 2000). The percentage of antagonized mGluRs is time-dependent at low nanomolar concentrations, but reaches a plateau at around 10 μM, the concentration we chose to use for our experiment (Howson and Jane 2003).

A growing number of studies corroborate NAAG's role in modulating synaptic plasticity. For example, Adedoyin et al. (2010) found that NAAG inhibits synaptic transmission and plasticity in the mouse amygdala, and Pöschel et al. (2005) found that NAAG reduces the magnitude of long-term potentiation via group II mGluRs. This mechanism is believed to target presynaptic group II mGluRs which decrease cAMP and protein kinase A when activated, leading to a lowering of L-type calcium channel conductance (Pöschel et al. 2005; Zhao et al. 2001). This produces a feedback mechanism whereby the release of NAAG also inhibits the future release of more neurotransmitter. However, few studies were found on the effect of NAAG on paired-pulse facilitation (PPF), which is a specific type of short-term synaptic plasticity as only a short period following the initial EPSP is affected. Because PPF depends on having a high enough presynaptic calcium concentration, it follows that NAAG may affect PPF.

The goal of this experiment was to explore the role of group II and III mGluRs in PPF, tested with NAAG and LY-341,495. We tested this by eliciting PPF in crayfish neuromuscular junctions in control, NAAG, LY-341,495, and LY-341,495 + NAAG solutions. Crayfish were used because they are simple to prepare and have easily accessible, exclusively glutaminergic neuromuscular junctions. We hypothesized that NAAG inhibits paired-pulse facilitation, measured by the decreased ratio of the amplitude of the second evoked EPSP to that of the first. This is because NAAG is an

agonist of mGluRs that decrease presynaptic calcium, an abundance of which is necessary for PPF to take place. In a similar vein, we also predicted that LY-341,495 would increase PPF by preventing the negative feedback mechanism that would usually inhibit release of additional neurotransmitter. Therefore, adding LY-341,495 should increase the ratio of the second EPSP amplitude to the first.

We found that the mean EPSP ratio of the LY-341,495 + NAAG condition was significantly higher than that of the control condition, while the LY-341,495 condition was only marginally significant compared to the control. However, we found no evidence that NAAG significantly changed the EPSP ratio. The difference between the control condition and the wash conditions were only marginally significant.

MATERIALS AND METHODS

Materials

Crayfish preparation

The crayfish (*Orconectes* sp.) was anesthetized in an ice water bath. We cut off the tail at the base. Using a scissor, we cut the tail along both ventral sides slightly above the line on the exoskeleton. After gently pulling and cutting off the ventral surface to remove the swimmerets, we gently pushed out the deep phasic flexor muscles, leaving the deep phasic and superficial extensor muscles attached to the exoskeleton. The tail was pinned down ventral-side-up on Sylgard in a 40 mL petri dish (Dow Corning Corporation, Midland, MI) and immersed in crayfish Ringer's saline (see *Solutions* below). The preparation was kept at room temperature (24 °C) throughout the experiment. Solutions were replaced with fresh Ringer's saline every 30 minutes to sustain the neuromuscular junction.

Solutions

Low-calcium crayfish Ringer's solution (5.4 mM KCl, 196 mM NaCl, 10.1 mM $MgCl_2$, 10 mM HEPES, 6 mM $CaCl_2$, pH 7.4) at room temperature was used for the control condition and as a base for the other solutions we made (20 μM NAAG, 10 μM LY-341,495, and a solution containing 20 μM NAAG and 10 μM LY-341,495). Concentrations were chosen based on departmental advice.

To make the 20 μM NAAG solution, we used a micropipette (Pipetman, Gilson Inc.) to measure 250 μL of 10 mM NAAG (Sigma-Aldrich Corporation, St. Louis, MO) and diluted it with 125 mL of crayfish Ringer's solution.

The procedure for making the 10 μM LY-341,495 solution was similar, except that we diluted 30 μL of 10 mM LY-341,495 salt (Tocris Bioscience, Bristol, UK) in 30 mL crayfish Ringer's saline.

For the solution containing 20 μM NAAG and 10 μM LY-341,495, we performed the same procedure as above, but used 30 mL of the prepared 20 μM NAAG solution to dilute the 30 μL of 10 mM LY-341,495 instead.

Electrophysiology

Recording electrodes

We made the microelectrodes used to measure cellular electric potential by pulling them from 1.2 mm borosilicate glass capillaries in a horizontal puller (PUL-1, World Precision Instruments). After filling them with 3 M KCl with a fine syringe and clearing away bubbles, they were placed in the right electrode holder (World Precision Instruments). These electrode holders were fastened in place by magnetic holders (Kanetec) and metal clamps (Thermo Fisher Scientific, Inc.) so they would not accidentally fall over. We illuminated the dissection with a Fiber-Lite Fiber Optic Illuminator (Model 190, Dolan-Jenner Industries, Inc.) and observed it through a stereomicroscope (Leica ZOOM 2000).

Suction electrodes

We made suction electrodes from unfilled recording electrodes by sanding off the pointed tips with sandpaper, so that the resulting hole was large enough to accommodate a nerve, and placed one in the left electrode holder (World Precision Instruments). The hollow electrode was connected to a syringe to target nerves to stimulate.

Recording

The recording electrode and a reference electrode were connected to the intracellular electrometer (Model 3160, A-M Systems Inc.), which was in turn attached to the AC/DC differential amplifier which channeled our data into the computer-based data acquisition program LabChart Reader (both by ADInstruments, Dunedin, New Zealand). The resistance of the recording electrode was between 10-20 Ω for the entire experiment.

The suction electrode and a ground electrode were wired to a square pulse stimulator (SD9, Grass Products, Natus Neurology). The settings for paired-pulse facilitation were 0.04 ms duration, 25 ms delay, and a threshold voltage just high enough to elicit EPSPs in the muscle cells. (Because the dependent variable of our experiment was the *ratio* of the EPSPs rather than their amplitude, the differences between crayfish and the voltages we used did not affect our results.)

Experiment

We began the experiment by collecting EPSPs from a crayfish tail soaked in control saline, waiting about 5 seconds between each stimulation to prevent short-term plasticity from influencing later results or the neuron from tiring out. Each trial was the mean of at least five EPSP ratios collected from the same nerve, same muscle, and same solution. After we had collected at least one trial, we switched to either 20 µM NAAG solution or 10 µM LY-341,495 solution. We repeated the same procedure as above, collecting at least one trial before replacing the solution once more with control saline. With this control saline, we were able to perform wash tests to determine if the chemical we had just applied had any lasting effect on synaptic plasticity that might influence our later results. Then, we replaced the control saline with solution containing 20 µM NAAG and 20 µM LY-341,495 and collected at least one more trial, before repeating the wash procedure.

Data analysis

We used LabChart Reader to calculate the amplitude of both EPSPs elicited by each twin-pulse stimulation according to the following formula:

$$amplitude = EPSP_{max}\text{-}EPSP_{min}$$

Then, we used Excel (Microsoft, Redmond, WA) to find the ratio of the second EPSP amplitude to the first:

$$ratio = \frac{EPSP_2}{EPSP_1}$$

We averaged the five ratios obtained from five twin-pulse stimulations we carried out for each trial. A trial was defined as at least five consecutive and successful paired-pulse facilitations in the same nerve, cell, and solution. Switching any of these three things starts a new trial.

To analyze this data, we used MiniTab 17 (Minitab Inc., State College, PA) to run one-way ANOVA tests ($\alpha = 0.1$) on the ratios calculated above to see if there were any significant differences between the means. If a significant difference was found, we followed up with a series of two-tailed *t*-tests to determine which conditions had significantly different results.

RESULTS

Summary

We induced paired-pulse facilitation (PPF) in a crayfish neuromuscular junction in the presence of NAAG, LY-341,495, or both to determine what effect these chemicals have on synaptic plasticity mediated by group II and III mGluRs. The dependent variable we measured was the ratio of the second EPSP amplitude to the first. (An example of PPF results is shown in Fig. 1). The independent variable was the presence or absence of each of the chemicals in the solution. First, we calculated the mean EPSP ratios of the control and experimental conditions (NAAG, LY-341,495, and LY-341,495 + NAAG).

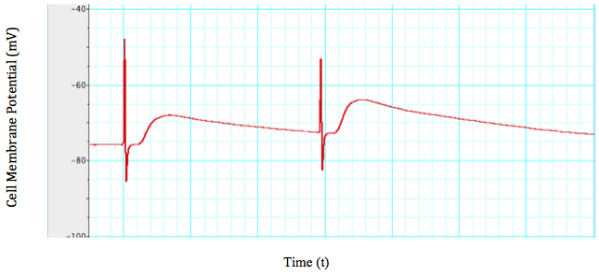

Figure 1. An example of paired-pulse facilitation data recorded for the control condition. The *x*-axis represents time, and the *y*-axis represents membrane potential (mV). The first EPSP was caused by the first stimulation, and then a second stimulation 0.05 ms after caused the second EPSP, which had a larger amplitude.

LY-341,495 and NAAG alone cause no significant change in paired-pulse facilitation

Compared to the mean ratio collected for the control condition (1.10 ± 0.08), that of the LY-341,495 condition (1.46 ± 0.19) was not statistically significant (Fig. 2, $p = 0.123$), but it is close enough to $p = 0.1$ to be considered marginally significant. Though not statistically significant, these results suggest a trend of LY-341,495 increasing PPF by blocking group II and III mGluRs.

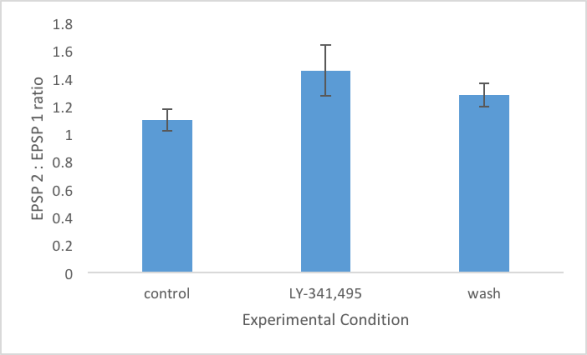

Figure 2. Mean ratio of PPF EPSP amplitudes in control, LY-341,495, and post-LY-341,495 wash conditions. We stimulated neuromuscular junctions with paired pulses in control ($n = 5$), LY-341,495 ($n = 5$), and post-LY-341,495 ($n = 5$) conditions. The *x*-axis designates the control, experimental, and wash conditions, and the *y*-axis represents the mean EPSP amplitude ratio (EPSP amplitude 2 : EPSP amplitude 1). Each trial was calculated from the means of at least five elicited PPF EPSPs. Error bars represent standard error of the mean.

However, the mean ratio from NAAG alone (1.15 ± 0.05) was not significantly different from the control at all (Fig. 3, p = 0.661). We were surprised at these results, since they contradicted our hypothesis that NAAG would decrease the EPSP ratio, as well as previous research into NAAG's effects on synaptic plasticity, which said NAAG lowers PPF by activating group II and III mGluRs.

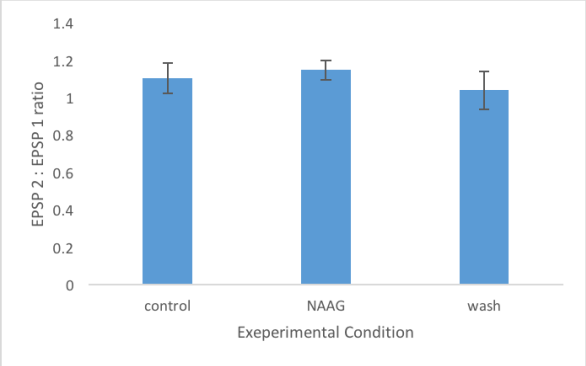

Figure 3. Mean ratio of PPF EPSP amplitudes in control, NAAG, and post-NAAG wash conditions. We stimulated neuromuscular junctions with paired pulses in control (n = 5), NAAG (n = 5), and post-NAAG (n = 4) conditions. The x-axis designates the control, experimental, and wash conditions, and the y-axis represents the mean EPSP amplitude ratio (EPSP amplitude 2 : EPSP amplitude 1). Each trial was calculated from the means of at least five elicited PPF EPSPs. Error bars represent standard error of the mean.

LY-341,495 + NAAG significantly enhances paired-pulse facilitation

The mean ratio collected under the LY-341,495 + NAAG condition (1.34 ± 0.06) was significantly different from that of the control condition (Fig. 4, p = 0.043). Therefore, we know that combining LY-341,495 and NAAG significantly increases PPF at the crayfish neuromuscular junction. However, based on the marginally significant results (p = 0.123) from the trial containing LY-341,495 alone, there is a visible trend showing that it is probably LY-341,495 by itself that is responsible for the change. Even though the LY-341,495 condition had a higher mean EPSP ratio than LY-341,495 + NAAG, we suspect that it was not statistically significant because its standard error (0.19) was larger than that of the LY-341,495 + NAAG condition (0.06).

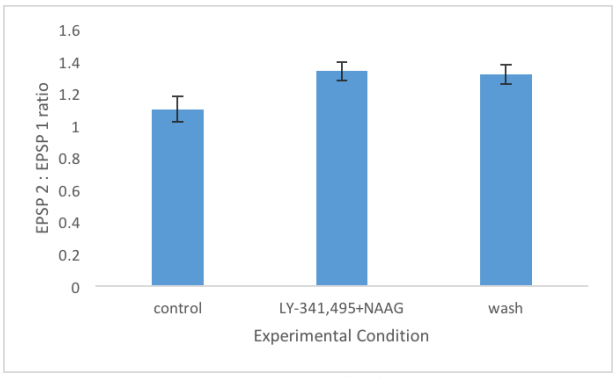

Figure 4. Mean ratio of PPF EPSP amplitudes in control, LY-341,395 + NAAG, and post-LY-341,495 + NAAG conditions. We stimulated neuromuscular junctions with paired pulses in control (n = 5), LY-341,495 + NAAG (n = 8), and post-LY-341,495 + NAAG (n = 3) conditions. The x-axis designates the control, experimental, and wash conditions, and the y-axis represents the mean EPSP amplitude ratio (EPSP amplitude 2 : EPSP amplitude 1). Each trial was calculated from the means of at least five elicited PPF EPSPs. Error bars represent standard error of the mean.

Other variables may have affected results

To determine whether our results could be influenced by unintended aftereffects of NAAG, LY-341,495, or tissue decay over time, we performed another one-way ANOVA test for our wash trials, comparing the means of the control condition with the means of each wash condition. The outcome of this test showed a marginally significant difference between the means (α = 0.1, F = 2.39, p = 0.116). The post-NAAG condition's mean was 1.04 ± 0.10, that of the post-LY-341,495 condition 1.28 ± 0.08, and that of the post-LY-341,495 + NAAG condition 1.32 ± 0.06. Because the means of the post-LY-341,495 and post-LY-341,495 + NAAG conditions are observably higher than that of the post-NAAG condition, we suspect that the strength and persistence of the blocker prevented us from washing it away, possibly influencing some of our results. This is shown by the persistence of high EPSP ratios after the LY-341,495 and LY-341,495 + NAAG steps even after the wash step (Fig. 2 and Fig. 4), in contrast to little if any lasting effects from NAAG (Fig. 3). However, we do not believe that these results were due to tissue decay, because wash conditions were collected on multiple dates at multiple points in time during the experiment.

DISCUSSION

Summary

The results of this experiment showed a trend of LY-341,495 enhancing short-term plasticity at the crayfish neuromuscular junction, as it increased the ratio of the second EPSP amplitude to the first in paired-pulse facilitation. Though the LY-341,495 + NAAG condition was statistically significant compared to control, the solution of LY-341,495 alone was only marginally

significant compared to control. Therefore, we cannot say with certainty that LY-341,495 itself increases PPF, but the observable trend in the data suggests this. (As mentioned in the Results section, the insignificance of the LY-341,495 condition may be due to the much higher standard error of those results.) However, the trend in the data is consistent with our hypothesis that LY-341,495 increases PPF at crayfish neuromuscular junctions through group II and III mGluRs, verifying the receptors' role in short-term synaptic plasticity. However, there was not enough evidence to accept the hypothesis that NAAG decreases PPF at crayfish neuromuscular junctions by the same mechanism. We had predicted that NAAG would activate the group II mGluRs that decrease presynaptic calcium concentration and thus affect PPF (Zhao et al. 2001), but our data did not support this hypothesis.

LY-341,495 may increase PPF

The LY-341,495 + NAAG results were statistically significantly higher compared to the control condition, while the LY-341,495 results were only marginally significantly higher. While we cannot absolutely accept our hypothesis that LY-341,495 increases PPF, the observable trend in the data suggests this to be the case. Although marginally significant compared to control, the trend that LY-341,495 increases PPF is consistent with what we predicted based on the mechanism described in Zhao et al. (2001), in which group II mGluRs reduce presynaptic calcium channel conductance (and thus the presynaptic calcium concentration on which PPF depends) when stimulated. If stimulating group II mGluRs is expected to decrease presynaptic calcium concentration, then antagonizing it should increase the concentration instead, facilitating PPF. However, it was also noted that even though only the LY-341,495 + NAAG condition was actually significantly higher than the control, it had a lower mean ratio (1.34) than the LY-341,495 condition (1.45). As mentioned in the Results section, we suspect that the only reason why the LY-341,495 condition was insignificant compared to control was because of its much higher standard error (0.19 versus LY-341,495 + NAAG's 0.06), which would affect how we calculated significance. Despite the fact that we cannot accept our hypothesis due to lack of statistically significant evidence, we consider it very likely that LY-341,495 increases short-term plasticity (in the form of PPF) at the crayfish neuromuscular junction.

Possible causes for NAAG results

However, we found it troubling that the effect of NAAG on the EPSP ratios of PPF was not significantly different from the control in any way. This clearly contradicts previous research that has not only demonstrated that NAAG decreases synaptic plasticity, but has explained the mechanism by which it does so (Adedoyin et al. 2010; Pöschel et al. 2005; Zhao et al. 2001). Other possible explanations for this discrepancy between the hypothesis and the results include flaws in our experimental design and other qualities of NAAG we did not account for. Ehrenberg and Walder (2010) found that not only did NAAG change the amplitudes of individually elicited EPSPs, it *increased* them in a dose-dependent manner. Though we had originally planned to test the crayfish tail with other concentrations of NAAG, this did not happen due to time and material constraints. Future experiments on this topic could determine whether NAAG's effects (if any) on PPF are dose-dependent, and whether they support our original hypothesis that NAAG decreases PPF. However, from the results of our experiment alone, we must with hesitation reject the hypothesis that NAAG decreases PPF at the crayfish neuromuscular junction.

Applications

Recent research suggests that dysfunctional glutaminergic systems may cause some types of schizophrenia. mGluRs, NAAG, and NAAG's peptidase GCP II in particular have been investigated as potential targets for new psychiatric treatments (Wierońska et al. 2015). GCP II is an enzyme that breaks down and removes NAAG from the synapse (Begeron and Coyle 2012). Schizophrenic patients exhibit less expression of group II mGluRs and GCP II than healthy controls (Ghose et al. 2009). The lack of GCP II allows NAAG to build up in the synapse where it cannot be removed or inhibited, thus reducing release of other neurotransmitters and negatively affecting synaptic communication (Rowland et al. 2013; Volk et al 2015). Our results suggest that LY-341,495 may cause increased neurotransmitter release at glutaminergic synapses. Therefore, adding LY-341,495 may counterbalance some of the effects of excessive NAAG and restore normal synaptic transmission in glutaminergic models of schizophrenia. Future research into drugs targeting the glutaminergic negative feedback loop of NAAG and mGluRs could promise valuable psychiatric treatments for future patients.

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