Pioneering Neuroscience, 2011, **12**, 25-28.

Metabotropic Glutamate Receptors do not Impact Post-Tetanic Plasticity at the Crayfish Neuromuscular Junction

WILL ELSAS, CHRISTI PETERSON, and SAM ROSEN
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

It has been suggested that metabotropic Glutamate receptors (mGluRs) play a role in reducing the symptoms of Schizophrenia. To further understand this disease and how mGluRs function, we studied the effects of group II and group III mGluRs on post-tetanic plasticity at the crayfish neuromuscular junction using intracellular recording. The crayfish tail was immersed in either a standard saline solution, a group II mGluR antagonist saline solution, or a group III mGluR antagonist saline solution. We expected that the addition of these antagonists would result in the inhibition of post-tetanic plasticity and a decrease in the amplitude of excitatory postsynaptic potentials (EPSPs) after stimulating the neurons at a high frequency, expecting that the group II antagonist would have the greater effect. However, our results showed no significant change in the difference between EPSP amplitude before and after the high frequency stimulation when the drugs were administered in relation to the control. This led us to conclude that mGluRs are not an influential player in creating post-tetanic plasticity in crayfish.

INTRODUCTION

Our experiment set out to show if metabotropic glutamate receptors, mGluRs, impact post-tetanic plasticity at the crayfish neuromuscular junction. Post-tetanic plastic is the phenomenon when a cell depolarizes more strongly after a bout of high-frequency stimulation than before the high-frequency stimulation. It is thought to be generated by the residual build-up of Ca2+ in the pre-synaptic cell, and due to the action of mGluRs. The buildup occurs because the active transport mechanisms in the cell take time to remove the Ca2+. mGluRs impact post-tetanic plasticity because they are only activated after high-frequency stimulation, and once activated, mGluRs begin a chain of secondary messengers that temporarily changes how the synapse responds to stimulation. The chain of secondary messengers depends on whether the mGluRs are on the presynaptic cell or the postsynaptic cell.

The review by Neale et al (2005) describes NAAGs involvement in decreasing apoptosis, its protection in stroke models, its part as an analgesic agent, and its role in reducing the effects of PCP that result in symptoms of schizophrenia. NAAG has been found to bind to mGluRs with a preference for group II mGluRs (Gafurov et al, 2001). Getting a better understanding of how mGluRs function will help possibly find a treatment for one of these diseases. To discover more about these molecules, we conducted an experiment to test if mGluRs are a cause of post-tetanic plasticity and if they are located on the pre- or post-synaptic cell.

Fricker et al (2009) found that NAAG acting on group II mGluRs does not have any significant effect on EPSPs or Long-Term Potentiation (LTP). In contrast, Adedoyin et al (2010) found that NAAG acts on group II mGluRs to decrease the EPSP, resulting in a decrease in pain. Similarly to Adedoyin et al (2010), Lea IV et al (2000) credits the interaction of NAAG and mGluR for blockading LTP in the hippocampus. These contradicting results are just one example of how the role of mGluRs is debated and still uncertain. This debate inspired our current research because it shows that the question of how mGluRs affect post-tetanic plasticity in various tissues is currently an unresolved question. According to Gafurov et al (2001), inhibiting group II mGluRs in the ventral nerve chord of a crayfish prevents the hyperpolarization of the glial cells while inhibiting group I and group III mGluRs had no significant effect on the membrane potential of the glial cells. This led us to focus on inhibiting group II mGluRs and observing the resulting changes.

The broad objective of this experiment was to fill in knowledge gaps about the crayfish neuromuscular junction and the role of mGluR. This knowledge will allow for a more detailed study of the neuromuscular junction in crayfish which could be useful in treating glutamate related medical conditions such as schizophrenia. We thought that the group II mGluR antagonist would decrease the magnitude of the post-tetanic plasticity more than the group III mGluR antagonist would, because studies in other organisms have shown that group II mGluR are more active than group III in mediating the release of glutamate (Neale et al, 2005).

When we applied known mGluR antagonists, then the magnitude of the post-tetanic plasticity did not change. We used intracellular recording to compare the

magnitudes of the EPSPs before the high-frequency stimulation (HF) with the EPSPs during HF as well as the first and fifth EPSP following HF when in control saline, saline with a known group II mGluR antagonist, and saline with a known group III mGluR antagonist. We also did some of these trials with paired-pulse stimulation, so we could measure the ratio (PPR) between the first and second pulses. This ratio shows if the change due to our treatments is presynaptic or postsynaptic, since any difference is due to the first cell altering how much neurotransmitter is released. Our experiment showed that blocking the mGluRs did not have any impact on post-tetanic plasticity.

MATERIALS AND METHODS

Preparing Crayfish

The crayfish were submerged in ice for at least 15 minutes prior to the experiment. After cutting the tail off, we cut along the lateral borders of the tail until we cut through the last segment so that we could remove the ventral surface and push away the muscle tissue, leaving four tissue bundles along the dorsal surface through the center of the tail. This was pinned to the dissection plate and submerged in crayfish saline.

Creating micro-electrodes

Using the micro-electrode puller PUL-1 (World Precision Instruments), we pulled 1.2mm glass pipettes and used a microfil syringe tip to fill them with 3M KCl, ensuring that no bubbles were present in the electrodes. We only used electrodes with a resistance of at least 4MΩ.

Preparing solutions

The control condition was a saline composed of 5.4 mM KCl, 196 mM NaCl, 2.6 mM $MgCl_2$ $6H_2O$, 10 mM sodium HEPES buffer, and 13.5 mM $CaCl_2$ $2H_2O$. The first drug added was the group II mGluR antagonist LY-341495. 20µL of the 5mM LY-341495 was mixed with 100mL of saline, creating a solution with a concentration of 1µM LY-341495. We let the crayfish sit in this solution for five to ten minutes before beginning recording to give the drug time to interact with the crayfish muscle cells. Our other variable was the group III mGluR antagonist DL-AP3. We combined 10µL of 10mM DL-AP3 with 100mL of saline creating a solution with a concentration of 1µM DL-AP3. We again let the crayfish sit in this solution for five to ten minutes. When changing the solution after the crayfish was in one of the drug solutions, we rinsed the tissue by letting it sit in standard saline for five to ten minutes and then replacing the saline with the next desired solution.

Stimulating and recording data

We stimulated the nerve cells and recorded the muscle's response to this stimulus with a micro-electrode inserted in the outer bundle of the extensor muscle. For each trial we penetrated a different cell with the micro-electrode. We collected data using single pulse stimuli as well as paired pulse stimuli. When using paired pulse stimuli, there was a 40 millisecond delay between the two stimuli of each pair.

Creating post-tetanic plasticity

To create a post-tetanic plasticity, an electrode stimulated the neuron at a frequency of about 0.2 Hertz. After approximately 30 stimuli, we increased the frequency to 2 Hertz. When recording paired pulses, we switched the stimuli to single pulse simultaneous when increasing the frequency. The frequency remained at 2 Hertz for an estimated 10 seconds then we returned the stimuli frequency to 0.2 Hertz. If the stimuli were originally paired pulse during a particular trial, the stimuli were returned to paired pulse at this time. We finished recording after around another 30 stimuli.

RESULTS

We investigated the role of mGluRs at the crayfish neuromuscular junction in inhibiting or promoting post-tetanic plasticity. Using intracellular microelectrodes within muscle cells and a suction electrode stimulating a nerve attached to those cells, we discovered that there was no significant effect on the post-tetanic plasticity after blocking various mGluRs. In all crayfish tested, post-tetanic plasticity was induced by first stimulating the nerve at .3 Hz for about 150 seconds to establish a baseline magnitude of the EPSPs. Then, the frequency of stimulation was increased to 3 Hz for ten seconds. Finally, the frequency was brought back down to .3 Hz for about another 150 seconds. These changes in frequency created the post-tetanic plasticity. Some of the trials were done with Paired Pulse stimulation, in order to calculate a Paired Pulse Ratio (PPR). This PPR shows if the change caused by blocking mGluRs is pre- or post-synaptic. In all trials, the magnitude of the EPSP was measured for each stimulation. Figure 1 is a typical graph showing how the magnitude of the EPSPs changed over time. The sudden increase in the middle around 80 seconds is due to the high frequency stimulation. After the high frequency stimulation, the magnitude of the EPSPs decreases slowly to baseline levels. The decay was generally fast enough that the 5^{th} EPSP after the high-frequency stimulation was almost entirely back to baseline levels.

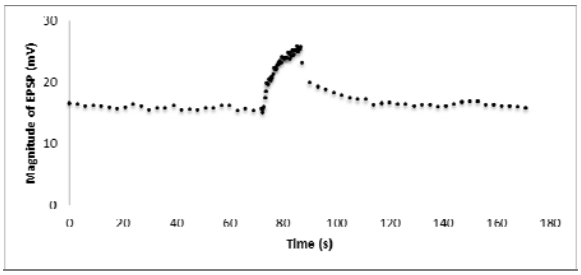

Figure 1. Magnitude of EPSPs over time. This represents one trial in eliciting post-tetanic plasticity. The data points from 0-80 seconds represent the magnitudes of the EPSPs at the low frequency stimulation of .2 Hz, the points from 80-90 seconds representing the high frequency stimulation of 2 Hz, and the remainder of the points representing the low frequency of .2 Hz again.

To discern the effects of each mGluR antagonist, one antagonist was added to the saline five to 10 minutes prior to eliciting a post-tetanic plasticity. After running a few trials, the saline with the antagonist present was removed and replaced by fresh saline. This effectively rinsed the antagonist out of the synapses in the crayfish. Then, the saline was replaced again by saline with the second antagonist in it. The control experiments did not have any antagonists present.

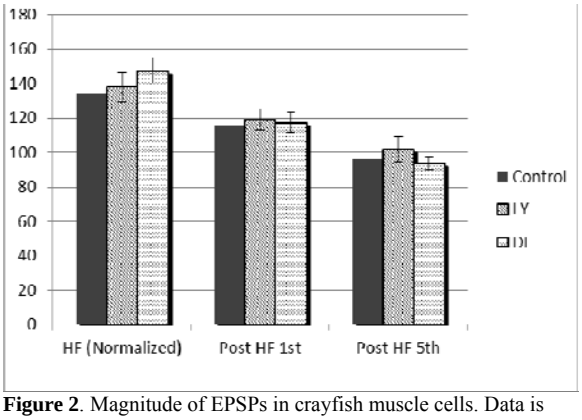

Figure 2. Magnitude of EPSPs in crayfish muscle cells. Data is normalized to represent percent of baseline magnitude EPSPs. Post HF 1st represents the mean magnitude of the first EPSP after the high-frequency stimulation; Post HF 5th represents the fifth. Each mean was calculated using 7-10 different trials, error bars stem from their respective means and represent ± one standard error.

Our results showed that introducing either of the mGluR antagonists has no effect on the magnitude of the post-tetanic plasticity or in how long it takes for the plasticity to decay back to baseline levels. Figure 2 shows the averaged data normalized to the baseline magnitude of the EPSP. During the high-frequency stimulation, immediately after the high-frequency stimulation, and five frames after the high-frequency stimulation, there was no significant difference between the three treatments. This disproves our hypothesis that mGluRs have an effect on post-tetanic plasticity. The PPR was calculated even though there was no change that could be located pre- or post-synaptically. It ensured that there were not canceling effects from the treatments, such as the pre-synaptic cell releasing more neurotransmitter but the post-synaptic cell becoming less sensitive to neurotransmitter. Since Figure 3 shows no significant differences between the PPR of the control and the two treatments, it can be concluded that there were no opposing effects.

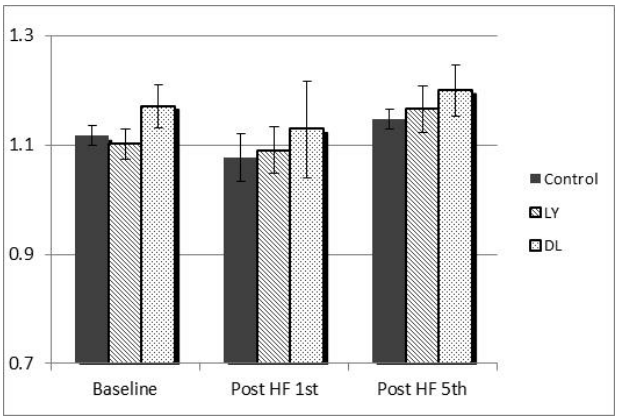

Figure 3. Paired Pulse Ratios. Data was calculated by dividing the magnitude of the second pulse by the first pulse. Baseline represents the pre-high frequency stimulation, Post HF 1st represents the first EPSP after the high frequency stimulation finished, and Post HF 5th represents the fifth. Each bar represents the mean of 4-7 PPRs with error bars representing ± one standard error.

DISCUSSION

While the analysis of our experiment led us to conclude that neither antagonist, when applied to the saline solution surrounding the crayfish cells, significantly affected the magnitude of the post-tetanic plasticity elicited by high frequency stimulation of a crayfish nerve, we could, in turn, also conclude that metabotropic Glutamate receptors have little to no effect on post-tetanic plasticity. Based on the results of previous studies involving mGluRs, we hypothesized that the presence of the mGluR antagonists would decrease the magnitude of the EPSPs after the period of high frequency stimulation in relation to those of the control condition in which no agonists or antagonists were added to the solution (Neale et al, 2005). Each of the three conditions – the two treatments and the control condition – followed very similar patterns: the high-frequency stimulation increased the amplitude of EPSPs to around 140% above the baseline level; the first EPSP that followed high-frequency stimulation dropped in magnitude, remaining around 120% above baseline; and the fifth pulse after the period of high-frequency stimulation showed, in all three conditions, a return to the baseline magnitude. There was very little variation among the conditions, leading us to deduce that the two experimental treatments had no significant effect.

Although the results reject the viability of our initial hypothesis, they do, however, illustrate much in regard to both group II and group III mGluRs, the antagonists LY-341495 and DL-AP3, and post-tetanic plasticity, leading us to revise our questions about the experiment and our future hypotheses.

There were many external variables that could not be controlled over the course of the experiment, which may have had confounding effects on the results and could have contributed – but did not necessarily contribute – to our rejection of the hypothesis. Many different crayfish were used throughout the experiment, all yielding different results, some slight and some dramatically distinct from the rest; some dissections elicited many EPSP recordings while others recorded nothing. When adding the drugs to the saline solution, we drained the reservoir of solution in which the crayfish was pinned and added the new solution with the additional antagonist, waiting 5-10 minutes for the cells to acclimate to the new environment. Our wait time was not well measured and thus, not very consistent, and maybe it was not even long enough for the cells to adequately detect the presence of the drug. In regard to the period of high-frequency stimulation, the length of time was also poorly measured and slightly inconsistent – an issue, which may have contributed to inconsistencies in determining the corresponding first and fifth post high-frequency recordings.

In a future study, along with changing the procedure to correctly control these potentially confounding variables, we will modify our research question and hypothesis to delve deeper into why exactly we saw no effect from either drug. We may also explore how altering the concentration of the antagonists in the solution might produce different results that illuminate the significant influence of one or both of the drugs. We may pursue an experiment that investigates the effect of different concentrations of a particular drug known to bind to mGluRs, ranging from low concentrations like those used in this investigation to much higher concentrations. We would perform several trials at each level, increasing the concentration of the antagonists in the saline solution after each round and allowing the cells to adequately acclimate to the new concentration before recording further data. Other paths might include investigating the effects of known mGluR antagonists different from those used in this experiment, first one drug at a time, and then some trials with more than one antagonist present in the solution at one time. So much can still be explored and so many questions must still be tested with regard to the crayfish neuromuscular junction; our experiment, while it did not significantly aid in our pursuit of better understanding of schizophrenia and possible treatment options, our investigation did provide a small steppingstone on the path to those answers. We must inquire much more about mGluRs; about Glutamate, NAAG, and other receptors; and about the diverse range of functions in which each plays a role. Whatever future experiments may be conducted, still quite little is known about the crayfish neuromuscular junction, so any new conclusions – whether groundbreaking or simply hypothesis-rejecting – provide us with new information and better understanding and guidance to apply to existing knowledge and even further future investigations.

ACKNOWLEDGEMENTS

We would like to give a big shout out to Professor Clark Lindgren, who provided extensive help and beneficial advice over the course of the experimental process. We also send thanks to Sue Kolbe, Ashley Millet, and Chris Kaiser-Nyman, who all played a large part in the success of our experiment and those of our fellow classmates.

REFERENCES

Adedoyin, M., Vicini, S., and Neale, J.H. 2010. Endogenous NAAG inhibits synaptic plasticity/transmission in the amygdala in a mouse inflammatory pain model. Molecular Pain **6**: 60.

Fricker, A., Mok, M.H., de la Flor, R., Shah, A. J., Woolley, M. et al. 2009. Effects of N-Acetylaspartyl glutamate (NAAG) at group II mGluRs and NMDAR. Neuropharmacology **56**: 1060-1067.

Gafurov, B., Urazaev, A. K., Grossfeld, R. M., and 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**: 227-235.

Lea IV, P. M., Wroblewska, B., Sarvey, J. M., and Neale, J. H. 2000. β-NAAG rescues LTP from blockade by NAAG in rat dentate gyrus via the type II metabotropic Glutamate receptor. Journal of Neurophysiology **85**: 1097-1106.

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