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Ketamine reduces EPSP amplitude and increases paired-pulse facilitation in *Procambarus clarkii*'s neuromuscular junction

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ABSTRACT

Ketamine is recently found to have effects on humans' synaptic plasticity that are similar effect antidepressant. Studying this drug's effect on invertebrate's synaptic plasticity can shed light into its influence on humans. Our research focused on ketamine's role in the synaptic plasticity of crayfish dorsal extensor's muscular junction. Specifically, we examined how ketamine affects the excitatory postsynaptic response in *Procambarus clarkii*. In our experiment, we applied paired-pulse stimulation on crayfish tail in 3 conditions: basic crayfish saline (control), ketamine solution (250μM and 500μM), and return to control. We examined the EPSP amplitudes and the percent change between all conditions. Our findings revealed that there was a reduction in synaptic excitation when ketamine was introduced but there was increase in facilitation of the second EPSP pulse. The difference between the two concentrations of ketamine, however, were not dramatic, revealing that 250μM of the drug may have been sufficient in blocking the receptors. We also deduced from our "return to control" data that ketamine did have a long lasting effect on the neuromuscular junction.

INTRODUCTION

Regulation of ketamine for human consumption and treatment is currently a controversial topic in the medical world since studies on how this drug affects the human nervous system, particularly synaptic plasticity, remain insufficient and inconclusive. Ketamine is known as an antagonist of N-methyl-D-aspartate (NMDA) receptors, which means that the drug blocks receptors of this type and disrupts their regular functions. It also affects other glutamate receptors, such the as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and the beta-adrenoceptors receptors.

Excessive consumption of ketamine causes hallucinations and dissociative mental states, both of which results in several deaths. However, numerous recent science journal articles have found ketamine's potential as an antidepressant, since it negates the changes in synaptic plasticity caused by depression (Marsden, 2013). Significant limitations of available treatments for depression, along with rapid antidepressant effects of ketamine (Martinowich et al. 2013, Browne and Lucki 2013), have spurred renewed efforts in the scientific community to study this drug's effect on synaptic plasticity more extensively.

Synaptic plasticity, the ability of synapses to strengthen or weaken over time, is one of the important neurochemical foundations for human behavior. Therefore, by studying synaptic plasticity, we can understand the rationale behind cognitive processes in human, such as perception, language, memory, reasoning and emotions. NMDA receptors are the predominant molecular device for controlling synaptic plasticity. NMDA receptors were thought to exist only in vertebrates (Mayer and Westbrook 1987), but evidence suggests that this type of receptors is also present in invertebrates' neuromuscular junction, specifically on the membrane of the presynaptic neuron (Dale and Kandel 1993).

The NMDA receptor interacts with glutamate, the most abundant excitatory neurotransmitter in the invertebrate's nervous system. Glutamate is released from the presynaptic cell (Schramm and Dudel 1997) when an action potential occurs and binds to non-NMDA receptors on the postsynaptic cells. This binding causes excitatory postsynaptic response (EPSP) – a temporary depolarization of postsynaptic membrane potential – in the postsynaptic cell. However, when the amount of glutamate becomes excessive in the synaptic gap, these molecules bind to NMDA receptors on the presynaptic membrane, which stops the release of additional glutamate. (Feinstein et al. 1998). In human, blocking NMDA receptors and influencing synaptic plasticity can cause various effects, ranging from temporary loss of consciousness to brain damage, depending on the concentration.

Although there are numerous studies on how ketamine affects rats' synaptic transmission and plasticity (Kamikaya et al. 2011; Riberio et al. 2014), similar studies on invertebrates like crayfish are scarce and yield inconsistent results. This is a gap in the current body of scientific knowledge. The nervous system of crayfish features similar neurophysiological and neurochemical mechanisms to organisms of higher complexity, which

allows for relevant analysis at the cellular and circuit level. Invertebrates such as crayfish have proven to be excellent models for the study of synaptic plasticity. Much of the information we now have about synaptic plasticity and glutamate receptors was discovered in work done on crayfish preparations (Dudel, et al., 1987; Shinozaki, 1988; Parnas, et al., 1994; Dudel, et al., 1997; Schramm, et al., 1997). Therefore, by studying the synaptic plasticity, and the factors that affect it, in crayfish will provide a better understanding of human's synaptic plasticity. Furthermore, the studies of ketamine on rats lack reliability, as they yield very different results. Some researches, like Kamikaya et al. (2011) claim that ketamine induce synaptic depression, while others, such as Narimatsu et al. (2002) came to the conclusion that ketamine cause synaptic facilitation in rat hippocampal.

Our research seeks to reconcile these findings, while giving insight on how ketamine affects the excitatory postsynaptic potential of crayfish. By examining one of ketamine's potential mechanisms, we hope to provide a framework for future research directions aimed at developing safe and efficient antidepressants from ketamine.

In this experiment, we examined the effect of ketamine on the EPSP of the crayfish species *Procambarus clarkii*. We applied paired-pulse stimulation on crayfish tails that were first submerged in basic crayfish saline (control), then in two different concentrations of ketamine (500 μM and 250 μM) and finally in the control solution once again. We then examined the difference in EPSP amplitude and percent change of second-pulse facilitation of crayfish muscle cells between the four conditions. The EPSPs in the crayfish dorsal extensor muscle cell, in response from the paired-pulse stimulation were obtained using the intracellular recording method.

We hypothesized that exposure to ketamine will result in an increase in EPSP amplitude and less facilitation - lower percentage change in EPSP amplitude between the first and second EPSPs. This is because ketamine, as an NMDA antagonist, will block NMDA receptors and prevent glutamate from binding to these receptors. Since the negative feedback loop discussed earlier will be disrupted, more glutamate will be released and bind to postsynaptic receptors, which in turn results in more depolarization and EPSP amplitude. Also, ketamine will cause a lower percentage change in the two EPSPs. Most of the postsynaptic receptors will have been occupied with glutamate after the first stimulation, so additional release of glutamate will not cause a much higher second EPSP. In the end, we had to reject our hypothesis because our results showed that ketamine reduces EPSP amplitude and increases paired-pulse facilitation.

MATERIALS AND METHODS

Preparation of the crayfish specimen

In this experiment, we used the crayfish species *Procambarus clarkiii*. For the preparation of the crayfish specimen, we followed the instructions for dissection in "Crawdad: A Lab Manual for Neurophysiology" (Wyttenbach, Johnson and Hoy, 2012), under the subheading "Muscle Resting Potential." We began our dissection of the tail by cutting the carapace along the ventral ridges of the shell. We then removed and discarded the superficial muscle to expose the dorsal extensor muscle. After muscle removal, both the nerves connected to the dorsal extensor muscles and the muscles themselves were accessible for observation.

Solutions

Our control condition is basic crayfish saline, consisted of 5.4 mM KCl, 200.7 mM NaCl, 12.3 mM MgCl26H2O, 5 mM Sodium Hepes Buffer, and 6.5 mM CaCl2*2H2O. Immersing the crayfish tail in this basic crayfish saline will prolong the life of the cells and preserve cell fatigue and integrity, which allow for experimentation.

To expose the crayfish dorsal extensor muscle to ketamine, we immersed the crayfish tail in ketamine solutions of either 250 or 500 μM. For the 250 μM ketamine solution, we mixed 99.5 mL of aforementioned basic crayfish saline and .5 mL of a 50 mM ketamine stock solution. For the 500 μM concentration, we mixed 99 mL stock saline with 1 mL of the same ketamine solution.

Making Microelectrodes for Intracellular Recording

We pulled two types of glass microelectrodes using the PUL-1 electrode-pulling machine, manufactured by World Precision Instruments. The recording microelectrode was filled with 3.0M of KCl and was tested for an optimal resistance of 5-10Ω using the A-M Systems Inc. Intracellular Electrometer. The suction electrode was sanded down to effectively capture the nerve that would receive the stimulation. Both microelectrodes were secured in micromanipulators to ensure stability when piercing the muscle cell or capturing the nerve.

Testing Timeline

We performed 4 trials, each with a different crayfish. In each trial, we tested 3 conditions: "control," "with ketamine" and "return to control." For the control, we submerged crayfish in basic saline. Then, we replaced the saline with either the 250 μM 500 μM ketamine solution. Finally, we removed the ketamine solution and submerge the crayfish tail in basic crayfish saline once again. We used the 250 μM ketamine solution for two trials, and the 500 μM for the other two.

Recording and Analyzing Data

To begin recording data, we captured a nerve using the suction microelectrode. We then pierced a deep extensor muscle near the suctioned nerve with a recording microelectrode by using a micromanipulator. We zeroed the junction potential, which was recorded in the extracellular solution, before entering the muscle cell. A resting membrane potential was found using the program LabChart. We then applied on the captured nerve a paired pulse stimulation with a frequency of 0.55 Hz, duration of .05 ms and delay of 40 ms. The stimulation's voltage magnitude varied from one trial to another because some nerves are more sensitive to stimulation than others.

We took between 60-90 EPSP recordings for each condition. Whenever we change the conditions (changing the solutions), we allowed a diffusion period of 15 minutes before data recording. Using a module of DataPad, we recorded the EPSP amplitude of both EPSP that result from the paired-pulse stimulation.

In order to analyze the data, the mean amplitude for the first and second EPSP were calculated for each trial. Then, we averaged the means for the trials that involved the same ketamine concentration. We applied the same method to the percent change in amplitude data. The percent change between the paired EPSPs were calculated using the following equation: Percent change in amplitude = ((Second EPSP amplitude - First EPSP amplitude) /First EPSP amplitude)*100

We used bar graphs to present our data. Our first graph compares the average EPSP recorded in control condition to that recorded in 500 µM ketamine solution. Another graph compares the percent difference in the two consecutive EPSPs of paired pulse between our aforementioned conditions. The purpose of these graphs is to show the effect of ketamine on facilitation. We also had similar graphs for the 250 µM concentration trials.

Initially, we planned to analyze the data using t-test, comparing the EPSP amplitude and the percent change between the paired EPSPs from one condition to another. These comparisons would help us to determine whether to reject our initial hypothesis or not. If there is a significant difference between one condition to another, we can conclude that ketamine has an effect on EPSP. However, since we did only two trials for each concentration of ketamine, we cannot perform a statistical test due to insufficient data. A t-test is only meaningful when used to compare the data of three or more trials.

RESULTS

Our research aimed to test the effects of ketamine, an NMDA receptor antagonist, on the crayfish. In our experiment, we used three different saline baths. We started out with the basic crayfish saline bath to record the baseline and then replaced the solution with either the 500µM concentration of ketamine or the 250µM concentration to the crayfish environment. Finally, we returned to our original control solution to observe any changes.

Ketamine reduces EPSP amplitude

When we switched to the 250µM ketamine solution, we saw a significant decrease in EPSP magnitude for both pulses when compared to control data (Fig. 2). The average magnitude for first and second pulse was 3.493mV and 4.605mV respectively. The 500µM concentration bath yielded a similar decrease in EPSP magnitude to the 250µM concentration. The average first- and second-pulse EPSPs were 3.860mV and 4.866mV (Fig. 1). Overall, 250 µM and 500 µM ketamine reduces EPSP amplitude by 65.70% and 70.23% respectively.

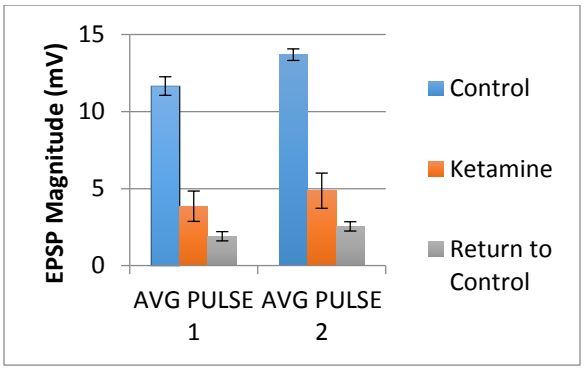

Figure 1. Average EPSP magnitude for three testing conditions. The blue bars represent control EPSP magnitudes, orange bars represent the 500µM ketamine concentration testing, and the gray bars represent the return to control data. Error bars represent the range of averages from two trials. N equals 2.

When we returned to the control saline bath, we saw EPSP amplitude that were smaller than that of the control data. Our first and second-pulse EPSPs were 1.913mV and 2.548mV for the 500µM concentration trials (Fig. 1) and 1.769mV and 2.179mV for 250µM trials (Fig. 2).

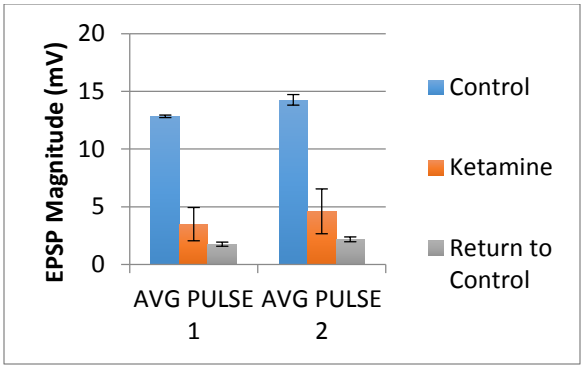

Figure 2. Average EPSP magnitude for three testing conditions. The blue bars represent control EPSP magnitudes, red bars represent the 250µM ketamine concentration testing, and the green bars represent the return to control data. Error bars represent the range of averages from two trials. N equals 2.

Ketamine increases paired-pulse facilitation

For all conditions, we observed a facilitation – an increase in amplitude – from the first EPSP to the second. Percent Change in EPSP is a way to mathematically represent facilitation. The average percent change between control pulses was 17.644% for the 500 µM trials (Fig. 3) and 11.083% for the 250 µM ones (Fig. 4).

When we switched to the 250 µM ketamine condition, the average percent change increased to 27.303% (Fig. 3). The average percent change in 500 µM ketamine trials was 31.293 %. Both concentration of ketamine resulted in a similar increase in percent change compared to the control.

Facilitation in the return to control test groups also occurred at a smaller level than both experimental concentrations with an average percent change of 20.056% for 500µM trial days (Fig. 3) and 21.416% for 250µM trial days (Fig. 4).

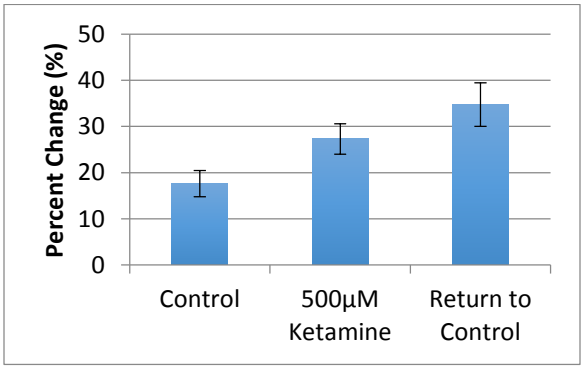

Figure 3. Average percent difference between first- and second-pulse EPSP magnitudes. Each bar represents the percent difference for a testing group: Control, 500µM Ketamine Concentration, and Return to Control. Percent difference was calculated using this formula: ((Second EPSP magnitude - First EPSP magnitude) / First EPSP magnitude)*100. Error bars represent the range of averages from two trials. N equals 2.

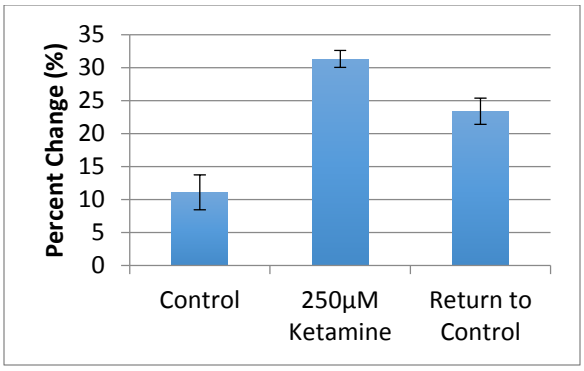

Figure 4. Average percent difference between first- and second-pulse EPSP magnitudes. Each bar represents the percent difference for a testing group: Control, 250µM Ketamine Concentration, and Return to Control. Percent Difference was calculated using this formula: ((Second EPSP magnitude - First EPSP magnitude) / First EPSP magnitude)*100. Error bars represent the range of averages from two trials. N equals 2.

250 µM and 500 µM ketamine solutions results in identical effects

We tested two different ketamine concentrations to determine whether different concentrations of ketamine result in significantly different changes. The effect of 250 µM and 500 µM ketamine turned out to be quite similar (Fig. 5).

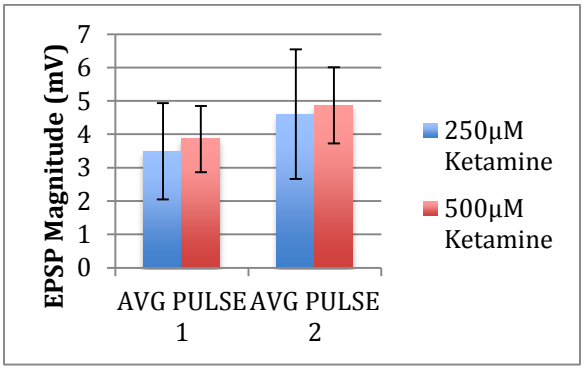

Figure 5. Average EPSP magnitude for 500µM and 250µM ketamine concentration testing. This graph compares the average of the average EPSP magnitudes of each experimental group. Error bars represent range of averages. N equals 2.

DISCUSSION

We hypothesized that exposure to ketamine would increase the EPSP amplitude and cause a lower facilitation between the two EPSPs in paired pulse stimulation. The results did not support both parts of our hypothesis, since there was a decrease in EPSP amplitude and higher facilitation when ketamine was added.

Since Feinstein et. al (1998) found that the binding of NMDA to NMDA receptor decreases the release of neurotransmitter and EPSP amplitude, we would expect

ketamine, as an NMDA antagonist, to increase EPSP amplitude. However, our results showed that ketamine caused a lower EPSP amplitude compared to that of the control, demanding further investigations into scientific studies on the drug ketamine.

The research of Leong (2002) can provide a rationale behind the lower EPSP amplitude for the conditions with ketamine. Leong's finding challenges the claim that ketamine is a selective NMDA receptor antagonist. Ketamine can also block non-NMDA receptors, such as the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), causing EPSP amplitude to decrease (Leong 2002). The concern with this inference is the inconsistency in the research subject. Leong experimented with a vertebrate while we did research on crayfish, an invertebrate. However, Craig et. al (2006) confirms the existence of AMPA in invertebrate's muscle membrane, and also concludes that AMPA receptors are the most prevalent type of glutamate receptor in invertebrates like crayfish. By blocking postsynaptic AMPA receptors, ketamine prevents glutamate from binding to these receptors, resulting in a low EPSP since the postsynaptic ion channels cannot open for ion transfer. Therefore, even though blocking NMDA receptors causes a higher EPSP, the decrease in EPSP caused by ketamine blocking AMPA receptors might have been so overwhelming that it resulted in an overall decrease in EPSP.

To rationalize the increase in paired-pulse facilitation, we continued to look into ketamine additional effects on receptors other than NMDA and AMPA. Besides NMDA and AMPA receptors, Ketamine also influenced other glutamate receptors. Narimatsu et. al (2002) concluded that ketamine causes a higher facilitation the second EPSP in paired-pulse stimulation by activating beta-adrenoceptors . The findings of Ishida et. al (1980) confirms that beta-adrenoceptors are present in crayfish. This study enables us to use Narimatsu's results in an attempt to explain the higher percent change (facilitation) caused by ketamine.

In order to extensively test the effects of ketamine on the crayfish neuromuscular junction, we used two different concentrations of the drug, 250μM and 500μM. From our data we found that there was not a big difference in the result between the concentrations. The 500μM concentration yielded a slightly smaller reduction of synaptic excitation compared to that of the 250μM. Nevertheless, when we analyzed the data of the 250μM ketamine condition, all the trends discussed above are consistent with those concluded from the 500μM condition. The molecular reasoning for this phenomena could be that the 250μM concentration of ketamine was sufficient to block most of the glutamate receptors. Thus a concentration double that amount would not have made much of a difference.

We noted that ketamine has a long lasting effect on the crayfish dorsal extensor neuromuscular junction because the EPSPs data (EPSP amplitude and percent change) recorded in the "Control" condition and "Return to control" condition are very different. We know that without ketamine, the crayfish EPSPs don't change over time (the data is not presented in Results). This observation means that the cell does not die over time and any changes to EPSP are induced by ketamine's neurochemical effects. Therefore, ketamine seems to have a permanent, irreversible effect on the neuromuscular junction of crayfish. An alternative explanation would be that the recovery time was not long enough. We recorded the EPSP for "Return to control" 10 minutes after replacing ketamine solution with basic crayfish saline, while other studies suggest that the minimum time for recovery is 4 to 5 hours, and may take up to 24 hours (Bioniche Pharma USA, 2009)

Initially, we wanted to examine the effect that ketamine has on NMDA receptors of crayfish. However, since ketamine also affects many other sites and receptors, our results were skewed and the effect on NMDA was not clear. Therefore, for future researches, we would block the AMPA and beta-adrenoceptors by including inhibitors of these receptors in the saline bath. Also, to determine whether ketamine has a permanent effect on crayfish's synaptic plasticity, we will allow a recovery period of more than 24 hours in the future.

This study offers a potential answer to the unique properties of ketamine as a non-selective antagonist that shows a direct effect on AMPA receptors. However, such research has not yet been pioneered in the science community. Thus with this reported literature of the extraordinary findings of ketamine's effects on the neuromuscular junction of crayfish, we wanted to provide a spark for subsequent extensive research to confirm our still limited understanding of the drug and its potential to alter the very core of human behavior.

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