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Ketamine reduces synaptic transmission and DF2 enhances recovery from ketamine-induced synaptic depression at crayfish neuromuscular junctions.

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ABSTRACT

Ketamine is a chemical that blocks N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-isoxazole propionic acid (AMPA) receptors and reduces excitatory postsynaptic potential (EPSP) amplitude. Due to this capability, ketamine is commonly used as an anesthetic; however, recent studies are showing its potential use as an antidepressant. DF2, an FMRFamide-related neuropeptide, creates a long-lasting enhancement of EPSPs, which may be able to counter the effects of ketamine. To see if DF2 enhances recovery from ketamine-induced EPSP depression, we induced EPSPs in crayfish tail extensor muscles. We stimulated the muscle in a normal physiological saline solution (Ringer's solution), a ketamine solution, a Ringer's solution with DF2 added, and finally, a ketamine solution with DF2 added. Our results show that ketamine reduced EPSP amplitude, and DF2 was not able to successfully recover the EPSP back to normal ranges after ketamine. Thus, although ketamine can decrease EPSP, DF2 was able to minimize ketamine's effects.

INTRODUCTION

As depression continues to plague the world, researchers are striving to find effective treatments for combating mental illness, an example being ketamine (Meisner, 2019). This mental disorder may require long-term pharmacological treatment, to which the body may become resistant over time (Chen, 2019). Additionally, it may take days or even weeks for the patient to begin seeing a significant change. For some individuals, this may be incredibly harmful, which is why it would be beneficial to introduce faster-acting medications (Chen, 2019). Traditionally, ketamine was a medication used as an anesthetic that can provide pain relief and sedation, among other uses. Ketamine effectively and rapidly blocks the glutamate receptors N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and beta-adrenoceptor receptors. Glutamate blockage reduces the EPSP amplitude found in postsynaptic cells. The reduced EPSP has drawn many researchers' attention to utilizing it as an antidepressant once more (Mayer & Westbrook, 1987).

EPSPs, or excitatory postsynaptic potentials, occur within postsynaptic neurons (Shepherd, G. M., & Hanson, P. I., 2014). A change in the postsynaptic membrane creates EPSPs. Change in the postsynaptic membrane encourages action potentials to fire by bringing the membrane potential closer to the threshold potential (Matthews, 2001). Generally, the average resting membrane potential is around -70 mV and occurs from having the intracellular space more negatively charged than its surrounding environment (Chrysafides, 2019).

DF2, an FMRFamide-related neuropeptide, creates a long-lasting enhancement of synaptic transmission (Friedrich, R. W., Molnar, G. F., Schiebe, M., & Mercier, A. J., 1998). Neuropeptides such as DF2 activates protein kinases, which causes more neurotransmitters to spill into the synaptic gap. These solutions have not been tested together extensively, so DF2 may be able to counter the effects of ketamine.

There is not extensive research on the effects of ketamine on neurons. Although this research is missing on humans, it should be tested extensively on other organisms first to evaluate its impact on humans for later use. By analyzing EPSPs in the presence of ketamine and DF2, we can study how ketamine could be used to alter long-term synaptic plasticity and its strength as a possible long-term antidepressant.

We tested the effect of ketamine on EPSPs in the crayfish neuromuscular junction and how DF2 impacts the long-term effects of ketamine. The overarching question we tested was, does DF2 enhance recovery from ketamine-induced EPSP depression? By looking at the synaptic plasticity of ketamine on crayfish ventral abdominal extensor muscles, we can gain insight into its possible effects on humans as an antidepressant (Hoang, Slattery, & Park, 2015). In addition to finding its potential impact on humans, we may gain an understanding of possible conditions needed to override its receptor blocking capabilities. We hypothesized that ketamine would decrease EPSP function, and DF2 will make minimal impact on the recovery of ketamine-induced EPSP depression. Our results showed that ketamine

decreased EPSP amplitude, and DF2 was unable to restore EPSP amplitude.

MATERIALS AND METHODS

Preparation of the Crayfish

Our instructor supplied us with the crayfish *Procambarus clarkii*. The crayfish were anesthetized in a container of ice water before dissection. We removed the tail of the crayfish with a pair of scissors. We then dissected the crayfish by cutting the lateral sides of the tail with scissors and peeling off the outer shell and muscles. We used our thumb to remove the remaining muscle mass in the dorsal portion of the tail, comprising the swimmerets, flexor muscles, and the digestive tract. Removing this muscle mass exposed the superficial extensor muscles and nerves. We pinned the tail with its exposed extensor muscles ventral side up in a sylgard-coated dish and covered it with 100 mL of Ringer's solution. Ringer's solution simulates the extracellular fluids in a crayfish that prolongs the lifespan of the exposed extensor muscles. The solution contained 5.4 mM of potassium chloride (KCl), 196 mM of sodium chloride (NaCl), 2.6 mM MgCl2-6 H2O, 10 mM HEPES, and 13.5 mM CaCl2-2 H2O at a pH of 7.4.

Solution Preparation

We acquired baseline data from each crayfish prior to collecting any data with the DF2 and ketamine solutions. The Ringer's solution comprised the baseline data for each crayfish. Finding this baseline data for each crayfish allowed us to later normalize the data. We prepared a DF2 solution with a 50 nM concentration to observe the effects of the enhancer on EPSP generation. We added the DF2 solution to the ketamine solution or ringer's solution with a micropipette when appropriate. We then prepared 100 mL of 50 μM ketamine solution by using 10 mL of 500 mM stock ketamine solution and diluting it with 90 mL of Ringer's solution. Once we collected data on each solution's individual effects on EPSP generation, we made the 50 μM ketamine solution the base solution in the dish and let the crayfish abdominal extensor muscles bathe in the base solution for 15 minutes.

From there, we generated EPSPs. Once the EPSPs were stable we added the DF2 solution and continued data collection. The duration for each experiment permits a 15-minute bathing time and another 30 minutes of data recording. After bathing in the ketamine solution for 15 minutes, we added DF2 to the sylgard-coated dish at the 10-minute mark of the 30-minute recording period, leaving 20 minutes for measuring the effects of the DF2 on the neuromuscular junction of the abdominal extensor muscles.

Arrangement of Laboratory Equipment

We pulled borosilicate glass capillary tubes with a 1.2-millimeter diameter with a microelectrode puller (World Precision Instruments, Pul-1). We filled the microelectrodes with 3 M KCl solution using a syringe with a microfil head attachment. After removing the air bubbles by flicking the microelectrode body, we placed the filled microelectrode into a microelectrode holder (E Series with Straight Body, Warner Instruments), which was inserted into a micromanipulator. We connected the microelectrode to an intracellular amplifier and placed a wire connected to the electrometer's negative terminal in the Ringer's solution.

Additionally, we created suction electrodes from microelectrodes by filing down the tip against sandpaper. These microelectrodes were filled with Ringer's solution and put on a separate micromanipulator. We connected the electrode to a plastic syringe that was later used to suck in a nerve for the duration of the experiment. With the nerve sucked up, we used a stimulator (Grass SD9) to apply an electrical pulse.

Intracellular Recording and Nerve Stimulation

Using the micromanipulator, we inserted the recording microelectrode into crayfish extensor muscles to measure the cell membrane potential in millivolts (mV) with microelectrodes connected to a DC amplifier (Model 1600 DC Neuro Amplifier, AM Systems). The output from the amplifier was fed into a Power Lab (AD Instruments, driven by Lab Chart software). To test the microelectrode, we inserted the tip of the electrode into the solution, zeroed the amplifier, and ran an electrode test to determine whether the resistance fell within the acceptable 5-20 M ohms (**Ω**). If the resistance was in an acceptable range, it permitted us to proceed with the experiment. For each solution, we recorded EPSPs for 30 minutes after the abdominal extensor muscles soaked in the designated solution for 15 minutes. We held the stimulus frequency at 0.4 hertz (Hz) and the duration at 3.5 milliseconds (ms). We tapped the intracellular recording electrode to maintain the resting membrane potential in the -50 to -70 mV range. We analyzed our data at a later time and displayed graphs using a spreadsheet program (Excel 2019, Microsoft).

RESULTS

The goal of our research was to see whether ketamine reduces the amplitude of EPSPs and if DF2 can revert the amplitude of the EPSPs to its prior range in the crayfish extensor muscles. The results of this experiment show if ketamine is capable of long-term potentiation at crayfish neuromuscular junctions. Our process consisted of inducing EPSPs in crayfish extensor muscles using Ringer's solution, then adding either a ketamine solution or DF2 activator to see how the EPSPs changed with the new substances.

We gave each of the substances an introductory period of 5-10 minutes before recording any data. This introductory period ensured that the solution was affecting the crayfish extensor muscles. After the introductory period ended, we stimulated the extensor muscles for 30 minutes. The substances we used were Ringer's solution, ketamine, DF2, and a combination of ketamine and DF2. We used the Ringer's solution as the baseline of our data, which allowed for us to determine the average amplitude of EPSPs for the duration of the experiment.

Our first trials involved the ketamine only solution. The solution consisted of 100 mL of 50 μM ketamine solution, which we created by using 10 mL of 500 mM stock ketamine solution and diluting it with 90 mL of Ringer's solution. The average percent change of all four trials was -39.1% however the error bar ranges from -60.5% to -17.7% to so it is possible that -39.1% doesn't represent the true average of the results of the ketamine solution.

The next step in our experiment was testing the effect of DF2 only solution on EPSP. We added 50μM of DF2 to Ringer's solution. The graph shows that the average percent change of EPSP using DF2 compared to the original baseline EPSP using Ringer's solution was 168.5%. However, with only 2 trials, the error bar was very large ranging from 14.3% to 322.7%, so the results are uncertain.

The last set of trials involved a ketamine solution with drops of DF2 activator added. We conducted 3 trials. The solution consisted of 100 mL of 50 μM ketamine solution and 50μM of DF2. Results show that the average percent change from baseline was -60.2%, however standard error suggests that the average percent change from the baseline ranges from -80.4% to -40.0% during the 30-minute trial period.

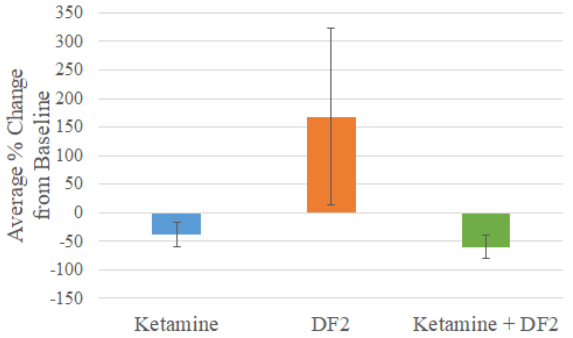

Figure 1. The average percent change compiled from the experiments conducted on ketamine, DF2, and ketamine and DF2 combined. Ketamine is seen to consistently decrease the amplitude of EPSPs throughout all of its experiments. DF2 is shown to have a very large error bar, indicating that the effects of DF2 are uncertain. p-value = 0.3741588

DISCUSSION

We hypothesized that exposure to ketamine would reduce EPSP amplitude and that DF2 would not significantly increase the EPSP to its prior range. Ketamine did in fact reduce the amplitude of EPSP and we found that DF2 was unable to increase the amplitude of the crayfish EPSPs, therefore didn't fully override the effects of ketamine.

Furthermore, due to the high variability of the DF2 efect, it's difficult to fully determine the effects of DF2 on the EPSP. Although DF2 did have a statistically higher average percent changes from the baseline, the error bar was so large that these results are uncertain. Granted the ketamine and DF2 solution caused the average percent change from the baseline to increase more than that of the ketamine solution alone.

Because DF2, an artificial neuromodulator, was unable to override the effects of ketamine in crayfish, it may be possible that natural neuromodulators in humans would be unable to override the effects of ketamine as well. These results may support research on the long-term effects of ketamine on depression. An experiment conducted by Hoang, Slattery, and Park supports our findings that ketamine has long lasting effects on the neuromuscular junction (2014). In their experiment, they examined the effect of ketamine on EPSP of crayfish, similar to our study. They, however, applied paired-pulse stimulation on crayfish tails for different concentrations of ketamine solution. Their results showed that ketamine generally decreased EPSP amplitude, which directly supports our trials involving the ketamine only solution.

If a follow-up experiment was conducted, we would record significantly more trials with the solutions used, especially DF2. Additionally, we would test the effects of the solutions as they are being introduced to the crayfish extensor muscles in order to more precisely see how these solutions affect the amplitude of the EPSPs over time. We would also test the effects of the solutions for a more

extended period of time to determine if the effects of ketamine or DF2 diminish after a certain number of hours. With these modifications to the experiment, it would help determine if these solutions are metabolized by the crayfish tail. The trends shown by ketamine could show its potential use as medicine.

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