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Evidence that D-Serine is a Co-agonist to NMDA Receptors at the Crayfish Neuromuscular Junction.

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ABSTRACT

N-methyl D-aspartate receptor (NMDAR), a glutamate receptor, is vital to neural cells, having been linked to the facilitation of many physiological processes such as memory, plasticity and neural development. Studies show that in brains, NMDARs rely on the neurotransmitter D-Serine to act as a co-agonist along with the neurotransmitter glutamate. Since little research has been conducted on the role of D-Serine in invertebrates, we sought to discover whether any evidence supports the role of D-Serine in co-agonistic facilitation of NMDARs in the crayfish neuromuscular junction. We measured the amplitudes of excitatory postsynaptic potentials (EPSPs) in the crayfish neuromuscular junction under two separate conditions – an increased D-Serine concentration and a D-amino acid oxidase (DAO) to deplete D-Serine. Average EPSP amplitudes significantly increased in the higher concentration of D-Serine and significantly declined in the DAO condition. These results provide support for our hypothesis that D-Serine acts as a co-agonist at the glutamate receptor in the crayfish neuromuscular junction.

INTRODUCTION

The role of N-methyl D-aspartate glutamate receptors (NMDARs) in neural cells is both complicated and diverse. As excitatory neurotransmitter receptors located in the synaptic junction between cells, NMDARs are involved in coordinating, facilitating, and mediating a plethora of physiological processes including memory formation, synaptic plasticity and development, and protein proteolysis. Like most other receptors, NMDARs require specific neurotransmitters to bind to them for activation; however, NMDARs require more than just their primary activator glutamate. These receptors require a co-agonist along with the neurotransmitter glutamate to become activated. Until 2006, the co-agonist for the n1 site on N-methyl D-aspartate receptor was thought to be occupied mostly by glycine, thus the secondary site on NMDARs was nicknamed the 'glycine site.' After much research demonstrated the higher affinity of the amino acid D-Serine to agonize the so-called 'glycine-site' (Martineau et al., 2006; Hashi-moto et al., 1995; Schell et al., 1997; Matsui et al., 1995), D-Serine was accepted as the more significant co-agonist of NMDAR. The binding of D-Serine to the glycine-site activates NMDARs, resulting in the opening of an ion channel, allowing the flow of Na^+ and small amounts of Ca^{2+} ions into the cell and inducing an excitatory postsynaptic potential (EPSP).

Still, questions remain on the role of D-Serine in the process of neural transmission in invertebrates such as crayfish. Although invertebrates collectively exhibit a remarkable diversity of glutamate receptors, there is no known study on the example and properties of an invertebrate NMDA receptor (Mayer and Westbrook, 1987). The absence is noteworthy because invertebrates provide some of the best examples of plasticity in the synaptic interactions among identified neurons (Byrne, 1987; Nguyen and Atwood, 1990). On the same note, to determine the absence of D-Serine sites on NMDARs in crayfish would demonstrate an evolutionary difference between invertebrates and vertebrates. Any difference would offer information that would assist in outlining the evolution of life on Earth.

In our research, we sought to discover if there is any physiological evidence that D-Serine acts as a co-agonist at the glutamate receptor of the crayfish neuromuscular junction. More specifically, we hypothesized that an increase of D-Serine concentration would lead to an increase in EPSPs and the depletion of D-Serine would result in a decrease in EPSPs, due to the special property of NMDARs. In order to deplete the intracellular level of D-Serine, we applied D-amino acid oxidase (DAO) to specifically degrade D-amino acids. In contrast, to increase the intracellular D-Serine concentration, we applied a concentrated dose of D-Serine to the extracellular saline solution. In a pioneer study depleting D-Serine from neural cultures, the same technique led to a 60% decrease in the spontaneous activity attributed to the postsynaptic NMDARs (Synder et al., 2000).

We observed the effects of these conditions on neurotransmission and compared the average amplitude of EPSPs in each condition with baseline recordings. Our data supported our prediction that D-Serine plays an

essential role for NMDARs activity in neural transmission in crayfish neuromuscular junctions (NMJ).

MATERIALS AND METHODS

Preparation of the Crayfish Superficial Extensor Muscles

Based on stretch receptor procedure #10 from the Cornell Crawdad website, we performed a dissection of the crayfish's lower abdomen in order to access the superficial extensor muscle cells on the dorsal interior of a crayfish's tail. Using the anesthetization process mentioned in alternative experiments, (Chon et al., 2002), a research lab assistant provided us with a specimen that had been cooled in ice for around 15 minutes. This anesthetization procedure slowed down the specimen's nervous system to the point of minimal activity, pain receptors inactive to the point where the crayfish would not feel significant pain for the remaining duration of our experiment. Securing the anesthetized specimen, we cut off the ventral side of the crayfish tail from the specimen by inserting scissors between the muscle and around the exterior shell and cutting through to the last segment, down to where the ventral side meets the flippers. In order to enable direct access to the dorsal interior muscle cells and nerves, we pushed the unnecessary muscle mass out of the dorsal side of the tail. We took care to avoid using the tip of the finger nail when removing the muscle mass to ensure that the superficial extensors muscles beneath this mass remained intact. With completion of the dissection, the superficial tail muscles and nerve extensions were visible and able to be studied. The remains of the crayfish torso and tail that were not used were placed back into buckets of ice to later be moved to the freezer and disposed of.

Electrode Construction

In order to observe the effect of D-Serine in crayfish neuromuscular junctions, we forged electrodes to both measure intracellular excitatory postsynaptic potentials (EPSPs) and stimulate them. We created sharp electrodes by placing 1.2 mm glass tubes in a microelectrode puller. These sharp microelectrodes were designed to pierce the superficial extensor muscle cells in a dissected crayfish and record intracellular potentials. In order to stimulate a consistent stream of EPSPs, we required suction electrodes so we could stimulate nerve cells that formed synapses on the muscle cells. By gently rubbing the tips of sharp electrodes on sandpaper until the tip was blunted, we made a few of our sharp electrodes into suction electrodes.

We filled the sharp electrodes with 3M KCL and checked for air bubbles before each attempted reading to avoid any interference. The sharp electrodes, placed in an electrode holder also filled with KCL, were then attached to our precision instrument set up.

Recording Intracellular EPSPs

The suction electrodes, attached to electrode holders and a micromanipulator, sucked up the saline fluids once the tip of the electrode entered into the solution. The electrode holder received signals from a Grass SD9 Stimulator, which was set to send twin pulses at a voltage between 2 and 3 volts for 0.2 ms at 0.9 Hz, with delay intervals of 20ms. The twin pulses provided us with both the average amplitude of the EPSPs from the extensor muscles and the changing ratio between the first and second EPSPs.

Using Scope V4.1.1, we zeroed the electrical signal from the sharp electrode once it entered the saline solution and tested the resistance levels. A resistance level between 5-10 MΩ was considered sufficient for our intracellular recordings. Looking in the bridge amplitude in SCOPE, we would be able to record EPSPs once a dip and plateau were visible, signaling our sharp electrode's entrance into the superficial extensor muscle cell. The resulting recordings from the twin pulses combined with the readings from the sharp electrode were taken in 1 minute intervals for 20 minutes.

Solution Preparation

In order to test for our variables, we needed specifically prepared saline solutions to submerge our crayfish specimen in. These solutions, designed to preserve the living crayfish tissue for as long as possible, were made based on a stock saline solution provided by our lab mentors. In each experiment, the dissected specimens were submerged in 100mL of this unaltered stock solution to generate a baseline for comparison when testing the role of increased and decreased D-Serine levels in the function of NMDA receptors in crayfish cells. The concentration of the increased D-Serine saline solution was calculated using a simple dilution formula: C1V1=C2V2. Based on this, 2 mL of a 500 μM thinned D-Serine stock solution was diluted into 98 mL of stock saline solution. According to the study conducted by H. Katsuki et al. (2007), the concentration of D-Serine could be increased to 100– 1000 μM. 25 mL of the prepared D-Serine dilution was exchanged with 25 mL of the stock solution that the crayfish specimen would be submerged in, ensuring that the saline solution would be comprised of 250 μM of D-Serine.

To address our secondary hypothesis, we also calculated and created a saline solution with the DAO.

This DAO saline would deplete the intracellular levels of D-Serine in our muscle cells. On the basis of the aforementioned formula, we introduced 100µL of 25 mM DAO into 100 mL of stock saline, diluting to 25 µM DAO solution. This solution was exchanged with the 100 mL stock solution upon the completion of the recordings of the 20-minute baseline period.

Statistical Analysis

Using Minitab, we conducted two Student's T-Tests to determine if the difference between our baseline EPSPs and manipulated EPSPs were significant. The letter "n" represents the number of the measurements done in a single muscle cell.

RESULTS

We hypothesized that an increase of D-Serine concentration would lead to an increase in EPSPs, and that the depletion of D-Serine would result in a decrease in EPSPs, both due to the NMDARs' co-agonist requirement. After we applied the crayfish preparation in the increased D-Serine and DAO conditions, we measured the EPSPs in each condition at the crayfish neuromuscular junction when stimulating the nerve. To stimulate EPSPs across the neuromuscular junction in our crayfish specimen, we applied paired pulses with a minor delay of 20 ms while each specimen was submerged in either a 250µM D-serine solution or DAO solution. Our measurements suggest that D-Serine plays a significant role in the activation of NMDARs, indicating that the neurotransmitters are a co-agonistic requirement for significant facilitation.

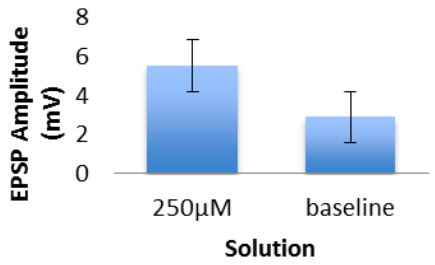

Figure 1. *A comparison of the mean amplitudes of the initial EPSPs stimulated down the superficial extensor muscle cells of crayfish specimen in a stock saline vs. a 250 µM concentrated D-Serine solution. A Student's T-Test was used to determine significance. The average EPSP amplitude for submersion in the stock saline was lower compared to when the D-Serine solution was introduced to the specimen (n=20, p<.05). Error bars represent standard error of the mean.*

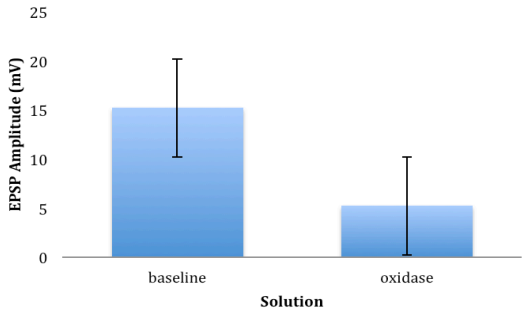

Figure 2*. The effect of DAO on average EPSP amplitudes measured in the superficial extensor muscle cells of a crayfish. Average amplitude significantly decreases in the DAO condition (p<.001, n=20). The mean amplitude of the DAO condition is 5.21 mV and the mean amplitude in the baseline condition is 15.18 mV. Error bars represent standard error of the mean. A Student's T-Test was used to determine statistical significance.*

When we measured the amplitude of EPSPs in increased D-serine solution (Fig.1), we noticed that the amplitude was approximately twice the amplitude of the control group. Comparing the amplitude in DAO condition with that in the control group, a very sharp 60% decrease was measured (Fig.2). A direct comparison between Fig. 1 and Fig. 2 suggests the presence of D-Serine plays a significant role in determining the amplitude of EPSPs, though the facilitation of these postsynaptic potentials is not entirely dependent on its presence. Based on the average amplitudes of our DAO samples, we can assume that NMDARs are still co-agonized by other neurotransmitters such as glycine. However, D-Serine still plays a much larger role is co-agonizing NMDARs than other neurotransmitters (Fig. 1).

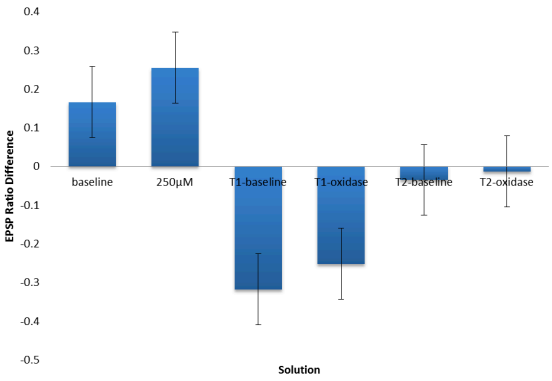

Figure 3. Average Ratio of Twin Pulses Quantal Contents in three sets--increased D-Serine, Trial 1 of and Trial 2 of DAO conditions. We recorded the first (m1) and second (m2) quantal contents at a voltage between 2 and 3 volts for 0.2 ms at 0.9 Hz, with *20ms delay intervals and calculated the ratio of (m2-m1)/ml to show the degree of facilitation. The error bars represent standard error of the each data set. (n=20)*

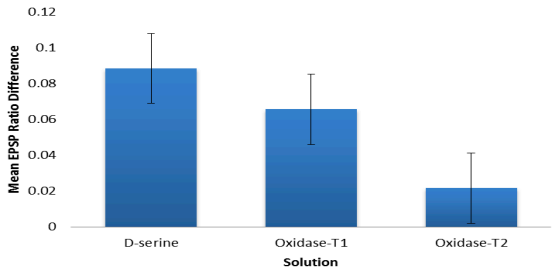

Figure 4. Average Ratio Difference of (m2-m1)/m1 in three sets at 20ms delay intervals at the crayfish neuromuscular junction. Error bars represent standard error of the each data set. (n=20)

Stimulation with twin pulses produced two subsequent EPSPs in our recordings, with first (m1) and second (m2) quantal contents, representing the strength of neurotransmitter release. Taking the ratio of (m2-m1)/m1, we sought to determine the degree of facilitation. As shown in Fig. 3, the ratio in increased D-Serine solution was positive in contrast to both DAO trials in which the ratios were negative. An increase (53%) in EPSP facilitation was shown upon the application of D-Serine. Compared to the sharp increase in increased D-Serine condition, lower increases were observed in both trials of DAO conditions. The average change in facilitation for each experimental condition (Fig.3) is shown to become less negative in comparison to the baseline. The ratio difference between the increased D-serine condition and the control group is the highest among all sets. Ultimately, the ratio differences indicate that the increased rate of facilitation is due to the presence of D-Serine, the decrease rate due to the lack of it.

DISCUSSION

In agreement with our hypothesis, the data demonstrated that the application of D-Serine significantly increased EPSP amplitude and the depletion of D-Serine by D-amino acid oxidase largely decreased EPSP amplitude. Through T-test calculations, we determined that our results were significant; therefore affirming that D-Serine co-agonizes NMDA receptors in crayfish neuromuscular junctions.

We observed that the EPSP amplitude doubled after the application of D-Serine, suggesting that D-Serine has strengthened NMDARs transmission. As we compare the amplitudes between DAO condition and the control group, the 60% decrease indicated that the depletion of D-Serine decreased the activity of NMDARs. Additionally, twin pulses gave two EPSPs with first (m1) and second (m2) quantal contents. During the application of twin pulses, the level of Ca^{2+} was elevated by the first nerve impulse; the residual internal Ca^{2+} combined with the newly available Ca^{2+} introduced by the second impulse, leading to an increased rate of release of transmitter. This initial elevation of Ca^{2+} in the first nerve impulse demonstrates that the changes are presynaptic. To continue, we took the ratio of (m2-m1)/m1 as a measure of the degree of facilitation. We observed that the ratio was affected by the application of both D-Serine and DAO conditions, indicating that neuronal D-serine may exert its affects presynaptically. The rising ratio in D-Serine condition reflected an improvement in D-Serine's ability to release neurotransmitter, indicating an increased facilitation of EPSPs in the superficial extensor muscle cells. The ratio difference we got in DAO conditions didn't suggest significant change compared to the ratio difference in D-Serine (Fig. 4), confirming D-Serine's great presynaptic influence in neurotransmission.

However, some of the data we collected was much different than predicted, suggesting possible points of error. The negative value of the ratio we received in our DAO baselines might be an effect of irregular or faulty instruments. There were times when our electrode holders were malfunctioning, causing us to spend a significant portion of time where we were incapable of recording. Combined with smaller inconveniences such as broken electrode tips, misplaced nerves, and electrical noise, the electrode holders could have been the beginning of a number of possible errors in our recordings. It is entirely possible that these faulty instruments may also have provided us with inaccurate information.

Also, there is the possibility that our preparation of D-amino acid oxidase may have affected other D-amino based receptors. The unintentional inhibition of other receptors may have altered our results to look as if DAO has a stronger effect on EPSPs than normal. Further research on the impact of DAO on neurotransmitters would determine if this suggestion is feasible.

Furthermore, although previous experiments have used glutamate to determine the presence of NMDARs in invertebrates (Pfeiffer-Linn & Glantz, 1991), there was no present research indicating that NMDARs in invertebrates were co-agonistically facilitated by D-Serine. Much like the research done in vertebrate neurons (Mothet et al., 2000; Stevens et al., 2003), our research demonstrates that D-Serine plays a significant role in propagating EPSPs in the neural cells of invertebrates. With this knowledge, although there is no distinct evolutionary change between the general development of invertebrates and vertebrate neurons, further research can be done on the particular distribution of D-Serine and other neurotransmitters within the cells of species whom appear much earlier on the evolutionary chain (Byrne, 1987; Nguyen & Atwood, 1990; Hille, 1984; Lunt, 1986). To be able to determine the point in which the co-agonist facilitation of NMDARs appears

evolutionary could offer information about the environmental conditions of the earth that would have favored these conditions.

Further research on the role on the distribution of neurotransmitters involved with NMDARs could also outline further psychological and technological innovations, especially considering psychiatric drug development.

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