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Fluoxetine Acts as an Antagonist at 5-HT2C Receptors in the Crayfish Neuromuscular Junction

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ABSTRACT

Fluoxetine (Prozac) is a frequently prescribed antidepressant, identified as a selective serotonin reuptake inhibitor. Prozac's function as an SSRI leads to the popular belief that its antidepressant mechanism is related simply to an increased serotonin level in the synapse, due to the blockage of the serotonin reuptake pump. However, while some previous research has suggested that Prozac acts as an agonist, other research has suggested that Prozac also acts as an antagonist of $5HT_2C$ receptors, a function apparently contradictory to its role as an SSRI. We sought to further elucidate Prozac's effect on $5HT_2C$ receptors in the crayfish neuromuscular junction. To determine if Prozac acts as an antagonist of $5HT_2C$ receptors in crayfish *Procambrius clarkii* neuromuscular junctions, we compared excitatory postsnyaptic potential (EPSP) amplitudes of control/5HT treatments, and control/Prozac and 5HT. Our results suggest that Fluoxetine does indeed act as an antagonist of $5HT_2C$ receptors in the crayfish neuromuscular junction.

INTRODUCTION

5-HT (Serotonin) is an indoleamine found in plants, animals, and humans (Marsden, 1996). In the human brain and spinal cord, serotonin levels affect a wide range of physiological activity, including sleep and wakefulness, thermoregulation, cardiovascular function, sexual behavior, and drug-induced hallucinatory states (Marsden, 1996). 5-HT acts on numerous receptors (all subtypes in the 5-HT "family"); the $5HT_2C$ receptor, specifically, has been associated with defensive and aggressive behavior in both crayfish and in humans (Panksepp et al, 2003). Most importantly to our investigation, low 5=HT levels in the brain have traditionally been associated with depression, and most antidepressants target the complex 5-HT systems of the human nervous system.

Fluoxetine, more commonly known as Prozac, was introduced into clinical use as a treatment for depression in 1988. Unlike previously used antidepressants, which acted unintentionally on other neurochemicals, Prozac appeared to act selectively on the 5-HT reuptake pump. Twenty-two years later, Prozac is now the most widely prescribed anti-depressant in the world, and has provided effective symptomatic relief for millions of individuals suffering from depression (Vaswani et al, 2003).

Prozac is known as a selective serotonin reuptake inhibitor (SSRI) because (among other effects) it blocks the reuptake of serotonin at chemical synapses. The increased short-term presence of serotonin in the synapse, in turn, indirectly influences numerous pathways and areas in the human brain. Beyond this increased presence of serotonin in synapses, however, the effects of Prozac are not well understood. The struggle to understand the direct mechanism of Prozac is due largely to the complexity of the 5-HT pathways and functions in the human brain. The role of 5-HT in crayfish has been similarly difficult to study, due to the numerous functions and pathways of 5-HT in the crayfish nervous system (Panksepp et al, 2003). And despite twenty-two years of prescribed usage, the direct mechanism by which Prozac exerts its anti-depressant activity is still unclear (Vaswani et al, 2003).

Specifically, as a serotonin (5-HT) reuptake inhibitor, Prozac is traditionally understood to indirectly keep the postsynaptic cell stimulated, causing a more frequent and high excitatory postsynaptic potential (EPSP). However, in addition to acting as an SSRI, Prozac has been shown to act as an agonist for $5HT_2C$ receptors in cultured astrocytes; to inhibit currents mediated by 5HT3 receptors; to potentially cause a downregulation of 5HT1 receptors -- and, as we will be investigating, to act as an antagonist and inhibit 5-HT binding to the $5HT_2C$ receptor (Ni et al, 1996).). Clearly, as demonstrated by the many functions and effects of Prozac in chemical synapses, the conventional understanding of Prozac's antidepressant activity as exclusively an SSRI is insufficient.

Other antagonists of the $5HT_2C$ receptor include N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (Ni, Camacho et al, 1997), as well as the drug agomelatine (Howland 2009). It should be noted that Agomelatine is a prescribed anti-depressant, and it is a

known inhibitor of the $5HT_2C$ receptor. Thus inhibition of $5HT_2C$ receptors and treatment for depression are not necessarily in opposition. However, Agomelatine also acts on melatonin levels in the brain, and its success as an antidepressant is unclear (Howland, 2009).

If Prozac acts as an antagonist at $5HT_2C$ receptors, as Ni et al (1996) demonstrate, EPSPs at the crayfish neuromuscular junction should be depressed. This effect is contradictory to its identified role as a reuptake inhibitor. However, these results contradict an earlier finding that Prozac serves as an agonist of $5HT_2C$ receptors (Chen et al, 1995), and it appears as if research in this area has not extended to invertebrates. Accordingly, our study will seek to further elucidate whether Prozac acts an antagonist or an agonist on the $5HT_2C$ receptor, as well as to extend the investigation to invertebrates. We hope, therefore, as Ni et al (1996) suggest, to gain further insight, through identifying Prozac's possible role as an antagonist, into the effect of such a common drug as Prozac on the human brain.

We hypothesize that Prozac will indeed act as an antagonist in the crayfish neuromuscular junction. We expect, therefore, that EPSPs of crayfish treated with 5-HT and Prozac will be smaller than the control of exclusively 5-HT. Moreover, because we do not expect 5-HT to be present in the crayfish neuromuscular junction after dissecting the animal, we expect Prozac to have no effect on EPSPs until we add 5-HT to the solution. Again: we hope to further understand the role of Prozac in animal synapses by perhaps solidifying its role as an antagonist in the crayfish neuromuscular

Our findings supported the hypothesis that Prozac is an antagonist in the $5HT_2C$ receptors: neither Prozac alone nor the mixture of Prozac and 5-HT did increase EPSPs compared to the baseline, in contrast to the 5-HT treatment, which did.

MATERIALS AND METHODS

Crayfish preparation

The specimen used was an adult crayfish, species *Procambrius clarkii*, obtained from Carolina Biological Supply (North Carolina, USA). The crayfish was kept on ice for anesthetization. The separated tail of the crayfish was used for our studies. We dissected the crayfish down to the muscle bundles along the dorsal part of the tail. The nerves within these bundles are the areas we stimulated and the EPSPs were measured in the muscles found in the same sections as the nerves. After pinning the crayfish to a 200 ml silicon bowl, we added Ringer's solution (Table 1); solution was then changed every fifteen minutes in order to keep the muscle cells responsive. We used one preparation for all of the measurements.

Chemical application

We studied the effects of serotonin (5-HT) and Prozac on EPSPs in the crayfish neuromuscular junction. We only recorded EPSPs if the resting potential was below -20 mV.

Chemical	Concentration (mM)
KCl	5.4
NaCl	196
$MaCl_2$	7.1
Hepes	10
$CaCl_2$	6

Table 1. Chemical composition of Ringer's solution. The pH was 7.4.

First we applied Ringer's solution (Table 1) to take baseline measurements. Then, in order to determine the effect of serotonin in the crayfish NMJ, we mixed 5-HT into the Ringer's solution to produce a final concentration of 10 µM. After washing the crayfish of 5-HT we then measured EPSPs under Ringer's solution again. Following that measurement, we studied the crayfish when it was treated with 10 µM Prozac in Ringer's solution. We then applied a 1:1 mixture of the serotonin and Prozac solutions to the crayfish tail. We finished with measuring EPSPs in a control solution again.

Electrophysiology

We used glass tubules made from glass tubes melted under heat, and filled each with 3M KCl. Glass microelectrodes were used to enter the cell and to measure the EPSPs through connection to a computer, running the program *Scope*. Additionally, we used a suction electrode which allowed us to suck up crayfish nerves and stimulate the muscles. Both electrodes were held by manipulators that allowed for movement of the electrode throughout the solutions and crayfish. We only recorded EPSPs if the resistance of the microelectrodes was at least 4 MΩ.

RESULTS

We studied the effect of Prozac on 5HT2C receptors in the crayfish neuromuscular junction. In order to do so, we measured the EPSP in the crayfish NMJ in Ringer's solution, in serotonin solution, in Prozac solution, and in a mixture of serotonin and Prozac solution. After this, we compared the change in EPSP between control and serotonin to the change between Prozac and Prozac with serotonin. Our goal in using this design was two-fold. First, by measuring EPSPs in this order of solutions, we

sought to determine if Prozac had an independent affect on the EPSP of crayfish neurons. Second, we sought to determine if Prozac had any effect on 5HT. Because of Ni et al's (1997) hypothesis that Prozac acts as an antagonist on 5-HT_2C receptors, we expected that the change from control to serotonin would be larger than the change from Prozac to the mixture of Prozac and serotonin.

EPSP increased when serotonin was applied, and stayed the same as the control when Prozac or the mixture of Prozac and serotonin was applied, as seen in Figure 1.

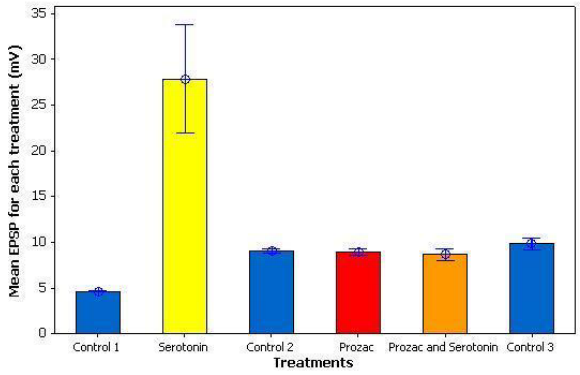

Figure 1. The average EPSPs measured in the crayfish NMJ in various solutions: Ringer's for control (4.59 mV, n=5), serotonin (27.87 mV, n=7), second control (9.06 mV, n=4), Prozac (8.94 mV, n=13), mixture of Prozac and serotonin (8.66 mV, n=16) and third control (9.82 mV, n=7). The average EPSP for serotonin is significantly higher than for control 1 (p=0.008) or control 2 (p=0.019). There was no significant difference between the controls, the Prozac and the mixture of Prozac and serotonin. The error bars indicate standard error of the mean. Student's t-test was used to determine statistical significance.

There was a statistically significant difference between the mean amplitude of the EPSP under baseline conditions and the mean amplitude of the EPSP in serotonin. On the other hand, comparing the mean EPSP amplitudes of the crayfish muscle cells in Prozac and in the mixture solution of Prozac and serotonin did not show any significant difference, suggesting that Prozac alone has no significant effect on EPSPs. It is suggested that the difference between the two control EPSPs was due to incomplete washing of the specimen after treatment with 5HT.

Based on the data, we can conclude that serotonin significantly increased the EPSP amplitude in the crayfish NMJ, while the mixture of Prozac and serotonin did not increase the EPSP, suggesting that Prozac was inhibiting the effect of serotonin.

DISCUSSION

We conclude that our hypothesis was supported: 5HT significantly increased the EPSP amplitude in the crayfish NMJ, while the mixture of Prozac and 5HT did not increase the EPSP. This suggests that Prozac was inhibiting the effect of 5HT. In other words: the lack of increase in the Prozac-5HT solution shown in Figure 1 explains that when 5HT is mixed with Prozac, 5HT does not increase to a large EPSP as in the 5HT solution. We hypothesize that this lack of increase is due to the blockage of $5HT_2C$ receptors by Prozac, for Ni et al (2007) have already suggested that Prozac acts as a $5HT_2C$ antagonist.

Prozac is prescribed frequently to individuals who struggle with depression. However, previous research has shown that Prozac is an antagonist to $5HT_2C$ receptors (Ni et al, 1997), and our results add further data to support this hypothesis. Prozac's role as an antagonist raises questions about the precise function of Prozac in NMJs: if Prozac antagonizes $5HT_2C$ receptors, then Prozac is slowing the flow of 5HT to the postsynaptic cell. This effect is contrary to the identified function of Prozac as a selective serotonin reuptake inhibitor, if the antidepressant function of Prozac is believed to be correlated to increased serotonin levels in the synapse. In other words, our results suggest that increased serotonin in the synapse may not be the direct mechanism, or may not be the only mechanism, by which Prozac acts as an antidepressant.

Thus, broadly speaking: the importance of our research supports the possibility that serotonin's antidepressant activity and effects may involve mechanisms other than or in addition to its function as a selective serotonin reuptake inhibitor. Specifically, our research suggests that Prozac acts as an antagonist on serotonin receptors.

Further research could attempt to identify these mechanisms, examining the effect of increased serotonin in the synapse. More specifically, further research could identify if Prozac also acts an antagonist of $5HT_2C$ receptors in mammals. Moreover, further research could determine if Prozac acts as an antagonist on other types of 5HT receptors. The exact number, nature and function of serotonin receptor subtypes in crayfish is still unknown, though researchers have identified that $5HT_1X$ is also present in the crayfish neuromuscular junction, and there are believed to be numerous others present (Spitzer et al, 2008). Our results do not differentiate between the variety of 5HT receptors present in the crayfish neuromuscular junction, since the EPSPs of the crayfish treated with general 5HT solution were significantly higher than crayfish treated with 5HT and Prozac. It is therefore possible that Prozac acts on serotonin receptor subtypes other than $5HT_2C$.

This experiment had its limitations. Time was the most prominent limitation, as we only had about twenty lab hours to record data. It was therefore vital that we were efficient and accurate in our methods. Second, we had a limited amount of samples to study because we gathered our crayfish from the same supply as seven other experiments. Most significantly, we were not able to pinpoint the precise type of 5HT receptor which Prozac appeared to have blocked. We were only able to observe the more general antagonistic role of Prozac on 5HT receptors; we relied on Ni et al's (1997) methods to focus on the specific blockage $5HT_2C$ receptors.

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