The Effect of COX-2 and FAAH Inhibition on WIN 55,212-2 Induced Spatial Memory Deficits



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Abstract

The hippocampus is a brain structure that is integral to memory processes. There is a high density of cannabinoid (CB) receptors in the hippocampus and many compounds that act on the endocannabinoid system have a negative impact on memory and learning. It is proposed that these deficits are caused by CB-1 receptor dependent increases in cyclooxygenase-2 (COX-2). When stimulated, a subunit of the CB-1 receptor can dissociate from the receptor complex and begin an enzymatic cascade which results in increased expression of COX-2. Studies suggest that this effect of CB agonists can be reversed through co-administration of a COX-2 inhibitor. In this study we attempted to ameliorate memory deficits caused by the CB agonist WIN 55,212-2 through co-administration of rofecoxib and URB-597. Task performance on a complex labyrinth water maze served as an objective measure of the subject's spatial working memory. Although previous research supported this theory, this study did not find a significant effect of drug treatment on either escape latency ($p = 0.87$) or errors made ($p = 0.89$).

Keywords: WIN 55,212-2, Cannabinoids, Spatial Working Memory, Cyclooxygenase-2, Fatty Acid Amide Hydrolase, Morris Water Maze

Introduction

Neural Correlates of Memory

The ability to form and retrieve memories is an essential function of an intact nervous system that provides the organism with the tools necessary for learning. Generally speaking, memories can be defined as recordings of an organism's experience which are then encoded and stored for later recall. These encoded memories influence how the individual will behave in similar situations and as such provides a basis for learning. The neural basis of memory has been subject of study for many years. The hippocampus is often seen as the primary brain structure involved in memory processes. Neurons within the hippocampus exhibit interesting behaviors which are essential to their role in memory. The activation or inactivation of neural circuits can influence the future activity of the circuit in a process known as synaptic plasticity. Long-term potentiation (LTP) is the most well studied form of synaptic plasticity and it is integral to memory formation. LTP is a stimulus-dependent increase in action potentials following repeated stimulation from a presynaptic neuron but it can also be achieved experimentally through electrical stimulation applied in rapid succession also known as tetanic stimulation.

N-methyl-D-aspartate (NMDA) receptors are a type of ionotropic glutamate receptor that are integral to LTP and knockout mice lacking these receptors in the CA1 region of the hippocampus are unable to induce LTP in the presence of a tetanic stimulus (Tsien, et al., 1996). This is because the NMDA receptor has two requirements for activation. One of which being the presence of the respective neurotransmitter, in this case glutamate, but the second requirement is that the post-synaptic cell must be depolarized. If the cell is hyperpolarized, an extracellular Mg^{2+} ion will block the ion pore regardless of whether glutamate is present or not. This gating of the NMDA receptor is why it serves such an

important role in the initiation of LTP because it renders it inactive unless a sufficient amount of neural activity is occurring in that area. Dendritic spines form on the post-synaptic cell as a result of this process which further improves the communication between cells.

The Endocannabinoid System

Cannabinoid (CB) receptors are a type of G-protein coupled receptor (GPCR) found throughout the nervous system and body as a whole. The word 'cannabinoid' is derived from *cannabis* which is a genus of plants which produce compounds which interact with CB receptors in a similar manner to endocannabinoids. Two species in this genus, *cannabis sativa* and *indica* are cultivated for their recreational and medical uses. Currently, there is evidence that compounds found in these plants may provide an effective therapeutic option in the treatment of chronic pain, seizure disorders, and even Alzheimer's disease (Chen, et al., 2013). The primary psychoactive compound in cannabis is Δ-9-tetrahydrocannabinol (THC). The behavioral effects of THC have been described as euphoria, analgesia, sedation, as well as alterations in cognition and memory (Paunescu, et al., 2011).

There are two well studied forms of the CB receptor. The first, referred to as CB-1 is also known as the central CB receptor as it is spread thoroughly throughout the brain with the highest density in the hippocampus, substantia nigra, and cerebellum (Moldrich & Wenger, 2000). The second is the CB-2 receptor which is primarily found to be expressed on immune cells. Currently, five endogenous compounds have been identified to bind to CB receptors but anandamide and 2-arachidonylglycerol (2-AG) are considered to be the two major endocannabinoids. These neuromodulators have been shown to elicit a wide variety of cellular and behavioral effects. One of which being that they appear to have potent anti-inflammatory effects on the cell (Chen, et al., 2013).

Upon stimulation, the subunits of the G-protein that is bound to the receptor will break off and act as second messengers within the cell. This induces an enzymatic cascade which alters the firing behavior of the cell. The $G_{i/o}$ subunit of the CB-1 receptor inhibits adenylyl cyclase which generally has an inhibitory effect on the cell (Turu & Hunyady, 2010). Another way the CB-1 receptor can alter behavior of the cell is by altering the function of ion channels. GPCR's differ from ionotropic receptors in that they do not have an ionic pore and exert their influence over ion channels indirectly through signaling cascades. The CB-1 receptor has been shown to activate inwardly-rectifying potassium channels and inhibit several types of calcium channels. These cellular effects are why it is often referred to as a modulatory system as it doesn't directly inhibit or excite cells, but acts in a slower and less specific manner to alter neurotransmission in the area.

CB receptors are located on the presynaptic terminal and engage in a process known as retrograde transmission. These receptors are located at both excitatory and inhibitory synapses which is important due to their modulatory role in the nervous system. Long-term depression (LTD) is a process counter to LTP in which synaptic activity is suppressed and activation of CB receptors can cause this to occur. When this occurs at an inhibitory site, it can facilitate LTP in excitatory synapses which receive input from the inhibitory site (Puighermanal, et al. 2016). In this manner the endocannabinoid system has a dual role in the facilitation of LTP in that it can selectively increase or decrease LTP depending on the location where these neuromodulators are released.

It is also important to recognize that Purkinje cells in the cerebellum have been found to express CB-1 receptors and it is proposed that hypomotility induced by cannabinoid agonists is caused by the modulatory effect they exert on these cells (Moldrich & Wenger,

2000). Anandamide is broken down in the synapse by an enzyme called fatty acid amide hydrolase (FAAH). The pattern of mRNA expression of this enzyme follows the same pattern as CB receptor expression with the highest densities in limbic and cerebellar structures which offers support to its role as the degradation mechanism for anandamide. Compounds like URB-597 inhibit FAAH and increase synaptic concentrations of anandamide. URB-597 has been shown to reverse memory impairments induced by traditional cannabinoid agonists (Piomelli, et al., 2006).

Induction of COX-2 via CB-1 Receptor

Current pharmacological research indicates that GPCR's can adopt different conformational shapes depending on the ligand which is bound to them. These different shapes can cause differences in the resulting signaling cascade. The most relevant example in this case being that THC appears to induce inflammation through a CB-1 dependent mechanism even though anandamide and 2-AG appear to decrease inflammation. This is because when THC binds to the receptor, the beta-gamma dimer of the G-protein breaks off and begins a signaling cascade resulting in increased expression of COX-2 (Chen, et al, 2013). Contrary to this is when an endocannabinoid interacts with the receptor and causes the alpha subunit to begin a cascade decreasing expression of the same enzyme (Tham, et al., 2007). COX-2 catalyzes a reaction in which prostaglandins are produced from arachidonic acid. The resulting chemicals act as inflammatory mediators and a beacon towards which glial cells such as astrocytes and microglia are drawn. These mediators can also stimulate the release of prostaglandins from endothelial cells found in the cerebrovascular system (Hein & O'Banion. 2009). The inflammation caused by this process has been demonstrated to impair hippocampal LTP and dendritic spine density in a COX-2 dependent manner evidenced by the reversal of this effect upon administration of a COX-2 inhibitor (Chen, et al. 2013).

Water Maze

Hippocampal LTP has been described as the synaptic mechanism by which spatial working memory is encoded and the Morris water maze (MWM) has been used extensively to study this phenomenon (Voorhees & Williams, 2006) (D'Hooge & De Deyn, 2001). In order to specifically assess spatial working memory, slight modifications in protocol from the traditional MWM approach are required. Voorhees & Williams (2006) explained a method in which by moving the platform to a different location within the maze on each testing day, the subjects will not be able to rely on previous experience in the maze in order to find the platform. In this way, the first trial each day is the only reference they have for how to escape the maze and unimpaired working memory would be evidenced by a shorter escape latency in subsequent trials. Because this protocol uses the first trial each day as the sample trial and relies on the subject's natural dislike of water to motivate escape behaviors there is no need to deprive them of food or water. Instead of using a traditional MWM in this study, a complex labyrinth water maze was used because it allowed for the analysis of a second dependent variable. In addition to escape latency, numbers of errors that the subject made during the trial were recorded. Errors were defined as more than half of the rat's body length occupying a path that did not lead to the platform. The rats would frequently cling to the corners of the maze and poke their head around but this was not considered to be an error.

Experimental Objectives and Hypothesis

In the present study, we sought to attenuate CB-1 dependent impairments in spatial working memory through two different methods. The first was direct inhibition of COX-2 with rofecoxib. The second method was through decreasing expression of COX-2 via increased endocannabinoid activity induced by URB-597. It is anticipated that the combined

effect of these methods will be more effective than either one on its own. In this manner, it was expected that the control group would have a shorter escape latency and make less errors than the groups receiving WIN 55,212-2. Additionally, it was expected that the group receiving all three drugs would outperform those that only received the CB agonist. Rofecoxib and URB-597 were not expected to influence maze performance in the absence of WIN 55,212-2.

Materials and Methods

Subjects

Seven female Sprague-Dawley rats were employed as subjects. The rats were born on 7/29/16 and their average weight was 311g; the lightest rat weighing 281g and the heaviest weighing 363g. Sprague-Dawley rats are known to be relatively easy to handle and have a long history of use in medical research. Animals were kept in a controlled environment with a 16.5/7.5 light/dark cycle and ad libitum access to food and water. Animals were acquired from the Allegheny College general animal colony and were housed in the Carnegie Hall animal colony located in the basement of the building. Behavioral testing occurred between 2/28/17 and 3/13/17 for the first three subjects and between 3/14/17 and 3/27/17 for the final four subjects.

Apparatus

The complex labyrinth maze consisted of a square plastic maze with several paths that would not lead to the platform at the end of the maze. The layout displayed in Figure 1 was preserved throughout the course of this experiment however the location of the platform was changed each day. Prior to each test period, the maze was emptied and refilled with warm water. This was necessary because WIN 55,212-2 has been shown to induce

hypothermia in higher doses. In order to avoid potential harm to the subjects, water temperature in the maze was monitored and kept above 70F.

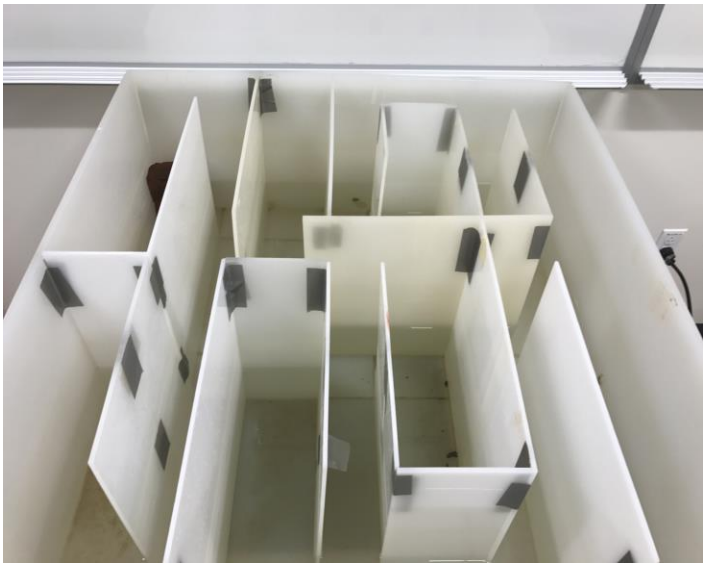

Figure 1: The layout of the complex labyrinth water maze. Water level was faded from near empty to half full for five of the seven subjects as they had difficulty swimming at the beginning of the experiment.

Since the drugs were in a suspension rather than a solution they were thoroughly mixed prior to being drawn into the syringe to ensure that a proper dose was administered. A Fisher Vortex-Genie 2 (Cat. # 12-812) was used to acquire an even distribution of particles in suspension. A Stoelting heating chamber (SN # 63011-301) set to 32°C was used to warm the subjects during each intertrial interval as well as after the final trial in order to avoid harm to the subjects.

Chemical Application

WIN 55,212-2 binds to the CB-1 receptor and elicits similar behavioral effects as THC. It was expected that this compound would impair the subject's ability to find the platform in the water maze. Cannabinoid induced hypomotility could pose an issue to this study seeing as it would be a confounding variable for the water maze task. However, previous research has determined that a dose of 1mg/kg of WIN 55,212-2 was sufficient to impair memory but not the subject's ability to move through the maze (Hasanein & Far

2015). 10mg WIN 55,212-2 was suspended in 1mL dimethyl sulfoxide (DMSO) and 9mL saline. This concentration was selected because the volume injected in milliliters was equal to the subject's weight in kilograms otherwise expressed as 1.0mL/kg. Subjects received four i.p. injections of this compound at a dose of 1.0 mg/kg. URB-597 inhibits FAAH which breaks down neurotransmitters that bind to CB receptors. The anticipated effects of this compound are that it will improve task performance in the water maze in treatments that also involved WIN 55,212-2. URB-597 has no established lethal dose and also it does not appear to present any liability for dependence after chronic administration (Hasanein & Teimuri Far. 2015). 10mg of URB-597 was suspended in 1mL DMSO and 9mL saline and injections were administered at a volume of 1.0mL/kg. Subjects received four i.p. injections of this compound at a dose of 1.0mg/kg.

Rofecoxib is a selective COX-2 inhibitor. This enzyme converts fatty acids to prostaglandins, which are a class of molecules that trigger inflammation in the body. This compound decreases inflammation by blocking the activity of COX and relieves pain. Chronic administration can cause renal, gastrointestinal, and cardiovascular issues but these effects were not observed in this study as the subjects were not be exposed to rofecoxib for a long enough period of time or high enough dosage to elicit them. This drug was also suspended in 10% DMSO and 90% saline and injected at a volume of 1.0mL/kg. Subjects received four i.p. injections of rofecoxib at a dose of 3.0mg/kg. Saline was used as a control and was not expected to elicit any effects in the subject. Each saline injection was administered at a volume of 1.0mL/kg.

Experimental Procedure

Prior to each injection, subjects were weighed in order to determine the volume of solution necessary to give the proper dose. If subject weight dropped below 80% of their original weight at any time during experimentation, the subject would have been pulled from testing until their weight recovered to above 80%. However, none of the subjects lost that amount of weight and so all subjects were deemed fit for experimentation each day. All drugs were administered via intraperitoneal (i.p.) injection. The first injection was WIN 55, 212-2 at a dosage of 1.0mg/kg. This dose was determined by Hasanein and Teimuri Far (2014) to impair spatial memory in rats while not inducing hypomotility which would have been a confounding variable for the water maze due to the fact that it relies on the subject's ability to explore the test chamber. The second treatment was 1.0mg/kg of URB-597 (Hasanein, Teimuri Far. 2014). The third treatment was rofecoxib at a dose of 3mg/kg (Padi, et al. 2004). The following treatment conditions were arranged to examine the effects of co-administration of the compounds above. These conditions consisted of the following consecutive injections at the same doses as described above: URB-597/rofecoxib; WIN 55, 212-2/URB-597; rofecoxib/WIN 55,212-2; and rofecoxib/URB-597/WIN 55,212-2.

In treatments involving less than three drug doses, saline injections were administered in place of the drug so each subject received three injections total on each day including control days. There was a 12 minute period between each injection as well as the final injection and the first water maze trial. All conditions had a total latency period of 48 minutes from the first injection in order to maintain the same time course throughout experimentation. Each subject received all seven treatments in different orders to rule out any order effects. Each day that a subject received one of these treatments was followed by a day of saline injections to serve as a control and to allow the subject to return to baseline

response rate for the next test period if necessary. Behavioral testing consisted of three trials per day in the complex labyrinth water maze. There was an intertrial interval of four minutes between each maze trial in which the subject was towel dried and placed in a heating chamber to prevent a potentially harmful decrease in body temperature. If the subject was unable to locate the platform within four minutes, they were gently guided to the platform and placed on the platform for 30 seconds before being dried off. These trials were recorded as failed but were not excluded from statistical analysis.

Experimental Design

This experiment was a within-subjects design where all subjects received all treatment conditions. The independent variable was the drug treatment group which consisted of eight levels. There were two dependent variables in this study; escape latency measured in seconds and the number of errors made during each trial. The order of these conditions was counterbalanced in a Latin square such that each subject received the treatments in a different order. A one-way repeated measures ANOVA was run for each dependent variable. All statistical tests were run using IBM SPSS to determine whether any significant differences between treatments and maze performance existed.

Results

A mixed ANOVA with trial number (Trials 1, 2, and 3) as the within-subjects variable and treatment group (control, rofecoxib, URB-597, WIN 55,212-2, rofecoxib + URB-597, rofecoxib + WIN 55,212-2, URB-597 + WIN 55,212-2, and rofecoxib + URB-597 + WIN 55,212-2) as the between-subjects variable was run on escape latency. Mauchley's test determined that the assumption of sphericity was violated (X^2 (2)= 7.30, p = 0.026) and as such a Greenhouse-Geisser correction was applied. The interaction between trial number

and treatment group was not significant $F(1.85, 12.98)= 1.61$, $p = 0.87$, $\eta^2 = 0.11$. Neither was the main effect of trial number $F(1.85, 12.98)= 2.18$, $p = 0.12$, $\eta^2 = 0.024$ or the main effect of the treatment group $F(7, 90)= 0.50$, $p = 0.83$, $\eta^2 = 0.037$.

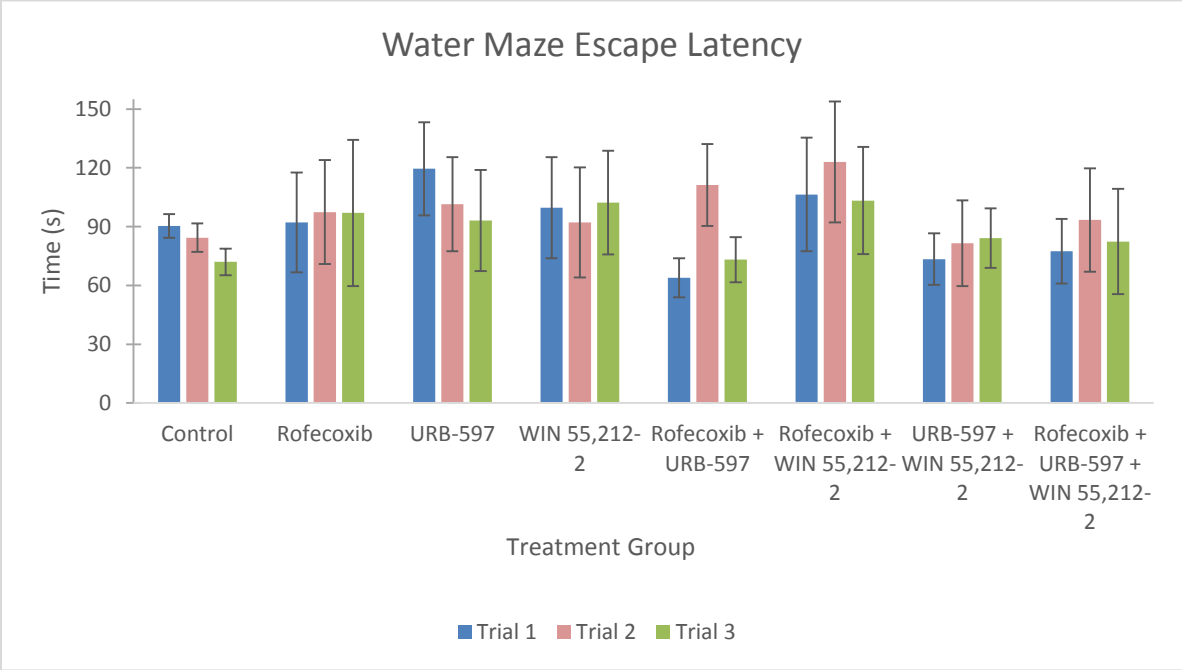

Figure 2: Mean escape latency for each trial and group is plotted above. Error bars represent standard error of the mean. No significant interaction between trial number and treatment group was observed.

Another mixed ANOVA was run with trial number (Trials 1, 2, and 3) as the within-subjects variable and treatment group (control, rofecoxib, URB-597, WIN 55,212-2, rofecoxib + URB-597, rofecoxib + WIN 55,212-2, URB-597 + WIN 55,212-2, and rofecoxib + URB-597 + WIN 55,212-2) as the between-subjects variable on errors made in the water maze. Mauchley's test determined that the assumption of sphericity was violated (X^2 (2)= 11.28, $p = 0.004$) and as such a Greenhouse-Geisser correction was applied here as well. The interaction between trial number and treatment group was not significant $F(1.79, 12.51)= 0.89$, $p = 0.56$, $\eta^2 = 0.065$ and the main effect of treatment group was not significant either

$F(7, 90)= 0.57$, $p = 0.77$, $\eta^2 = 0.43$. However, the main effect of trial number was significant $F(1.79, 12.51)= 48.50$, $p = 0.001$, $\eta^2 = 0.35$. Across all groups, significantly more errors were made on the first trial ($M = 4.43$, $SD = 2.27$) than on the second trial ($M = 2.78$, $SD = 1.77$) and significantly less errors were made on the third trial ($M = 1.90$. $SD = 1.58$) than on the preceding two.

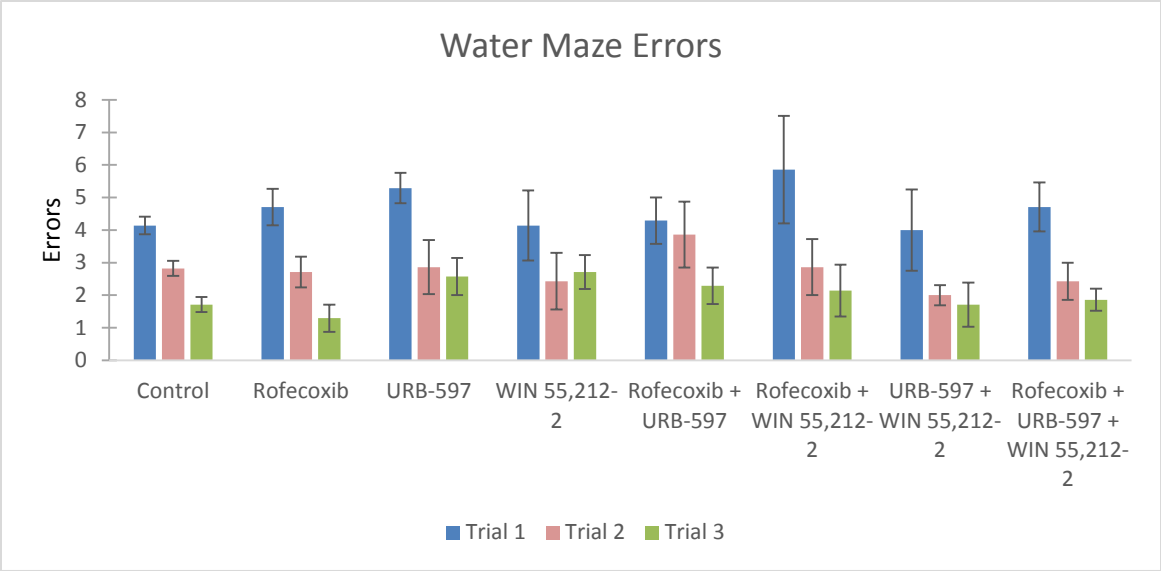

Figure 3: Mean number of errors for each trial and group are plotted above. Error bars represent standard error of the mean. There was no significant interaction between trial number and treatment group.

Discussion

Overall, the results of this experiment were inconclusive. WIN 55,212-2 should have impaired maze performance according to previous research yet no statistically significant difference was observed between the days that subjects received WIN 55,212-2 in comparison to control. Additionally, there was no evidence to support the hypothesis that URB-579 or rofecoxib improved CB receptor induced impairments in maze performance. The only significant result indicated that trial number had an effect on the number of errors the subject made. This means that less errors were made in successive trials. This was the

expected result however since the interaction between trial number and treatment group was insignificant, this study did not provide any evidence that the treatment groups had an effect on maze performance. Subjects treated with saline and all three drugs had a slightly shorter escape latency than those treated with WIN 55,212-2 which was the expected result, however these differences were not large enough to be statistically significant.

While previous research has assessed the effect of COX-2 and FAAH inhibition on cannabinoid induced impairments in spatial memory, there is a lack of research on what these drugs would do in combination with one another. The results of this study are incongruous with previous work indicating that COX-2 inhibitors can improve impairments in maze performance caused by cannabinoid agonists (Chen, et al. 2013). As well as research indicating that FAAH inhibitors elicited the same effect on these impairments (Hasanein & Teimuri Far. 2015). It is possible that an interaction occurred between rofecoxib and URB-597 that prevented these effects from occurring but further research must be conducted in order to determine whether this interaction exists.

Limitations

This study had many limitations which may have prevented a significant effect from being observed. First of all there was a reasonable difference between how fast each individual rat was able to complete the maze especially during the first few days. In total there were 17 failed trials in which the subject was unable to find the platform before the four minute cutoff time. The subjects would occasionally spend the majority of the trial period fixed in one spot and this led to several of the failed trials. It is possible that this immobility was caused by WIN 55,212-2 as it has been previously observed to cause this (Hasanein & Teimuri Far. 2015). However, immobility was observed in subjects not treated with WIN

55,212-2 so it is not likely to be the reason this occurred. Since trial failure was specific to the subject and not the treatment group it's possible that that preexisting differences between the rats could have skewed the results (Voorhees & Williams. 2010). A pretreatment period could have potentially minimized their effect on the data. It appears that the rofecoxib + WIN 55,212-2 group had the worst maze performance out of any group which is inconsistent with previous research however since failed trials were not excluded from data analysis, these data points could be responsible for this observation.

Previous researchers have used a 30 second intertrial interval when assessing spatial working memory (Varvel & Lichtman). In this study a four minute intertrial interval was required because the rats needed to be dried off and warmed before being put back in the maze. It is possible that the length of the intertrial interval prevented an effect from being observed because working memory doesn't last very long and the subjects may have forgotten the path that led to the platform in this time. Another limitation that impacted the results of this experiment was the small sample size. Since each day that a drug treatment was administered was followed by a control day, there was a much larger sample size for the control group than the experimental groups and this is reflected in the smaller standard error in this group relative to the others. This is further supported by the fact that there was a clear pattern of responses present in the control group that was not seen in the other groups.

Future Directions

Further research will definitely require a larger sample size or perhaps another round of drug treatments to increase the number of measurements of each group. These alterations would likely lead to more meaningful results because they would decrease the standard error and change the mean to a number that is more representative. If increasing the sample size

does not provide significance, then it would lend support to the possibility of a detrimental interaction between rofecoxib and URB-597. It may be beneficial to employ a different behavioral test which does not require drying off the subjects after each trial such as a radial arm maze which is also used to assess spatial memory in rats. A delayed match-to-sample schedule of reinforcement could be used to examine working memory but this would require either food or water deprivation in order to be effective. Another way to go about this experiment would be to use a between-subjects design. While this would increase the impact of individual differences on the results in addition to increasing the required sample size, it would afford several opportunities to the researcher. For example, it would be possible to examine whether the drug treatments caused physiological differences in the subjects. A staining procedure could be used to examine the effect of cannabinoid agonists on glutamate receptor expression in the hippocampus. Since THC has been shown to decrease expression of these receptors through pro-inflammatory signaling (Chen, et al. 2013) in a manner that is reversible through COX-2 inhibition, it would be interesting to find out if URB-597 has this same effect on glutamate receptors.

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