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Efficacy of Intranasal Orexin A Administration: A Clinical Approach of Attention

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Neuroscience from The College of William and Mary

By

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Abstract

Orexins are a class of neuropeptides that are associated with homeostatic functions, such as food intake, sexual behavior, and the sleep/wake cycle. Orexins also play a pivotal role in cognitive processes such as attention and memory. In the present experiment, we employed a visual attention task to assess the effects of intranasal administration of orexin A in rats. This task required the rats to discriminate between trials when a visual light was illuminated from trials when the light was not illuminated. We tested the efficacy of this administration technique to see its viability as a therapeutic in attention disorders. Intranasal orexin A decreased accuracy in the visual sustained attention task, specifically on trials when the light was not illuminated. This task performance decline may be due to the over-activation orexins, which in turn hyper-excites the acetylcholine-releasing neurons, a neurotransmitter system known to be critical for attention.


Introduction

Attention dysfunction is a symptom of a variety of diseases, including but not limited to, Autism (Allen & Courchesne, 2001), Attention Deficit Disorder (ADD) (Barkley et al, 1992), Alzheimer's Disease (Perry et al, 2000), and Schizophrenia (Diwadkar et al, 2014). Attentional dysfunction can have a significant negative impact in people’s lives, thus, treatments for these deficits are important. Attention is associated with the basal forebrain, hippocampus and parts of the thalamus (Posner & Petersen, 1990). With these regions of the brain being so important for different physiological functions it can be assumed that by changing the attention processing can also alter sensory processing and ultimately motor behaviors of the subject as well. The basal forebrain contains acetylcholine-releasing neurons, cells that are necessary for normal attentional processing. The hippocampus and thalamus are both used in memory formation and emotional processing; the thalamus is also the “relay” center, as all sensory information (besides olfaction) go through this structure (Posner & Petersen 1990).

Alzheimer’s disease (AD) is a debilitating disease that more than 5 million people live with to date, and about 16 million people will be diagnosed with the disease by 2050 (Alzheimer's Association, 2017). AD is the sixth leading cause of death in America, and the onset of this disease typically occurs at 65 years of age and the prevalence increases in older groups (Alzheimer’s Association, 2017). AD is a neurodegenerative disease that is characterized, during early stages, by a decline in cognitive processing, especially attention and memory. As the disease progresses patients need more caregiving because of their diminishing ability to remember and complete daily activities. Thus, development of
therapeutics to help alleviate the symptoms are of high importance for patients and caregivers.

Research demonstrates, in ADD and similar disorders there is frontal lobe hypoactivity (Barkely et al, 1992). The frontal lobe is critical for normal executive functioning, such as selectively attending to environmental stimuli, inhibitory control (e.g. not doing something), working memory and cognitive flexibility. All of these different diseases that have attention problems have distinct causes and progress because of different mechanisms. However, enhancing the activity of the core neural circuitry underlying attention may have beneficial effects for multiple conditions characterized by aberrant attentional processing.

**Cholinergic System**

Acetylcholine-releasing neurons located in the basal forebrain that project to the cortical regions of the brain, are essential for attention task performance (Sarter et al, 2005). Research has shown cholinergic transmission in sensory areas (i.e. vision and audition) enhances the cortical processing of thalamic inputs (Sarter et al, 2005). Enhanced cortical processing is viewed as the ability to narrow the attention on the incoming stimuli while simultaneously filtering the irrelevant noise. This increased cortical processing helps with better controlled attention processing and working memory, potentially leading to more efficient long-term memory storage. The cholinergic system can also suppress the retrieval of internal cues (Sarter et al, 2005), which may distract an individual from target detection in the environment, therefore showing how this system is able to help focus the attention of the subject.
Cholinergic inputs to the prefrontal cortex also mediate the filtering of irrelevant information (Sarter et al, 2005). Thus proper modulation of this system is important to be able to distinguish between different signals or cues.

Orexins and Associated Receptors

Neurotransmitters are usually at most a couple of amino acids in size. But orexins are in the class known as neuropeptides, which consist of more amino acids, with orexin A being comprised of 32 amino acids. Orexin A has two helices connected by a loop and then it has a hairpin-looped tail. Four cysteine residues create the two disulfide bridges that hold the hairpin structure in place. As this structure is very stable and has few charged residues it is able to bypass most lipid bilayers (Yin et al, 2015). This structure does not possess many large aromatic or large/bulky residues thus it is also able to pass through most cell membranes, making this neurotransmitter able to adapt to many different cellular environments.

Orexins have two receptors, orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R). Both of these receptors engage GPCR signaling. Both have similar homologies, as they both are helix bundles that have a hydrophobic pocket (PubChem, 2018). The receptor will make contact with the hairpin loop of the orexin A ligand and start a GPCR mechanism cascade event. Orexin A has affinity for OX1R and OX2R, and Orexin B has affinity only for OX2R (Holmqvist et al, 2004). The specificity of these neurotransmitters makes it better for targeting specific receptors so that we know the direct effect of specific receptor activation. We wanted to look at the general effects of orexin administration. So, we picked orexin A, for we did not need to know specificity of the receptor action at this time.
Orexinergic System and Attention

The orexinergic system is shown to have significant influence on homeostasis by regulating processes such as, eating, drinking, sleep/wake cycle, and body temperature (Tang-Christensen et al, 1996). In addition, research has also demonstrated that orexin neurons can regulate attentional performance (Zajo et al, 2016). Orexin receptor activation will subsequently stimulate a GPCR mechanism (Gs or Gi) to then activate adenylyl cyclase to create more cyclic AMP (cAMP). If more cAMP is produced then this will then activate both PKC (protein kinase C) and PLC (phospholipase C). The increase in these two proteins leads to two different cascades, but the end result for both is an increase of calcium, which will help with increasing conduction of the neuron. Increase in conduction of neurons has been shown to improve mental abilities and flexibility. Therefore orexins maybe used in a therapeutic sense to help increase neuronal firing to cholinergic cortical regions (Sarter et al, 2005).

Orexins may be able to facilitate neural processing in other ways. For example, orexins may induce neuroplastic effects. These effects can increase of firing rate and potentially stimulate synaptogenesis. Increased synaptogenesis associated with increased connectivity, which can lead to more efficient biological systems (Bonci and Borgland, 2009; Borgland et al, 2006)

The ability of orexins to influence the cholinergic system has been well studied, but the best administration technique has not been concluded. Intravenous administration is limited by how easily drugs to cross the blood brain barrier or that neurosurgery can cause tissue damage. One viable low risk option would be to do intranasal administration (Thorne et al, 2004). The nasal passages are easily able to absorb most water and lipid
soluble drugs. The nasal pathway can be mapped to specific brain areas, being able to go to the cortices and as deep as the hippocampus and basal forebrain (Lochhead & Thorne, 2013). Thus, intranasal administration potentially allows for a high concentration of orexins to be able to exert its effects on the cholinergic projections (Lochhead & Thorne, 2013).

**Intranasal administration**

Intranasal (IN) administration was previously thought to be an inferior way to administer drugs compared to intravenous (IV) or intraperitoneal (IP) administration. However, it has been shown that there was actually more Orexin A in the cerebral spinal fluid (CSF) of rats who got IN administration compared to IV administration (Lochhead et al, 2011). Thus, IN administration may be more useful for getting orexin A into the brain compared to IV administration.

In patients with varying diseases they need to be treated with an assortment of drugs, some taken orally, some taken via IP injection into the abdomen, and some have to be administered intravenously. Most of these methods have low absorption rates and are unable to get to their targets as easily. Because the nose is lined with a mucosa membrane on the inside it can easily have drugs that are lipophilic diffuse across it. Thus, using this as a drug transport seems like a reasonable alternative to other administration methods.

Drugs can more easily pass through the nasal membrane with little disruption of tissue and can go directly to the brain. IV and IP injections disrupt tissue and other cell types while also being painful or even have detrimental impacts for the patient. Oral medications (e.g. pills or liquid medication) are not easily metabolized by the body, and
once they get to the target site they are not usually in the highest concentration (Lochhead J. & Thorne G., 2013). Also, if you want to raise the concentration of the drug pre-administration you run into the problem of causing too much of the drug in the periphery, possibly leading to side effects. Thus using IN administration seems much more beneficial for its many advantages. Such advantages include, non-invasive/reduced infection rate, bigger surface area (approximately 160 cm² for humans), rapid absorption (can bypass the blood brain barrier), and the drugs avoid elimination through first-pass metabolism in the hepatic system.

There are of course some drawbacks of IN administration, such as the active mucociliary clearance, low pH of the nasal passage, and you must use smaller volumes of the drugs (Lochhead J. & Thorne G., 2013). Mucociliary clearance describes the cilia of the respiratory tract expelling incoming foreign substances; low pH can denature or change the conformations of drugs. Lastly, the nasal passage can only uptake so much liquid at once, limiting the volume of a particular drug.

**Neuroinflammation**

Neuroinflammation is the activity of pro-inflammatory factors, which will then cause cells to take up more water and become “inflamed.” This activation has negative consequences because inflammation decreases neural plasticity, which can impact a variety of processes, including cognition (Lynch, 2015). Neuroinflammation will also cause microglial activation, due to the fact that inflammation in time will cause the neurons to undergo apoptosis (Duffy et al, 2015). Microglia can be beneficial for removing cellular debris, but constant activation can be deleterious. Microglia have reactive oxygen species
(ROS) as byproducts, which will cause further disruption of cells leading to more inflammation, which in turn will cause more activation of these microglia (Duffy et al, 2015).

Activation of orexin receptors can down-regulate microglia, which helps lessen the neuroinflammation (Duffy, 2015). With less neuroinflammation there will be less reactive oxygen species and cellular debris can hinder the firing rate. Allowing firing rates to speed up will lead to an improvement of cognition. Without excitotoxic effects, the neurons should be properly functioning to then help them fire faster which subsequently will allow for better attention scores.

Neuroinflammation can be activated by different factors, such as internal pro-inflammatory factors or exterior insults, such as surgery or brain trauma. A study conducted in 2007 proved that neurosurgery will increase interleukin 1beta (IL-1β) RNA in rats post surgery. IL-1β is a pro-inflammatory factor that has been shown to decrease neuron viability, which leads to apoptosis (Roscyzk, 2008). The same study also took a survey showing that the elderly who got neurosurgery have more cognitive deficits post surgery.

**Measuring Attention in Animal Models**

There are several different methods for measuring attention. Depending on what type of attention one wishes to study different tasks are used. There is sustained, spatial, divided, and alternating attention (Yantis & Johnston, 1990). The Morris Water Maze is used to help asses spatial attention as the rats are trying to locate a submerged platform be able to stay above water. This test helps understand how rodents navigate a tank with
minimal cues to be able to find the platform (Svensson et al, 2017). Researchers can use a Simultaneity Test DIAT-SHIF to be able to measure the ability of the subject to shift between two different stimuli (CogniFit, 2018). An example of an alternating attention task is cooking a meal, as you read the recipe and cooks the meal.

In this study we measured the sustained attention of rats. We are able to measure attention by using operant box conditioning to quantify the rats’ responses. Once the rat gets into the box there are two extended levers, which the rat will have to identify as the correct response. The goal of this experiment is to test whether orexin A changes the accuracy of responding in this attention task.

The Present Experiment

The goal of the experiment was to test the effects of IN orexin A administration on attentional performance. There are two possible outcomes for the experiment. First, orexin A could enhance attention, consistent with a previous finding with intracranial orexin A infusions. An alternative possibility is that activation of the orexin receptors via this administration method will lead to hyper-excitation. If there is too much saturation of the orexin receptors we will see the over-excitatory inputs to the cholinergic system, which will cause deficits in attentional performance.

Material and Methods

Subjects

12 male FBNF1 hybrid rats (National Institute of Aging Colony), weighing 320 grams to 490 grams, at 3 months old starting at the beginning of the study. The rats were housed
in pairs in a temperature and humidity-controlled vivarium, with a 12hr light/dark cycle. All behavioral testing and drug administration occurred during the light phase. All rats were water restricted throughout the experiment, only receiving water as a reward in the attention task and then, 30 minutes after the test session, rats were given *ad libitum* tap water access for 10 minutes. The rats were tested 6-7 days a week, and on non-testing days they would receive 20 minutes of water access. Food was available *ad libitum* throughout the study. All procedures were approved by the Animal Care and Use Committee at the College of William and Mary.

**Apparatus**

Rats were trained in one of four chambers, each located within a sound-attenuating box (Med. Associates, Inc.). Each chamber included a water dipper that could be raised to afford access to tap (0.01mL). Two levers were located on either side of the dipper. A panel light was above each lever and another light was above the dipper. A house light was on the opposite side of the chamber from the dipper and panel lights. Illumination levels of these chambers have been measured and described (Burk, 2004). Behavioral testing programs and data collection was monitored on a personal computer using Med-PC version IV software.

**Attention Task Procedure**

Testing took place between 9:00am to 4:00pm daily. Rats were trained initially to press an extended lever using a fixed ratio-1 (FR-1) schedule of reinforcement. Rats moved to the next phase of training once they received 120 rewards per session for three sessions.
In the second stage of training, there were two trial types, a signal trial and a non-signal trial. On a signal trial the center panel light would illuminate (1 second) and on a non-signal trial the light was not illuminated. A press on one lever was considered correct on signal trials and a press on the other lever was considered correct on non-signal trials. If the rats pressed the correct lever for a trial they receive water access for 3 seconds via the water dipper. The left lever was considered correct on signal trials for half of the animals and the right lever was considered correct on non-signal trials half of the rats and the rules were reversed for the other half of the rats. Each session lasted for about 45 minutes. The inter-trial interval (ITI) was 12 seconds. Once the rats reached at least a 70% accuracy on signal and non-signal for three consecutive days with a maximum of 60 omissions (failure to press either lever) they would move to the final stage. Before drug infusions began they moved into the last stage of training. In this task the signal duration varied between 500 ms, 100 ms, or 25 ms, and the ITI (9±3 seconds) was shorter and varied to create more attention demand (Zajo et al, 2016). Each training session consists of 162 trials (81 signal trials and 81 non-signal trials). For signal trials each of the three signal durations were presented for 27 trials within a session. Each trial was present in blocks of 18 (9 non-signal and 9 signal with 3 of each signal duration), and trial types were selected at random without replacement. Once reaching >70% success on all trial types then they move onto drug infusions and distracter test.

**Drug Administration and Behavioral Testing Sessions**

Orexin A was prepared a day before the first drug administration session and kept in the freezer until drug administration began. Mixing 500 µg of Orexin A (Tocris) with
140.4 mL of saline solution for a final concentration of 1000 nM. Then a second dilution was performed, taking 1mL of stock solution and mixing 9 mL of saline for a final concentration of 100nM. Before drug infusion days the rats were acclimated to the intranasal administration technique by infusing saline 3 days a week for two weeks. A volume of 0.25 µL of saline into each rats’ nostril by placing a pipette immediately near the nostril entry and administering the drug. Then the rats were intranasally administered 0.25 µL of orexin A (High dose= 1000 nM; Low dose= 100nM; Vehicle= Saline) approximately five minutes before they were put through their attention test. Rats received a total of three infusion sessions, receiving each drug dose once, in an order that was randomly assigned for each rats. On drug administration days the rats performed a “distracter” version of the task. The distracter version of the task was the same as pre-drug administration training, except that the houselight in the back of the chamber was flashed (1 second on/1 second off) during the second block of trials (trials 55-108) during the testing session. The first and third trial blocks were the same (no houselight flashing) as during pre-drug administration training

Behavioral Measures and Statistical Analysis

The number of hits (H), misses (M), correct rejections (CR), false alarms (FA), and omissions were recorded for each animal on every testing day. The relative hits were calculated as such: \[H/(H+M)\]; relative correct rejections were calculated as such: \[FA/(FA+CR)\]. During the drug infusion days, the baseline version of the test, where the houselight is continuously illuminated, occurred for the first 54 trials (block 1). Then for the next 54 trials (block 2), the houselight was flashed as a distracter. The houselight was
continuously illuminated for the final 54 trials in a testing session (block 3). Omissions were calculated separately from measures of accuracy. The hit rate, correct rejection rate, and omissions were determined for each block of trials in the testing session.

Repeated-measures Analyses of Variance (ANOVAs) were conducted. The factors in each analysis included drug dose (saline, 100mM, 1000mM) and block (block 1, block 2 when the distracter was presented, and block 3). For analyses of hits, signal duration (500, 100, 25 ms) were included. Significant main effects and interactions were assessed with follow-up t-tests or additional ANOVAs to clarify the basis of the significant effects. An alpha level of .05 was used to determine statistical significance.

Results

For hits, a dose X block X signal duration ANOVA yielded significant main effect of block interactions (F(2,22)=9.614, p=0.001). Subsequent t-test indicated that the hit rate was higher in block one compared to block three (t(11)=4.607, p=0.001). Hits were also higher during block two compared to block three although this result was not significant when the Bonferroni correction was applied.

Correct rejections (CR) reflect accuracy on non-signal trials. A dose X block ANOVA was conducted to assess effects of orexin A administration on CR. There was a significant dose X block interaction (F(2,22)=7.291, p=0.004). Follow-up one-way ANOVAs including the factor block for each dose (vehicle, low dose, high dose) yielded a significant effect of block when the low dose was administered (F(2,22)=7.721, p=.003), but not when vehicle or the high dose were administered. Subsequent paired t test shows that, for the low dose,
accuracy is higher during block 1 compared to block 3 \(t(11)=4.737, p=0.001\), and that correct rejections are higher during block 2 compared to block 3 \(t(11)=2.617, p=0.024\).

An omission is when a rat does not press any lever when both of the choice levers are extended. A dose X block ANOVA did not yield a main effect or interaction involving dose, indicating that drug treatment did not differentially affect the rats' response rate.

**Discussion**

The results suggest that IN administration effectively activates orexin receptors, but that the doses in the present experiment were sufficiently high to impair attentional performance. We did not see significant effects for the doses on the hits (see figure 1); we saw a general trend of worsening scores, but no dose difference. This might be because there is a saturation effect with the activation of the orexin receptors. The same amount of receptors might be activated in both doses, thus the doses should be adjusted accordingly. One possibility is that orexin A was able to be easily absorbed so we saw declines in task performance due to over-activation of orexin receptors. We tested this method of administration with orexin so that we can observe the impact that the high molarity of orexins have on direct administration via intranasal injections. The nasal cavity is made of a mucosa membrane, which allows entry to the brain immediately after administration. Labs have shown that following an IN injection of orexin A leads to a 5 to 8 fold increase in concentration of orexin A in the brain compared to IV injections (Dhuria et al, 2009). We see that the attention scores of the rats are severely impacted by the orexin A administration sessions compared to the vehicle (saline).
We see that, in a measure of visual attention, accuracy on non-signal trials decreases following orexin A administration compared to the vehicle (saline). One potential reason for this effect is that orexin neurons were over excited, which has shown to significantly decrease the accuracy of the attention performance of the rats. Previous research has shown that cholinergic system hyper-excitation leads to decreases in accuracy on non-signal trials in the present attention task (Sarter, M., & Bruno, J., 2004).

For the correct rejections, orexin A does not produce significant differences in accuracy compared with vehicle administration during the first block of trials within a testing session. The rats also displayed similar scores for the second block as well. However, during the third block, when the rats were recovering from the distracter, the low dose rats showed a sharp deficit going into their last block compared to the vehicle rats (see figure 2). The vehicle-treated rats were able to recover from the distracter, as their performance is similar on the first and third block of the trials in the attention task. The most interesting result from this experiment was the effect of the low orexin A dose on the rats’ correct rejections. The rats at the low dose actually displayed higher hit percentages in the first block than the other two dose groups. They also did better on block 2, but when it came to block 3 they did very poorly. This might have to do for the fact that orexins are helping the rats at first with this low dose or producing a bias to press the hit/false alarm lever. Once orexin A is in their system long enough and attentional demands are altered via distraction, we see a sharp decline in their accuracy. Their omissions get higher as well; thus showing a corollary effect of how the orexins being metabolized can affect their attention (see figure 3).
**Future Directions**

This experiment suggests that IN orexin A administration can affect cognition. Several different future directions arise from these results. First, histological experimentation to explore the cellular receptor system of the basal forebrain and hippocampus regions with regard to orexin receptor activity needs to be examined. For example, staining for orexin 1 receptors and acetylcholine receptors to see if there is correlation in the synaptic densities of the varying dose groups. This staining has been done in other studies prior (Fadel et al, 2001), but in this study we would be looking at the impact of intranasal administration. We would be specifically looking at if there were any up or down regulations of orexin receptors or acetylcholine receptors. This will help further our understanding of how the activation of the orexin receptor can impact neural plasticity of the cholinergic and orexinergic systems.

Many drugs are known to impair attentional performance and effect Ach functioning. For example, cannabinoids are a specialized endogenous neurotransmitter as well as an exogenous molecule found in plants and synthetic compounds. Just like orexins they can impact for attention and homeostatic activity (Huang et al, 2007). But unlike orexins, cannabinoids are shown to only be detrimental for attention and memory. From this experiment we then want to take this new information about the ability of intranasal administration and apply it to another system of interest. For example, it would be interesting to test whether cannabinoid receptor agonist-induced attentional impairments can be reversed by IN orexin A (Ramírez et al, 2005). IN orexin A administration may be able to normalize depressed cholinergic functioning induced by cannabinoid receptor agonists (Crespo et al, 2009). This treatment plan would be beneficial for people who have
underactive orexin systems (such as people who are habitual cannabis users or people who have developed AD). In contrary, this treatment plan would not be good for people who have overexcited Ach-releasing neurons, for it would over activate it too much and lead to cognitive difficulties.

With the largest growing demographic in the country being the elderly, and tending toward cognitive challenges and underactive cholinergic system, it becomes imperative to develop treatments to facilitate cognitive processing. If we can attenuate age-related cognitive decline or improve the symptoms in pathological conditions, such as AD in a timely fashion, then we are able to cut out a lot of taxpayers’ dollars. Financials aside, these less invasive treatment options are very beneficial not only for the elderly but for the whole population.

In closing, orexin A may be useful to normalize low acetylcholine levels. This experiment shows the efficacy of intranasal administration and how to use this method so that we can have better and more efficient drug administration treatments.
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**Figures:**

*Figure 1 Hits*

No significant differences with the doses of orexin on the hits, but there is a declining trend in the hits with the orexin doses.
There are significant deficits transitioning from the 2\textsuperscript{nd} block to the 3\textsuperscript{rd} block for the low dose. The higher dose shows a similar trend, but is not significant. Lastly, the vehicle there is a recovery of CR after the 2\textsuperscript{nd} block.
Figure 3 Omissions

There are no significant changes on omissions, but there is a trend of more omissions with both of the orexin doses.
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