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The Effect of Intranasal Orexin A on the Interaction between Social Isolation Stress and Attention

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The Effect of Intranasal Orexin A Administration on the Interaction between Social Isolation and Attention

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Abstract

Orexins are neuropeptides that have been shown to have an association with processes such as stress and attention. However, limited studies have been done on orexin's role in the intersection of stress and attention. In this experiment, Sprague Dawley rats were isolated at 21 postnatal days, isolated at 35 postnatal days, or placed into group-housing. Varying doses of orexin A or saline were administered intranasally into the rats. Their attentional performance was measured in a sustained attention task. An ANOVA was carried out on the hit rates, correct rejection rates, and omissions of socially isolated rats and group-housed rats. Intranasal orexin A did not significantly affect the attentional performance of either group-housed or isolated rats. There was no significant difference between the rates of hits or omissions between isolated rats and group-housed rats. However, isolated rats had significantly lower correct rejection rates than group-housed rats. These findings suggest that social isolation can cause attentional dysfunction, but intranasal orexin administration is insufficient to affect this dysfunction. Nonetheless, the study was limited in actually assessing the role of the orexinergic system in isolation-induced attentional dysregulation. Future studies should look at whether the orexinergic system is involved in this stress-induced attentional dysfunction, via the use of orexin receptor antagonists, and whether acute social isolation produces similar effects.

Introduction

The neuropeptides known as orexins have been shown to have an association with processes such as sleep, hunger, and stress. There are two types of orexins, orexin A and B, and two orexin receptors, the orexin 1 receptor and the orexin 2 receptor. Orexin receptor 1 has a higher affinity for orexin A, while orexin receptor 2 has a similar affinity for both orexin A and B (Sakurai et. al., 1998). These proteins were specifically expressed in the lateral hypothalamus

(Sakurai et. al., 1998). The lateral hypothalamus has been found to have orexinergic neurons that project to areas such as the locus coeruleus, the basal forebrain, the bed nucleus of the stria terminalis, the reticular formation, and the paraventricular nucleus of the thalamus, which are all neuroanatomical structures that are involved in arousal and stress (Peyron et al., 1998). The orexinergic system was first found to have a role in appetite, since when orexins were administered centrally, food consumption was increased in rats (Sakurai et. al., 1998). Subsequently, arousal was shown to be modulated by orexins. Orexin knockout mice showed symptoms similar to narcolepsy, such as random sleep attacks, and modafinil, an anti-narcoleptic drug, has been shown to activate the orexinergic system (Chemelli et al., 1999). Narcoleptic dogs also have been found to have a mutation in the gene for the orexin 2 receptor (Lin et al., 1999). Narcoleptic humans also have significantly less orexins detected in their cerebrospinal fluid (CSF) (Nishino et al., 2000). Sleep was also induced by orexin 1 and 2 receptor antagonists in rats, dogs, and humans (Brisbare-Roch et. al., 2007). Based on these findings, it has been well established that the orexinergic system modulates appetite as well as the sleep-wake cycle, but it plays a role in other behaviors as well.

Orexins, Stress, and Cognition

The orexinergic system also has a role in the stress reaction in rats, and is activated by stressful stimuli. Infusion of orexin A into the ventricles of the brain can induce behaviors corresponding to stress such as face-washing and grooming (Ida et. al., 2000). Swimming stress has been shown to increase the expression of cFos, a protein released in active neurons, in orexinergic neurons, which suggests that stress activates orexinergic neurons. Orexin 2 receptor antagonists reduced the increase of plasma adrenocorticotropic hormone (ACTH) induced by swimming stress (Chang et. al., 2007). Corticotropin release hormone (CRH) neurons release

CRH, leading to the release of ACTH from the pituitary gland. This release of ACTH facilitates the release of adrenaline from the adrenal gland. CRH neurons located in the paraventricular nucleus of thalamus have been demonstrated to express orexin 2 receptors. Stimulation of orexinergic neurons increases cFos in CRH neurons (Trivedi et. al., 1998; Bonnavion et al., 2015). Orexins facilitate anxiety. In the aforementioned study by Ida et. al., infusions of CRH antagonists decreased face-washing and grooming induced by orexin A (2000). Clinical studies have also produced similar data: patients with panic anxiety have higher orexin in their cerebrospinal fluid (CSF) (Johnson et. al., 2010). Orexin 1 receptor (OX1R) antagonists decrease anxiety-like behavior in rats as measured by their reaction to exposure to cat odor (Vanderhaven et. al., 2015). Orexin 1 receptor antagonists also reduced anxiety-like behavior induced by lactate measured by the open field test (Johnson et. al., 2010). These studies suggest the orexin 1 receptor plays an important role in stress and anxiety-like behavior.

Orexins also play a role in general cognition. For instance, attentional performance on a sustained attention task decreased in rats when they were given the orexin 1 receptor antagonist SB-334867, and increased when orexin A were systemically administered into the prefrontal cortex (Boschen et. al., 2009). These effects on attention are thought to be mediated through acetylcholine. Intranasal orexin A increased the release of acetylcholine in the medial prefrontal cortex (Calva et. al., 2018). The medial prefrontal cortex is a part of the frontal cortex that is significant in attentional processing in the brain (Sarter et. al., 2001). Orexin B administered directly into the medial prefrontal cortex also increased attentional performance on sustained and divided attention tasks, which implies a possible role of orexin 2 receptors in attentional processing (Lambe et. al., 2005). These results suggest that orexinergic neurons may mediate attention by projecting directly to the medial prefrontal cortex and binding to both orexin 1

receptors and orexin 2 receptors. Other than projecting to the prefrontal cortex directly, orexinergic neurons in the lateral hypothalamus could project to other areas in the brain that project to the prefrontal cortex. For instance, orexin neurons in the lateral hypothalamus project to the paraventricular nucleus of the thalamus (PVT) which in turn projects to the prefrontal cortex (Huang et. al., 2006). Besides attentional performance, hippocampal memory is also modulated by orexins. Orexin 1 receptor antagonist administration into the CA1 subregion of the hippocampus affected performance of rats on the Morris water maze (Akbari et. al., 2006). Degeneration of orexinergic neurons significantly decreases social memory, as well, and intranasal administration of orexin A recovered social memory deficits (Yang et. al., 2013).

Stress itself can impact cognition. A study by Kambali et. al. separated rat offspring from their mother from postnatal days 4 - 14 as a stressor. After maternal separation stress the rats were evaluated on attention, spatial learning, and social anxiety. The researchers found that maternally separated rats had higher attentional performance and spatial learning than rats that were not maternally separated, implying that maternal separation can actually increase cognitive performance (Kambali et. al., 2019). A meta-analysis of 113 studies of humans under acute stress demonstrated that stress prior to and during memory encoding impaired memory, but stress after encoding actually improved memory (Shields et. al., 2017). However, acute social isolation impeded social recognition memory in rats (Leser et. al., 2015). These findings suggest that stress can have various effects on cognition, either improving or impairing it depending on the type of cognition and the relative time of the stress. However, limited studies have been done on orexin's role in the intersection of stress and attention. In one study by Eacret et. al., the effect of orexin and social defeat stress on object recognition memory. In this study, the orexinergic system was activated via a DREADDs virus, in which a receptor for a ligand was embedded in

orexinergic neurons in the lateral hypothalamus. Orexinergic activation itself did not significantly increase novel object recognition memory, but under social defeat stress, it significantly decreased memory (Eacret et. al., 2018). This finding is aberrant since orexins typically increase cognitive performance, and stress can increase cognitive performance as well. However, the same study has not been done measuring attentional performance.

Social Isolation

Social isolation during adolescence has been shown to induce a stress response in rats as well as affect their adult behavior. Social isolation at 21 days of age in rats has been shown to increase release of stress hormones such as ACTH and corticosterone, as well as increase anxiogenic behavior on the elevated plus maze and startle reflex amplitude in male rats (Weiss et. al., 2004). Two studies isolated male Long-Evans rats at 28 postnatal days, but not at 21 days, and measured their performance in the elevated plus maze, which has two closed armes and two open arms elevated several feet above the ground. Less time spent in the open arms indicates more anxiety-like behavior in the rat. Socially isolated rats in both studies spent significantly less time in the open arms of the maze, suggesting that social isolation can cause anxiety-like behavior in adulthood. These two studies also showed that social isolation increased ethanol intake in male rats, which is induced by stress (McCool and Chappell, 2009; Chappell et. al., 2013). These results suggest that social isolation can act as a strong stressor with long-term effects, and that the date of isolation can vary from 21 - 30 days and increase stress. Isolation at 21 - 30 postnatal days is thought to be a stressor because at 21 - 30 postnatal days play behavior is expressed in rats. Play behavior often peaks at and then declines after 32 - 40 days of age (Panksepp, 1981). Isolation after 30 postnatal days produces inconsistent effects, with some studies showing anxiogenic results, and others showing the opposite. For instance, Long-Evans

rats isolated at 30 days of age showed no increase in anxiety-like behavior in the elevated plus maze nor did it increase ethanol intake (Butler et. al, 2014). In another study by Thorsell et. al., isolation at 45 days actually had anxiolytic effects in the open field test (2006). However, female rats socially isolated from days 30 - 50 showed increased climbing in the forced swim test and preference for sucrose (Hong et. al., 2012). This study might indicate that stress has differential effects on male and females.

Social isolation has also been used to model schizophrenia in animals, as socially isolated rats show schizophrenia-like symptoms. For instance, in a study by Wilkinson et. al., rats were either isolated at 21 postnatal days or group-housed, and then they were tested for prepulse inhibition. Prepulse inhibition is a phenomenon when the response to an auditory stimulus is inhibited because of the exposure to a smaller auditory pulse beforehand. This is thought to be a measure of sensorimotor gating, which is often deficient in schizophrenic patients. Socially isolated rats showed significantly less prepulse inhibition than group-housed rats. They also had higher extracellular dopamine levels in the nucleus accumbens when given amphetamine, which is another schizophrenia-like symptom (1994). Another study examined the effects of social isolation on auditory gating, in which there is a diminished response to the second of paired clicks because of sensory filtering. Auditory gating is deficient in patients with schizophrenia, since they cannot suppress a response to the second click. Sprague-Dawley rats were isolated at 21 postnatal days or group-housed, and then they were tested with a paired click stimulus. Isolated rats had a higher response to paired clicks, suggesting that social isolation leads to an impairment in auditory gating (Stevens et. al., 1997). These findings suggest that social isolation can be used as an animal model for schizophrenia.

Neural mechanisms involved in attention

Attentional processing is an aspect of cognitive performance relying on the ability to shift focus to certain aspects of an environment and integrate it into memory. One brain structure thought to be involved in attention is the basal forebrain, which is below the striatum and behind the frontal lobe. Basal forebrain lesions disrupted attention in monkeys, demonstrating that the basal forebrain is necessary in attentional processing (Voytko et. al., 1994). The basal forebrain has been found to project to the prefrontal cortex with glutamatergic, GABAergic, and cholinergic neurons (Henny and Jones, 2008). Acetylcholine is a neurotransmitter used throughout the central nervous system that plays a role in attentional processing. During a sustained attention task, increased acetylcholine is released in the prefrontal cortex (Himmelheber et. al., 2000). Furthermore, increased acetylcholine release in the prefrontal cortex has been shown to be associated specifically with attentional processes and not other processes such as reward delivery and lever pressing, which are associated with the sustained attention task (Arnold et. al., 2002). 192-IgG saporin, an antibody which selectively impairs cholinergic neurons, can decrease attentional performance in a sustained attention task when injected into the basal forebrain, suggesting that cholinergic projections from the basal forebrain to the prefrontal cortex mediate attentional processes (McGaughy et. al., 1996). Other neurotransmitters can modulate the release of acetylcholine in the prefrontal cortex. For instance, glutamatergic neurons from the basal forebrain also modulate cortical acetylcholine release, so they probably play a role in attention as well (Fadel et. al., 2001). Attentional performance is also mediated by the orexinergic system. When orexin 1 receptor antagonist SB-334867 was injected into the basal forebrain, attentional performance in the sustained attention task decreased. Moreover, when orexin A was administered into the prefrontal cortex, attentional performance increased (Boschen et. al., 2009). Attentional performance was also improved with infusions of orexin A

into the lateral ventricle, suggesting that orexinergic projections from the lateral hypothalamus to the basal forebrain are involved in attentional processes (Zajo et. al., 2016).

The present experiment

In this experiment, Sprague-Dawley rats were either socially isolated at 21 days or 35 days or placed in group-housing. Each subject was given varying doses of orexin A (10nM, 100nM) and saline via intranasal administration. Their attentional performance was then measured with a sustained attention task. Intranasal orexin A administration has previously demonstrated significant effects in restoring attentional performance in rats (Calva and Fadel, 2018). The aforementioned article studying social defeat stress and activation of orexin neurons showed that orexin activation under stress would decrease novel object recognition memory (Eacret et. al., 2018). It would make sense that intranasal orexin A during social isolation stress would have similar effects on attention. We hypothesized that intranasal orexin A administration would either decrease attentional performance in rats that are socially isolated.

Methods

Subjects

A total of 24 Sprague-Dawley rats (14 male, 10 female) bred in the humidity- and temperature-controlled animal vivarium at William & Mary were used in this experiment. The rats were housed in plastic tubs with the dam and siblings until social isolation or group housing. The rats were either socially isolated or group housed on postnatal day 21 or postnatal day 35. For the rest of the duration of the experiment, socially isolated rats were singly housed, while group-housed rats were placed in groups of four to five rats in a cage. All rats were given food ad-libitum, while water was restricted to ten minutes a day, since water was used as a reward in the sustained attention task. Isolation only restricted physical contact, so the rats could still see,

hear, and smell each other. Before the animals were trained for the attention task, they were handled each day for seven days.

Apparatus and Training

Rats were trained in one of 20 test chambers in a sound-attenuating cubicle. In each chamber, there were two retractable levers, a water port with a water delivery dipper (0.01 mL), three panel lights, and a house-light on the opposite side of the chamber to the panel lights and water port. The only panel light that was used was above the water port.

During initial sessions to shape lever pressing, the retractable levers were presented at all times. The dipper would deliver water whenever the subject pressed a lever. To avoid bias for one lever, five consecutive presses on the same lever resulted in discontinuation of water delivery until the subject pressed the other lever. When the rats received water 120 times for three sessions of this first training task, they would be trained in the next task. The next task involved signal and non-signal trials, for the beginning of which the levers are retracted. For a signal trial, the panel light would illuminate for either 500 ms, 250 ms, or 100 ms. The levers were then extended into the chamber. For half of the rats, during a signal trial, a press on the right lever was considered a hit. If it pressed the left lever, the trial was scored a miss. For a non-signal trial, the panel light stayed unlit, and the rat was required to press the left lever for the trial to be considered a correct rejection. If the rat pressed the right lever during a non-signal trial, it was considered a false alarm. For half the rats, the rules of the task were reversed so that a left lever should be hit during a signal trial and the right lever during a non-signal trial. If the rat did not press the lever with 3 seconds of lever extension, then the trial was scored as an omission. In order to be eligible for the next training stage, the rats must have had a 70% hit rate and 70% correct rejection rate on signal trials with 500 ms signal duration and 20 or fewer omissions. The

final stage of training is the same as the second stage except the signal durations were 250 ms, 100 ms, and 80 ms. In order to be eligible for testing, the rats must achieve a 70% hit rate and 70% correct rejection rate on signal trials with 250 ms signal duration and less than or equal to 20 omissions.

During drug administration sessions, there were three blocks for the sustained attention test. The first block of the test is the normal task, which is the same as the second stage of training. The second block of the test is the distractor task, in which the houselight turns on during the trials. The third block is the recovery task, which is the same as the final training stage.

Drug administration

Before drugs were administered, the rats were acclimated to intranasal administration to avoid stress during administration. Each rat was given 25 uL of saline in each nostril every day for seven days. After acclimation, all rats received three drug administration sessions in a randomized order: 25 uL of saline, 10 nM orexin-A (Tocris Bioscience), or 100nM Orexin-A was micro-pipetted into each nostril during these sessions. The amount of time between intranasal administration and the sustained attention task for each subject varied from one minute to sixty minutes, although it was usually sixty minutes. There was at least one session of drug-free testing in between drug administration sessions.

Behavioral analysis

The number of hits (h), misses (m), correct rejections (cr), false alarms (fa), and omissions were collected for each block of 54 trials. The relative number of hits ($h/(h+m)$) and of correct rejections (cr/(cr + fa)) was calculated for each block of trials within a session. The lever press latency, the time from when the levers were extended until a press was recorded, was

collected on each trial. Finally, the photocell response latency, the time from a lever press until the water port photocell was broken,was also collected for all correct responses.

Statistical analysis

Data were analyzed with mixed factor ANOVAs, which included factors dose, group, signal duration (where appropriate), and block. Significant interactions were followed up by one- or two-way ANOVAs followed by *t* tests. Data were analyzed with SPSS 19.0 for Windows (SPSS, Chicago, IL, USA). A level of $\alpha = 0.05$ was used as the criterion for statistical significance.

Results

A total of 24 rats were included in this experiment. There were 10 group-housed rats, 7 rats isolated at 35 postnatal days, and 7 rats isolated at 21 postnatal days. We carried out an ANOVA for hit rates for the factors of group (group-housed, 35 day isolation, 21 day isolation), signal duration, block, and dose of orexin A. We also carried out an ANOVA for correct rejection rates and number of omissions with the same factors except signal duration.

For hit rates, we found a significant interaction between signal duration and block ($p < 0.005$). However, we found no significant interaction effect of block x signal duration x orexin dose x group on hits or correct rejection rates ($p > 0.05$). We also found no significant interaction effect of block x signal duration x group, and no significant interaction effect of block x signal duration x dose. There was also no significant difference between the attentional performance of group-housed rats, rats that were isolated at 21 postnatal days, or rats that were isolated at 35 days. For correct rejection rates and omissions, we found the same results.

We carried out more ANOVAs on hit rates and correct rejections, but instead we combined the rats that were isolated into one group regardless of time of isolation. For hit rates,

we had the same results (Fig 2). However, the correction rejection rates of group-housed rats were significantly higher than those of isolated rats ($F(1, 23) = 4.415$, $p < 0.047$; Fig. 1). There was no significant difference of omissions between groups (Fig 3).

Discussion

Intranasal administration of orexin A did not affect the rate of hits in socially isolated rats nor in group-housed rats. It also did not affect the rate of correct rejections in either group. However, there was a significant decrease in correct rejections in socially isolated rats compared to group-housed rats, although there was no significant difference when the isolated groups were divided into rats isolated at 21 days of age and rats isolated at 35 days of age. These findings suggest that social isolation can impact attentional processes measured by the sustained attention task, but intranasal orexin A administration is not sufficient to affect the impact of social isolation.

There was no significant difference between the hit rates of isolated rats and group-housed rats. This finding is important because it suggests there was not a side or lever bias in socially-isolated rats. Thus, the lower correct rejection rate of socially isolated rats can be interpreted in terms of changes in attentional processing. The decrease in the correct rejection rates of socially isolated rats could indicate a dysregulation in the cholinergic system in the prefrontal cortex. Other effects of social isolation have been shown to be mediated by acetylcholine. For instance, rivastigmine, an acetylcholinesterase inhibitor, restored prepulse inhibition, a measure of sensorimotor gating that shows deficits in models of schizophrenia, in socially isolated rats. These effects are probably mediated by the actions of the muscarinic acetylcholine receptor (Higashino et. al., 2015). Attentional deficits in socially isolated rats could thus be caused by dysregulation of the cholinergic system as well.

Although this experiment showed that intranasal orexin A administration was not sufficient to restore attentional performance of isolation-reared rats to levels comparable to group-housed rats, it was not sufficient to completely understand the role of the orexinergic system in social isolation's effects on attention. Previous studies have used other means besides intranasal administration of orexins, such as DREADD viruses delivering receptors onto orexinergic neurons in the lateral hypothalamus (Eacret et. al., 2018). These methods increase the release of orexins into the synapse specifically from the lateral hypothalamus, while intranasal orexin A administration is less specific. Intranasal orexin A administration has previously been demonstrated to recover cognitive performance due to sleep deprivation in nonhuman primates (Deadwyler et. al., 2007). However, sleep deprivation probably affects the orexinergic system differently than social isolation. Furthermore, if the orexinergic system is overactive in isolated rats, intranasal orexin A may have no effect because orexin receptors in the synapse are already saturated due to high endogenous orexin release. Other methods to activate or inactivate the orexinergic system, such as orexin receptor antagonists or immunotoxic lesions to orexinergic neurons in the lateral hypothalamus, may be better at determining exactly how the orexinergic system is affected by social isolation. Similar methods applying to acetylcholine, such as acetylcholinesterase inhibitors or acetylcholine antagonists, might have also determined whether the cholinergic system plays a role in how social isolation affects performance on the sustained attention task. This study also did not directly assess the difference in activity of cholinergic or orexinergic neurons between isolated and group-housed rats. Immunohistochemical methods such as cFos and ChAT staining would ascertain whether cholinergic neurons in the prefrontal cortex and orexinergic neurons in the lateral hypothalamus are active.

In this experiment, some rats were isolated at 35 postnatal days of age, which is closer to the end of adolescence in rats. This presumably would not have the same effects as social isolation at 21 postnatal days, because it does not interfere with the critical period as much and is not as stressful. We do not know that rats isolated at 35 days were properly stressed for the experiment, although there were no significant differences in correct rejection rates between group-housed rats, rats isolated at 21 days, and rats isolated at 35 days, but there was a difference when the isolated rats were combined into one group. It might have behooved this study to measure stress in another way, such as measuring burrowing behavior or using another behavioral assay to study anxiety-like behavior such as the elevated plus maze or social interaction test. These measurements would provide an independent measure of the stress level for the socially isolated rats.

Another limitation of the study was the time between orexin administration and testing in the sustained attention task. Although intranasal administration is usually the fastest of all methods of administration, it can still take hours for all of an entire drug dose to circulate to the brain. Time between orexin administration and the task varied between a couple of minutes to about an hour. This variation was due to scheduling conflicts. If the task is done only minutes after intranasal administration, it might not be enough time for the orexin A to get to the brain, and we would not properly see the effects of orexin A administration on attentional processes. Thus, this time could act as a confounding variable. It would have been better if there was a set amount of time between the time that orexin A was administered and the time that the rat starts the task, to control for that variable.

Future studies could use immunohistochemistry to measure the activity of the cholinergic and orexinergic systems in socially isolated rats, as previously mentioned. Another possible

experiment could use orexin 1 receptor antagonists on socially isolated and group-housed rats in order to ascertain if the orexin 1 receptor plays a role in the effect of social isolation on attention. Other behavioral assays to measure attention, such as an attentional set shifting task or an auditory attention task as opposed to the visuospatial attention task used in this experiment, could be used in future studies to study the effect of social isolation and orexin A administration. In this experiment, socially isolated rats were isolated for the duration of the study, which would be considered chronic social isolation. The effect of acute social isolation, which could be from 21 to 35 postnatal days of age, on attention and the orexinergic system could be a subject for future experiments, and it could be compared to the effect of chronic social isolation and assess the effect of resocialization on resulting attentional deficits.

This experiment examined the interaction between stress and the orexinergic system and its effect on attentional processes. The findings of this study shows that social isolation can significantly affect attentional performance, but intranasal orexin A administration did not impact those effects. Thus, intranasal orexin A administration might not be a feasible method to treat attentional deficits due to stress.

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Figures

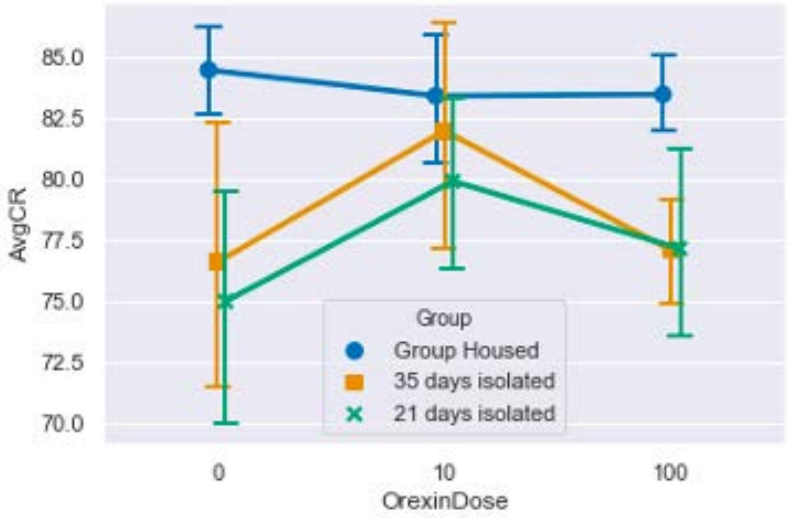

Figure 1. The average correct rejection rates versus dose of orexin A (0nM, 10nM, 100nM). Orexin A dose did not affect the correct rejection rate for isolated and group-housed rats. Group-housed rats had significantly higher correct rejection rates than socially isolated rats ($p = 0.047$). The points represent mean correct rejection rates and the bars span two standard deviations from the mean.

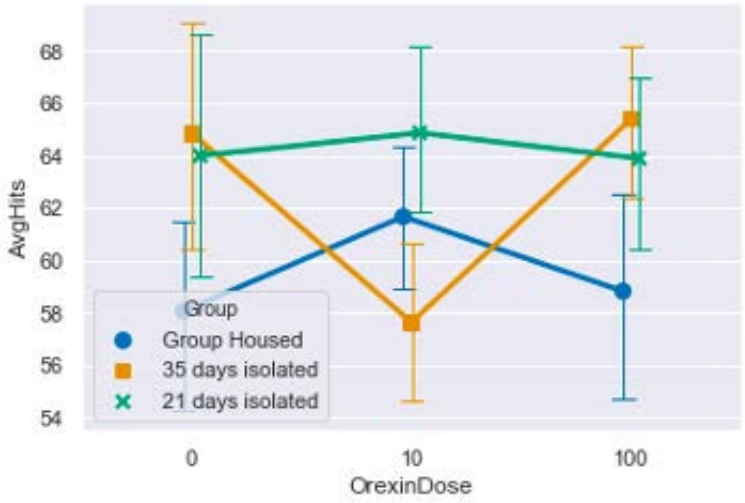

Figure 2. The average hit rates versus dose of orexin A (0nM, 10nM, 100nM). Orexin A dose did not affect the hit rate for isolated and group-housed rats. There was no significant difference between hit rates of group-housed and isolated rats ($p > 0.05$). The points represent mean hit rates and the bars span two standard deviations from the mean.

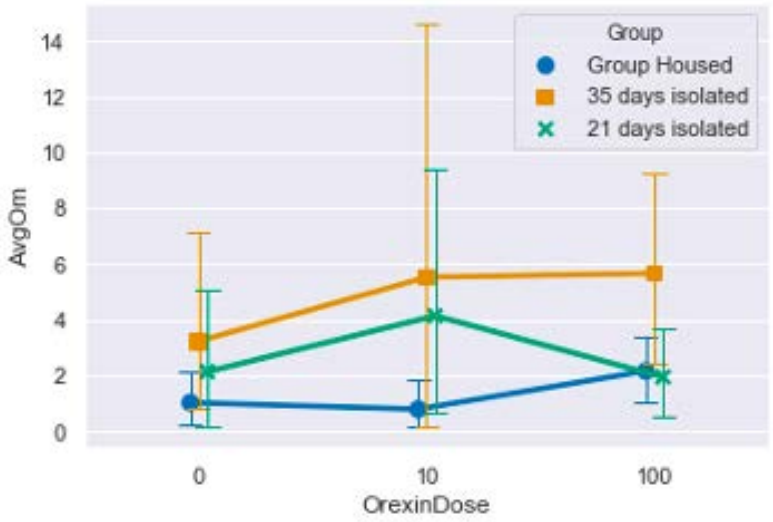

Figure 3. The average number of omissions versus dose of orexin A (0nM, 10nM, 100nM). Orexin A dose did not affect the number of omissions for isolated and group-housed rats. There was no significant difference between the number of omissions of group-housed and isolated rats ($p > 0.05$). The points represent mean number of omissions and the bars span two standard deviations from the mean.