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Orexin signaling during social defeat stress influences subsequent social interaction behaviour and recognition memory

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ABSTRACT

Orexins are neuropeptides synthesized in the lateral hypothalamus that influence arousal, feeding, reward pathways, and the response to stress. However, the role of orexins in repeated stress is not fully characterized. Here, we examined how orexins and their receptors contribute to the coping response during repeated social defeat and subsequent anxiety-like and memory-related behaviors. Specifically, we used Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) to stimulate orexins prior to each of five consecutive days of social defeat stress in adult male rats. Additionally, we determined the role of the orexin 2 receptor in these behaviors by using a selective orexin 2 receptor antagonist (MK-1064) administered prior to each social defeat. Following the 5 day social defeat conditioning period, rats were evaluated in social interaction and novel object recognition paradigms to assess anxiety-like behavior and recognition memory, respectively. Activation of orexin neurons by DREADDs prior to each social defeat decreased the average latency to become defeated across 5 days, indicative of a passive coping strategy that we have previously linked to a stress vulnerable phenotype. Moreover, stimulation of orexin signaling during defeat conditioning decreased subsequent social interaction and performance in the novel object recognition test indicating increased subsequent anxiety-like behavior and reduced working memory. Blocking the orexin 2 receptor during repeated defeat did not alter these effects. Together, our results suggest that orexin neuron activation produces a passive coping phenotype during social defeat leading to subsequent anxiety-like behaviors and memory deficits.

HIGHLIGHTS

- DREADDs stimulation of orexins during repeated social defeat produced a passive coping phenotype and subsequent deficits in social interaction and recognition memory
- OX2R antagonism prior to each defeat did not alter these deficits, thus, these effects are not OX2R mediated
- Elevated orexin neuron activity is associated with behavioral vulnerability to social defeat stress

1. INTRODUCTION

Orexins, also known as hypocretins, are hypothalamic neuropeptides that are known to be involved in the control of arousal [1–3], stress [4–6], reward [7], food intake [8,9], and anxiety [4,10,11]. Orexins are synthesized from a precursor molecule, prepro-orexin, and cleaved into two highly structurally related peptides, orexin A and orexin B [12,13]. These peptides bind to two G-protein- coupled receptors, the orexin 1 receptor (OX1R) and the orexin 2 receptor (OX2R) [12,14]. Orexin A binds with a similar affinity to both orexin receptors, whereas orexin B binds to OX2R with a significantly higher affinity relative to OX1R [14]. OX1R and OX2R have different mRNA expression patterns in brain regions associated with stress responses. For example, only OX1R is expressed in the central amygdala and CA1 region of the hippocampus while only OX2R expressed in the paraventricular nucleus of the hypothalamus, rostral nucleus accumbens, CA3 region of the hippocampus. Both orexin receptors are expressed in the paraventricular nucleus of the thalamus (PVT) although OX1R is more prevalent [15]. Differential expression of orexin receptors in these brain regions could mediate different aspects of arousal, memory, and the stress response.

Orexins have been implicated in the acute stress response [4,16], but there have been limited studies examining the contributions of orexins to repeated stress [17,18]. This is an important area of study, as repeated stress can increase the likelihood of developing stress-related illnesses such as depression and post-traumatic stress disorder (PTSD). Indeed, patients with stress-related illness such as depression, PTSD and panic disorder have altered levels of orexins in their cerebrospinal fluid (CSF) [19–21] suggesting that orexin regulation is important in the development of these disorders. Both orexin receptors influence cardiovascular and locomotive responses to stress [22]. During acute stress, OX1R antagonists attenuate stress-induced arousal [23], while OX2R antagonists decrease stress-induced ACTH [17,24,25]. Experiments investigating orexin signaling during repeated stress have found that orexins regulate the facilitation of the hypothalamic-pituitary-adrenal (HPA) response to a novel stressor in an OX1R dependent manner, [18], and OX2R regulates the HPA response to repeated restraint stress in conditions of elevated orexin release [17]. However the specific roles of OX1R and OX2R in the behavioral consequences of repeated stress are not clear, therefore we used an OX2R specific antagonist to elucidate the role of OX2R during and after repeated social stress.

In the present studies, we determined the role of orexins and their receptors in mediating responses to repeated social defeat. Specifically, we injected rats with a DREADDs-containing virus with an orexin promoter and used clozapine-N-oxide (CNO) to stimulate orexin neuron activation prior to each of 5 days of social defeat. We then performed behavioral testing on days 6-8 without orexin manipulation, to examine if stimulating orexins during social defeat modulates subsequent anxiety-like and memory-related behaviors. This virus combined with a CNO injection is sufficient to increase orexin CSF levels compared to controls [17]. To differentiate roles of OX1R

and OX2R activity in orexin-induced effects during repeated stress conditioning, we additionally used MK-1064, a selective orexin 2 receptor antagonist [17,26] in combination with CNO-induced orexin neuron activation. Examining the roles of orexin receptors during orexin neuron activity has relevance for studying patients with panic or anxiety disorders that exhibit elevated orexin levels in CSF and may respond differently to repeated stress than the healthy population [10].

2. MATERIALS AND METHODS

2.1 Animals

Adult male Sprague Dawley rats (225-250g upon arrival) were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were singly housed with ad libitum food and kept on a 12-h light-dark cycle with lights on at 0615 and lights off at 1815. Experiments adhered to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the Children's Hospital of Philadelphia Research Institute. All experiments were performed during the light phase when endogenous orexin levels are low to allow for maximal efficacy of DREADDs-mediated orexin neuron stimulation [27], and for differentiation of OXR-mediated signaling utilizing the OX2R-selective antagonist, MK-1064 [26].

2.2 Experimental Design

Rats were injected with hM3D DREADDs virus targeting the prepro-orexin promoter into the lateral hypothalamus to target orexin-expressing cells, as in our previous study [17]. For further information, see below. The virus expressed for four weeks while animals remained in their home cage before the start of the experiment (for experimental paradigm see **Figure 1A**). In brief, rats underwent 5 days of repeated social defeat in a modified paradigm from Miczek [28] as described previously [29–31] or were exposed to a novel cage for 30 minutes as a control. For each defeat, rats were placed into the home cage of a Long-Evans retired breeder from Harlan (Indianapolis, IN, 500-1000g body weight). A wire mesh divider was placed between the intruder and the resident either after the display of a social defeat, or after 15 minutes in the case of non-defeated controls. Daily latencies were recorded for each rat and rats that did not display defeat by 15min were assigned a latency of 900s. Animals remained in the resident's home cage subject to the sight and smell of the resident but without physical contact for the remainder of a 30 minute period. Each experimental rat was exposed to a novel resident to avoid habituation to the same aggressor over time. Animals were removed from the study if they had physical indications of bites or other injuries that persisted. Rats were administered either vehicle or OX2R antagonist MK-1064 90 minutes prior to each day of defeat, and either vehicle or clozapine-N-oxide (CNO) 60 minutes prior to exposure to every defeat (or the novel cage) each day (see Drug section below). Neither CNO nor MK-1064 were administered before behavioral tests on Day 6-8. A total of 63 rats were used in this experiment, for control groups Vehicle/Vehicle (n=7), Vehicle/MK-1064 (n=8), CNO/Vehicle (n=7), and CNO/MK-1064 (n=7) and defeated groups

Vehicle/Vehicle (n=6), Vehicle/MK-1064 (n=9), CNO/Vehicle (n=6), and CNO/MK-1064 (n=7). Average latency to be defeated was calculated as the average over the five days. Rats were randomly placed into experimental groups at 7-9 per group. 5 total rats were removed from the study that had less than 30% of orexin cells that were colocalized with GFP (DREADDs viral tag), and 1 rat was excluded from the study due to physical wounding during defeat. Rats underwent the social interaction test on Day 6, 24 hours following the last defeat session. On Day 7 and 8, rats were trained, and then tested, respectively, in the novel object recognition test. Rats were excluded from Novel Object analysis if they did not explore either object on training day, or if they only explored one object on training day. No drugs were administered during behavioral testing in the social interaction or object recognition tests. Body weight was measured prior to the first defeat and after the Novel Object recognition test to examine sustained effects of orexin manipulation during social defeat. Rats were sacrificed 1h after the start of the Novel Object Test. Brains were collected and flash frozen for assessment of viral expression, and adrenal glands and spleen were immediately weighed for analysis.

2.3 DREADDS viruses

The Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) virus (10^9 titer, 1ul bilaterally) was stereotaxically injected into the lateral hypothalamus at coordinates: 2.5 mm caudal to bregma, 1.8 mm from mid-line and 8 mm ventral to dura mater. Animals remained in their home cage while the virus expressed for 4 weeks, a timeframe in which the hM3D DREADDs is optimally expressed and as in our previous study [15], before experiments began.

Representative immunofluorescent images of the DREADDs virus transfecting orexin cells are shown in **Figure 1B.** Briefly, brains were collected and flash frozen, sliced at 20um on a cryostat between bregma coordinates -2.12mm to -3.30mm and placed directly on microscope slides. Brain slices were fixed in 4% paraformaldehyde for 30 minutes, then washed 3x5 minutes in Phosphate Buffered Saline (PBS, pH 7.4) with 0.3% Triton-X (Sigma-Aldrich; St Louis, MO) and incubated with Orexin A (1:250, sc-8070; Santa Cruz Biotechnology, Santa Cruz CA) and Green Fluorescent Protein (GFP) (1:500, ab290; AbCam, Cambridge, UK) in PBS with 5% Normal Donkey Serum (EMD Millipore; Billerica, MA) and 0.3% Triton-X overnight. GFP antibodies interact with the mCitrine tag on the DREADDs virus [32], therefore cells stained with GFP are used as markers of cells transfected by the DREADDs virus. The next day, slides were rinsed 3x5 minutes in PBS then incubated for 1h 30 minutes in AlexaFluor 488 Donkey anti-goat and AlexaFluor647 Donkey anti-rabbit (1:200, A-11055 and A-31573; Life Technologies, Carlsbad, CA). Slides were rinsed and coverslipped, then images were obtained with a Leitz DMR microscope (Leica Microsystems; Buffalo Grove, IL). Anterior to posterior distribution throughout the Lateral Hypothalamus is depicted in **Figure 1C.** In this study, the majority of GFP stained cells were found in the mid-posterior region of the Lateral Hypothalamus (Bregma -2.5mm to -3.3mm).

2.4 Drugs

MK-1064 is a selective OX2R antagonist [26] and was dissolved overnight at 30mg/ml in 20% Vitamin E d-a-tocopherol polyethylene glycol 1000 succinate (Vitamin E-TPGS) (Sigma-Aldrich; St Louis, MO). The 20% Vitamin E-TPGS solution that MK-1064 was dissolved in was used as Vehicle comparison [17]. Rats were administered either 20% Vitamin E-TPGS or 30 mg/kg MK-1064 at 1ml/kg by oral gavage 90 minutes before social defeat began. The dose and timing of MK-1064 administration was chosen based on a previous study where this antagonist effectively decreased the HPA response to acute and repeated restraint stress (Grafe et al, 2017 Neuroscience paper). Previous literature has indicated that a 20mg/kg dose of MK-1064 affects sleep measures in rats up to 8 hours after injection, but that the drug has a moderate clearance with half-life values in an appropriate range for once-nightly dosing [26]. Moreover, in this same study, radiolabeled MK-1064 showed nearly 100% of the dose was recovered after 48h in rats, so it is unlikely there are long-lasting effects of this drug. All animals received a vehicle injection of 20% Vitamin E 24h before the start of the experiment to acclimate to the oral gavage procedure before the experiment began. Clozapine N-Oxide (Tocris; Ellisville, MO) was dissolved in saline and 8% DMSO at 5mg/mL, and saline with 8% DMSO was used as vehicle. CNO was injected intraperitoneally at 2mg/kg 60min prior to the start of social defeat. Dose and timing were based on studies that show CNO efficacy in rats between 30min and 4 hours after injection [5,17,33–35] .

2.5 Social Interaction

The social interaction test occurred between 0900h and 1300h in a 70cm x 70cm arena. Rats were placed in the arena with another male Sprague Dawley rat of similar size and weight. Rats were allowed to interact in this arena for 15 minutes, and were videotaped and analyzed by two trained experimenters blind to conditions. The latency to interact (time in seconds until experimental rat explores stimulus rat) and total time interacting (number of seconds that the experimental rat explores stimulus rat) were assessed.

2.6 Novel Object

The day following the social interaction test (Day 7), rats were tested in the Novel Object Recognition Test in the same arena used for social interaction. Rats were placed in the arena with two visually identical objects in the afternoon of Day 7 and allowed to explore the two objects for 5 minutes. If rats did not explore either object or developed a preference for one object over the other on this training day they were excluded from analysis. The following morning (Day 8), one of the objects was replaced by a novel object and rats were again allowed to explore for 5 minutes [36]. Rats will generally spend more time with the novel object than the familiar object due

to their propensity for novelty, and this also indicates that the memory for the familiar object must exist [37]. The lack of this preference for a novel object is interpreted as a decrease in recognition memory as the animal can no longer recognize the familiar object [37]. Both tests were videotaped and analyzed in Noldus Ethovision XT 10 with nose-point tracking. Object zone was defined as 50% larger than the outline of the object itself. Preference index (percent of time spent with novel object) was calculated as time spent with the novel object divided by time spent with both objects x 100. Preference index and total exploration time of both objects were analyzed, as well as total mobility. Mobility was defined as the change in pixels in the arena between frames and scored with the mobility setting in Noldus Ethovision XT 10.

2.7 Statistical Analysis

A two-way ANOVA (vehicle or MK-1064 and vehicle or CNO) was used within the defeated and non-defeated groups separately to elucidate the effects of both MK-1064 and CNO. When necessary, Tukey's multiple comparisons tests were used. Tests were considered significant with an α value of less than 0.05. GraphPad Prism 6 (GraphPad Software, La Jolla, CA) was used to conduct statistical tests. Data are presented as the Mean ± standard errors of the mean. We followed up with t-tests between non-defeated rats and defeated rats when necessary for interpretation.

3. RESULTS

3.1 Orexin neuron activation reduces latency to defeat

Our prior work indicates that a shorter latency to defeat is associated with a passive coping strategy and a vulnerable phenotype whereas the longer latency to be defeated is associated with an active coping strategy and a more resilient phenotype (Wood et al, 2010, Chen et al, 2015, Pearson-Leary et al., 2017). Average latency for rats to exhibit a supine defeat posture over the 5 days of defeat was determined to assess coping style and stress vulnerability. There was a significant main effect of CNO ($F(1, 25) = 5.610$, $p = 0.026$) across the 5 days of defeat, indicating that animals injected with CNO to stimulate orexins exhibited lower latencies to be defeated than animals injected with vehicle (**Figure 1D**). This effect was not statistically evident on the first day ($F(1, 25) = 2.743$, $p = 0.1102$). There was no significant main effect of MK-1064 treatment across the 5 days and no significant interaction. The results were further probed and latencies between rats injected with vehicle/vehicle were higher than in rats injected with vehicle/CNO but there were no differences between the two groups administered MK-1064. These results suggest that OX2R does not mediate the passive coping strategy that occurs with orexin stimulation

3.2 Orexin neuron activation during defeat reduces subsequent social interaction

To assess the impact of orexin neuron stimulation and OX2R blockade during defeat on subsequent anxiety-like behavior, rats were tested in the social interaction test. Reductions in time spent interacting and increases in latency to interact in the social interaction test are validated measures of increases in anxiety-like behavior in rodents [38–40] (**Figure 2**). In control rats undergoing sham treatment in place of defeat conditioning (No Defeat), neither orexin stimulation by CNO, nor OX2R inhibition by MK-1064 had a significant effect on the time spent interacting in the non-defeated control rats (**Figure 2A, left**). A significant main effect of the OX2R antagonist on interaction latency, however, was observed in control rats receiving sham conditioning ($F(1, 23) = 7.643$, $p = 0.011$), and MK-1064 produced an increased latency to interact regardless of CNO treatment. This effect was due primarily to the significantly increased latency in the animals that received MK-1064 treatment without orexin stimulation (**Figure 2B, left**).

In rats receiving defeat conditioning, CNO induction of orexin signaling induced a main effect trend towards a decrease time spent interacting ($F(1,24) = 3.880$, $p= 0.061$), while the OX2R antagonist showed no effect on interaction time (**Figure 2A, right**). Unlike control non-defeat animals, MK-1064 administered defeat conditioning did not affect subsequent interaction latency (**Figure 2B, right**). In summary, orexin activation during social defeat subsequently decreased social interaction time (one measure of anxiety-like behavior) after repeated social defeat and this was not mediated by the OX2R.

3.3 Orexin neuron activation during defeat impairs subsequent novel object performance

In order to assess how altered orexin signaling during defeat affects subsequent recognition memory, the hippocampally-based novel object recognition test was performed 3 days after the end of social defeat. We first examined total object exploration time on training day (with two identical objects), and found no significant differences between treatment groups in either the no defeat or defeat conditions. Thus, neither social defeat nor orexin manipulation appear to affect anxiety to novel objects in general. Preference for a novel object on test day was determined by calculating preference index for the novel object compared to a familiar object (see **Methods** for more detail on this paradigm). As orexins could potentially affect arousal and activity, the time spent exploring and time spent mobile in the test were also quantified on test day.

Neither orexin stimulation nor the OX2R antagonist had any effects on novel object preference in control, non-defeated animals. However, there was a main effect of CNO on preference index in defeated animals ($F (1, 15) = 5.716$, $p = 0.030$) indicating that orexin stimulation during defeat reduced subsequent discrimination of a novel object (**Figure 3A**). The orexin receptor antagonist did not have an effect on object preference in defeated animals. Thus, orexin activation during social defeat reduced object recognition memory and this was not mediated by the OX2R.

Orexin stimulation by CNO did not have an effect on total time spent exploring in either the non-defeated and the defeated rats. However, there was a trend for a main effect of the OX2R antagonist in total time spent exploring in control non-defeated animals ($F(1, 23) = 3.458$, $p = 0.0758$) (**Figure 3B**). It appears that OX2R inhibition during control sham conditioning increased subsequent exploration, an effect not observed following defeat conditioning in the presence of MK-1064. Thus, while stimulating orexins throughout defeat decreased novel object preference, this was not due to a general increase in exploration. Further examination in Noldus Ethovision XT of movement in the novel object test showed that there were no significant differences in mobility between any groups (**Figure 3C),** supporting the idea that the decrease in novel object preference observed following orexin stimulation during defeat was not due to changes in total movement. In summary, orexin activation during defeat reduced preference for a novel object and this was not mediated by OX2R.

3.4 Body and Organ Weight changes in response to orexin manipulation and social defeat stress

Body weight was measured prior to the first defeat and after the Novel Object recognition test to examine sustained effects of orexin manipulation during social defeat (Figure **4A**). In non-defeated controls, there was a significant effect of CNO ($F(1, 24) = 7.812$, $p= .0100$) and a significant interaction effect ($F(1, 24) = 4.809$, $p = 0.038$) on body weight gained during the study. Post-hoc tests revealed that CNO significantly reduced body weight gain compared to vehicle in non-stressed rats. Furthermore, MK-1064 reduced body weight gain in CNO treated animals significantly more than in vehicle treated rats. There were no effects of CNO or MK-1064 in body weight gain in defeated rats. Additionally, it is apparent that defeated rats gained less weight overall than non-defeated rats, consistent with our previous study [29], and confirmed by t-tests between non-defeat and defeat groups (Figure 4A).

We also assessed weight changes in the adrenal glands and in the spleen. There was a trend for a significant main effect by MK-1064 in non-defeated controls on adrenal weight ($p=0.082$; **Figure 4B**). Rats treated with both CNO and MK-1064 treatments had the highest mean adrenal weight in this non-defeated group. However, no changes in adrenal weights were observed in defeated rats. Similarly, there was a trend for both a main effect due to MK-1064 ($F(1, 15) = 4.355$, $p = 0.054$) and an interaction effect ($F(1, 15) = 4.1$, $p= 0.061$) on spleen weight in non-defeated rats ($F(1, 15) = 4.355$, $p = 0.054$; **Figure 4C**). Specifically, while CNO treated rats had a higher spleen weight, MK-1064 reduced this measure. However, there were no effects on spleen weight in socially defeated rats.
These results suggest that orexin stimulation without concomitant stress reduces body weight gain overall. Blocking OX2R exacerbates this effect. Increases in spleen weight after orexin stimulation are reversed by orexin 2R blockade. Overall these data suggest that OX2R mediates the effects of orexin stimulation in non-stressed

rats but that neither orexin stimulation nor OX2R mediate such effects in defeated rats.

3.5 *Testing the effects of CNO on behavior in Non-DREADDs expressing rats*

A control experiment was performed to assess possible off target effects of CNO in non DREADDs-expressing rats. Specifically, a separate cohort of rats (n=16) was injected with either vehicle or CNO for five consecutive days, followed by testing in the social interaction test and novel object recognition test, as performed in our initial studies. The data indicate that social interaction time did not differ between vehicle- and CNO-treated groups (350.75±20.7 vs 375.75±18.8 seconds, p = 0.387, t-test, n = 8/group). Moreover, novel object preference did not differ between treatment groups (62.39±6.2 vs 57.05±4.9, p = 0.528, t-test, n = 8/group). Novel object exploration also did not differ between vehicle- and CNO-treated groups (27.57±3.9 vs 35.93±4.2 seconds, p = 0.181, t-test, n = 8/group). Lastly, distance moved did not differ between treatment groups (1449.56±99.1 vs 1287.71±154.5 cm, p = 0.402, t-test, n = 8/group). In summary, CNO treatment in non-DREADDs expressing rats did not cause significant changes in the social interaction or novel object recognition test when compared with vehicle-treated animals.

4. DISCUSSION

This study was designed to elucidate the role of orexins and OXR activity in coping with repeated social defeat, and the subsequent behavioral consequences of modulating orexin signaling during social defeat. Specifically, we aimed to first uncover the influence of orexin signaling and OXR activity on coping strategy during social defeat (as measured by defeat latency), whether these effects persisted to modulate subsequent anxiety-like behavior (measured by social interaction) and short-term memory (measured by the novel object recognition test). To accomplish this goal, we stimulated orexin neuron activity in both control and repeatedly defeated rats utilizing a DREADDs (CNO)-mediated approach prior to daily defeat. Further, an indication of the role of OX2R activity during defeat conditioning was assessed by using the OX2R-selecitve antagonist, MK-1064, in the presence and absence of CNO-induced orexin neuron activation prior to each defeat. At the end of 5 days of social defeat exposure or novel cage (control condition), rats were tested without any drug exposure in the social interaction test to assess anxiety-related behavior and the novel objection recognition test to assess episodic memory.

4.1 Orexin manipulation in the absence of social defeat has little effect on subsequent anxiety behaviors, and memory performance

There were no effects of exogenous orexin stimulation in the absence of stress on social interaction time, or novel object recognition performance. This suggests that the stress regulatory circuitry must interact with orexins to increase anxiety-like behaviors and to disrupt object recognition memory. One possible mediator

underlying this interaction is Corticotropin Releasing Factor (CRF). CRF is released in the brain after a stressor and CRF neurons facilitate the release of orexins [41]. Given the relationship between stress induced CRF and orexins and our lack of effect in non-stressed animals, it is likely that some level of potentiation from CRF is required for stimulation of orexins by CNO to induce stress vulnerability. Together, these results indicate that orexin stimulation in the context of no ongoing stress has no effects on coping behavior, social interaction time, or recognition memory and suggests that orexins only modulate these behaviors under increased levels of stress.

Social defeat decreases orexin mRNA [42], indicating that the non-stressed rats exposed to the novel cage control would have increased orexin tone compared to defeated rats. Increased orexin tone makes orexin antagonists more effective [43] but would explain why CNO-induced orexin activation does not affect behaviors in non-defeated animals. This is supported by our data showing that MK-1064 treatment to block the orexin 2 receptor starkly increases latency to interact in the novel cage controls, but not defeated rats. The novel environment could increase endogenous orexin tone, the social defeat paradigm likely reduces orexin levels, or a combination of both effects.

OX2R has a greater role in arousal/sleep than OX1R [44,45], and although the selective OX2R antagonist MK-1064 was not observed to induce sleep during the novel cage environment of the conditioning paradigm, it remains possible that the compound induced sleep in hours thereafter when the animals were not under observation. This greater amount of sleep may have somehow affected subsequent evaluation of social interaction latency. MK-1064-induced sleep or decreased arousal is less likely to occur to the same extent in the experimental group receiving the social defeat conditioning stimulus and could explain the difference between control and experimental groups.

In control non-stressed animals, however, body weight gain decreased as a main effect of CNO treatment (orexin neuron activation), an effect that was observed days after the end of CNO treatment. We also observed an increase in spleen weight after CNO treatment in the non-defeated controls, suggesting a role for orexins in immune signaling under control conditions that persists beyond CNO treatment. In addition, in these non-defeated rats, OX2R antagonism prevented the increase in spleen weight by CNO, therefore additional non-OX2R orexin neuron signaling may have the capacity to increase spleen weight under non-stress conditions. Further studies should examine the role of OX2R in regulating orexin-mediated immune signaling.

4.2 Orexin activation during social defeat induces a passive coping strategy, and produces subsequent anxiety-like behaviors and impairment in recognition memory

The results indicate that orexin neuron activation during social defeat stress is important in regulating both coping during social defeat and subsequent anxiety- and memory- related behaviors. More specifically, orexin stimulation induced

passive coping behavior as measured by a reduction in the latency to social defeat that affected subsequent behavior. Passive coping is associated with increases in anxiety- and depressive-like behaviors in socially defeated rats [29–31] consistent with the large literature in humans linking passive coping with increased incidence of depression [46–49]. Blocking OX2R did not affect latency to social defeat in either the absence or presence of CNO, suggesting that OX2R is not involved in coping strategies during defeat to subsequently impact anxiety-like and memory-related behavior. Although orexin antagonists are known to promote sleep, they still allow arousability to salient stimuli [50,51], thus, it is unlikely MK-1064 influenced arousal during the defeat itself. In support of this, no induction of sleeping behavior was observed in rats during the defeat conditioning, but changes in sleep/wake occurring at other times cannot be ruled out as 24-hour polysomnography was not specifically assessed in these experiments. In summary, these findings point to the importance of orexin neuron activity in mediating coping strategies to repeated social defeat having the potential to impact subsequent behavior.

Our data indicate that orexin neuron activation during defeat conditioning has the capacity to decrease subsequent social interaction time, consistent with an anxiety-like phenotype in rats that have a decreased social defeat latency [30]. This effect occurred regardless of OX2R inhibition with MK-1064, suggesting that orexin stimulation during defeat conditioning has the capacity to reduce social interaction time through non-OX2R mechanisms. Thus, as mentioned with the lack of effect in non-defeat conditions, perhaps CRF or another stress related peptide must synergize to create this effect.

We used the Novel Object test to assess the extent to which orexin signaling manipulations during social defeat affect subsequent recognition memory performance. This test assays spatial and recognition memory and depends on hippocampal [52] and entorhinal cortex structures [53]. The hippocampus plays a role in both memory and regulation of the stress response [54]. Results indicated that orexin stimulation by DREADDs reduced preference for the novel object in defeated rats. Thus, additional exogenous orexin neuron activity has the capacity to influence coping behavior that subsequently impairs recognition memory. Blocking the OX2R with MK-1064 did not alter novel object recognition, indicating that endogenous OX2R activity during social defeat has little impact on recognition memory, and that orexins only modulate spatial and object memory performance under increased levels of stress.

We have previously shown that MK-1064 blocked the CNO-induced body weight decrease during restraint [17], however, in this case, we saw no difference in body weight in our CNO treated socially defeated animals. It is possible orexin effects on body weight are highly dependent on the specific type of stress and effects may be mitigated during a psychological stressor. Social defeat is a more intense and different type of social and psychological stress than restraint, which could account for different patterns of orexin-mediated stress-induced body

weight changes.

4.3 Summary

In summary, orexin activation during social defeat decreased defeat latency to defeat, which has been associated with a vulnerable phenotype; OX2R blockade did not affect defeat latency, suggesting OX2R does not mediate processes in stress coping behavior. This vulnerable phenotype promoted by orexin activation was associated with subsequent changes in anxiety-like behavior and impaired episodic memory performance. Our dose of CNO does not impact social interaction behavior or novel object recognition behavior in the absence of a DREADDs virus, demonstrating that behavioral results can be attributed to changes in orexin signaling, not CNO or clozapine itself [55]. Given the therapeutic potential of manipulating the orexin system for arousal disorders [56] and the link between insomnia and depression [57], it is important to understand the role of orexin signaling in stress coping strategies having the potential to impact subsequent behavior.

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