Exploration of viral strategies to target the Suprachiasmatic Nucleus of the Hypothalamus with inhibitory DREADDs to understand circadian regulation of the brain

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Abstract

Circadian rhythms are generated by the presence of molecular clocks in all cells. Molecular clock timing is controlled by the suprachiasmatic nucleus of the hypothalamus (SCN). Lesions of the SCN result in a lost ability to align molecular clocks to the time of day. It is unknown how the SCN accomplishes this communication with molecular clocks throughout the body due to its very limited range of neuronal projections. A possibility is the recruitment of a systemic hormone with the ability to affect every cell of the body. There is evidence from prior Spencer Lab studies that the hormone CORT (cortisol in humans, corticosterone in rodents), secreted by the adrenal glands, does this. A loss of CORT production due to adrenalectomy (ADX) in rats causes molecular clock disruption. If CORT is injected at the normal peak time of secretion in ADX rats, these rats experienced restored molecular clock function. However, in ADX rats that received CORT 12 hours after peak secretion, molecular clocks did not align their timing to the CORT signal as would be expected if CORT mediates communication of SCN time-of-day signals. The molecular clocks in the SCN were not affected by ADX alone or either treatment. Due to this, the SCN was thought to compete with untimely CORT secretion to prevent circadian alignment. To be able to eventually test the hypothesis that CORT acts separately from the SCN to regulate molecular clocks throughout the body, this honors thesis project sought to develop a method to inhibit the SCN with temporal control and with more neuronal specificity than lesions. Intersectional viral strategies to deliver inhibitory Designer Receptors Exclusively Activated by Designer Drugs (DREADD) were used. The SCN was targeted with an adeno-associated virus (AAV) that expresses a Cre-dependent inhibitory DREADD. Several different SCN projection sites were injected with a retrogradely transported AAV that expresses Cre. The SCN was also targeted with non-Cre dependent viral constructs to

affirm optimal stereotaxic coordinates and effectiveness of different viral serotypes. Despite limited success at expressing inhibitory DREADDs within the SCN, there was consistent DREADD expression in the subparaventricular zone of the hypothalamus (SPZ), which receives the bulk of SCN innervation and which serves as an anatomical relay for the SCN as it is known to be involved in polysynaptic communication between the SCN and other brain regions. An additional experiment already in progress has targeted the SPZ for inhibitory DREADD expression to test if this would be a viable alternative strategy to use in the future to study the role of CORT, independent of SCN output, in aligning molecular clocks.

Introduction

Circadian rhythms are endogenously generated oscillations in behavior and/or physiology that occur over approximately 24-hours. All known organisms have a version of circadian rhythmicity, from prokaryotes like *E. coli* to more complex, multicellular species like humans (Bhadra et al., 2017). Activities such as experiencing hunger or undergoing bowel movements would be disadvantageous to organisms if experienced during inappropriate times (e.g. periods of sleep). Circadian rhythms manage these issues by aligning organism function to timing signals from the environment (e.g. light, temperature) to best conduct activities (Sharma, 2003). In inconsistent (e.g. shift work) or constant environmental signaling situations (e.g. pulling an all-nighter), misalignment between internal time and environmental time-of-day signals can occur (e.g. the circadian clock signaling that it's time to sleep, even though environmental time is the middle of the active phase as occurs in jet lag). Chronic misalignment is associated with development and severity of mental illnesses such as anxiety, depression, and post-traumatic stress disorder (PTSD) (Barnadas et al., 2015; Li et al., 2013). In a post-mortem human brain tissue study examining genes that oscillate in rhythmic fashion over 24 hours, those diagnosed with major depressive disorder (MDD) had significantly less rhythmic expression of genes in brain regions associated with emotional regulation when compared to healthy controls (Edgar & McClung, 2013). Understanding the mechanisms underlying circadian rhythms may present more effective methods to treat these disorders.

Circadian rhythms are generated by a molecular clock composed of transcription-translation feedback loops that occur in all cells of the body (Partch et al., 2014). In mammals, the primary feedback loop is made up of a positive arm comprised of Brain and Muscle ARNT-Like 1 (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) proteins, and a

negative arm constituting Cryptochrome (CRY1/2) and Period (PER1/2/3) proteins (Takahashi, 2017). BMAL1 and CLOCK form a heterodimer and act as transcription factors which bind the E-box in the promoter region of *Cry* and *Per* genes. *Cry* and *Per* subsequently have upregulated transcription and translation. CRY and PER join a roughly 50-protein complex that includes a nuclear transport protein (Aryal et al., 2017). This complex travels back into the cell's nucleus to interfere with BMAL1:CLOCK driven transcription. *Cry* and *Per* transcription is downregulated as a result. CRY and PER are then degraded, allowing BMAL1:CLOCK to bind the E-box and reinitiate *Cry* and *Per* transcription (Eide et al., 2005). This fluctuation of gene expression takes place over a period of approximately 24-hours but when it peaks during the day (i.e. timing of the molecular clock) is adjusted daily from external light cues (Emery et al., 1998; Travnickova-Bendova et al., 2002). Since one cycle of gene expression takes 24 hours, circadian behavior and physiological processes are timed to 24 hours.

Though close, circadian rhythms do not have an exact 24-hour period (Duffy & Czeisler, 2009). In addition, timing of the circadian system must be flexible as molecular clocks need to be properly entrained to changing local light/dark cycles. For example, plane travel across multiple time zones typically induces symptoms of jet lag due to mistiming in molecular clocks and the actual time of day, disrupting appetite, sleep, and other processes (Arendt, 2009). Molecular clocks are not permanently set, and adjustments are made to accommodate new environmental light cycles.

Not all cells have direct access to photic or other environmental cues to adjust their timing. Alignment of timing is controlled by the suprachiasmatic nucleus of the hypothalamus (SCN) (Rusak & Zucker 1979). Organisms lose the ability to align their molecular clock cycles with environmental light signals if the SCN is damaged or lesioned (Gall et al., 2016). The SCN

receives light information from the retina via the retinohypothalamic tract (RHT), allowing for entrainment, or the alignment of the SCN's molecular clock to environmental time-of-day signals like cycles of light and dark (Canteras et al., 2011). The SCN's clock acts as the main "time-keeper" of all molecular clocks of the body. It is unknown how the SCN coordinates this circadian information to the molecular clocks of all cells, as the SCN has few direct projections outside of local hypothalamic regions. It may accomplish this far-reaching signaling through recruitment of a systemic hormone.

A candidate molecule for propagation of timing signals from the SCN to cells throughout the body is CORT (cortisol in humans, corticosterone in rodents). CORT is a steroid hormone of the glucocorticoid class that is readily able to enter cells and interact with intracellular glucocorticoid receptors (GRs). Some clock genes have a glucocorticoid response element (GRE) in their promoter regions (Dong et al., 2011), and PER2 and CRY(1/2) proteins facilitate the binding of GRs to these response elements (Schmutz et al., 2010; Lamia et al., 2011) which may allow CORT to regulate clock timing. The secretion of CORT is regulated in a circadian manner, with the greatest secretion in the period directly preceding wakefulness, with secretion then decreasing progressively throughout the active phase (Spencer & Deak, 2017). CORT secretion is the main output of the hypothalamus-pituitary-adrenal (HPA) axis. The SCN interacts with the HPA axis by projections onto the subparaventricular zone of the hypothalamus (SPZ). The SPZ is the primary relay for circadian information of the SCN in the brain and extensively innervates the paraventricular nucleus of the hypothalamus (PVH), the starting point of the HPA axis (Vujovic et al., 2015). Notably, the SCN is one of the few regions in the body that lacks GRs (Balsalobre et al., 2000), which gives credence to the SCN utilizing CORT for entrainment of other cells in the body without disordering its own molecular clock. The SCN

may use the HPA axis and CORT to undertake entrainment of the brain and body's molecular clocks. Deficits in CORT are of particular interest due to CORT's irregular secretion and associated disruptions of the HPA axis in those with mental illnesses (Spencer & Deak, 2017).

Baseline cortisol secretion was found to be higher in those with an anxiety disorder compared to healthy controls (Mantella et al., 2008; Heinze et al., 2016). CORT secretion and HPA axis function displayed blunted circadian regulation which correlated to severity of anxiety (Adam et al., 2017). Chronic stressors elevate circulating CORT and result in diminished synaptic connectivity and apoptosis of neurons in the prefrontal cortex (PFC), a brain region important for emotional regulation (van Bodegom et al., 2017). The above results when taken together, suggest that there may be an interplay between circadian rhythms, CORT, and mental illness. Although these associations have been established, there is a lack of research delving into CORT's influence on clock gene expression and rhythmicity.

The rhythmic expression of CORT could be used to entrain molecular clocks throughout the body to the time of day and engage their activities accordingly. Adrenalectomies in rats (lesions of adrenal glands; ADX) have been conducted to measure CORT's effects on molecular clocks in cells of the PFC (Woodruff et al 2016). ADX alters daily core clock gene mRNA amplitude and shifts the phase of their transcription, with effects differing on the organs or tissues sampled (Nakamura et al., 2011; Pezuk et al., 2012). ADX rats demonstrate a loss in rhythmicity of molecular clock genes *Per1*, *Per2,* and *Bmal1* in regions of the PFC (Woodruff et. al 2016). To test whether clock gene expression in the PFC was contingent on the presence of CORT and/or time of day administered, ADX male rats received daily intraperitoneal (ip) injections of CORT or vehicle that were given at the normal peak secretion time (in-phase) of CORT in these rats. The in-phase CORT group restored rhythmic clock gene expression in the

PFC. Because CORT was able to restore normal clock gene profiles in ADX rats when given in-phase, it was questioned if the alignment of these genes could be shifted by injecting CORT anti-physically (12 hours after peak CORT) in ADX rats. Anti-phase cohorts were compared to in-phase and vehicle injection receiving ADX rats. The anti-phasic CORT cohort had abolished rhythms of clock gene expression that were significantly worse than the vehicle group. The clock gene expression amplitude had declined, and circadian rhythms flat-lined in the PFC of ADX anti-phase CORT rats. The SCN retained rhythms of *Per1*, *Per2*, and *Bmal1* in all ADX cohorts (Woodruff et. al 2016).

In the experiment, rats retained functional SCN activity which may have prevented alteration to rhythms in the PFC with anti-phasic supplementation of CORT. Competing circadian alignment signals from both CORT and the SCN could have contributed to the flattening of clock gene expression observed. To examine this hypothesis, a method to suppress the SCN would have to be employed.

One such method would be through lesioning. Chemical and electrolytic lesions to the SCN result in permanent impairment of molecular clock timing in all cells (Weaver 1998). These lesions often result in unwanted damage to other brain regions due to the small size of the SCN (Kalsbeek et al., 2000; Kriegsfield et al., 2004). Chemogenetic techniques, such as the intersectional viral approach to neuronal expression of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), can provide better specific targeting of manipulations to regions of interest. DREADDs are mutated human muscarinic acetylcholine G-protein coupled receptors (GPCRs) (Smith et al., 2016). DREADDs allow for controlled excitation or inhibition of cells because once expressed in cells, these receptors require the biochemically inert ligand clozapine-*N*-oxide (CNO) to exert their actions, providing an element of temporal control that

lesioning lacks. DREADD transgenes (including a promoter and fluorescent reporter) are contained in Adeno-Associated Viruses (AAVs) that are surgically delivered to regions of interest. AAVs are widely utilized as a viral vector for transgene incorporation due to their wide array of serotypes and ability to evade immunological responses (Haery et al., 2019). Cre-dependent DREADDs have a double-inverted open reading frame (DIO) that places the DREADD into an inverted, inactive orientation flanked by LoxP sites. The presence of Cre recombinase is required to cleave the LoxP sites around the DREADD gene to reconfigure it for proper transcription (Smith et al., 2016). Eukaryotic cells do not natively express Cre, therefore the Cre transgene has to be introduced to organisms to induce DREADD expression.

Cre recombinase inclusion can be accomplished by injections of viral constructs containing the Cre transgene. Retrograde-traveling AAV (rgAAV)-Cre is injected into neurons that receive innervation from the AAV-DIO-DREADD injected region. The retrograde AAV-Cre is taken up by axon terminals from projecting neurons and is retrogradely transported into the cell's nucleus, where it can then deliver the Cre transgene. Although Cre may be expressed in all cells that project to the AAV-Cre injected region, only neurons with AAV-DIO-DREADD that projected to the AAV-Cre injection site will express the DREADD protein (Roth 2016). To prevent the unspecific effects that lesions introduce, inhibitory DREADDs dependent upon Cre to express were used to achieve SCN shutdown. Microinjection of a DREADD that is not Cre-dependent into the vicinity of the SCN will likely diffuse to and infect adjacent brain regions. Use of an intersectional viral strategy may ensure that DREADD expression is restricted to SCN neurons.

The SCN has sparse projections across the brain. Further still, the SCN has the majority of its projections within hypothalamic/thalamic regions that are relatively close in proximity,

which is troublesome as retrograde-AAV-Cre injections may overlap with AAV-DIO-DREADD injections and prevent specific targeting of the SCN. This project explored several different SCN projection sites as candidates for microinfusion of the retrograde-AAV-Cre, in order to produce inhibitory DREADD expression selectively within the SCN. The parateneal thalamus (PT) and the paraventricular nucleus of the thalamus (PVT) are regions that demonstrated dense efferent projections (Chen & Su, 1990; Watts et al., 1987) and were thought to be sufficiently far from the SCN. Due to these criteria, the PT and PVT were initially chosen to receive retrograde AAV-Cre injections with inhibitory AAV-DIO-DREADD being injected into the SCN to test the viability of further experiments with DREADDs in the SCN. Experiments also used a non-Cre-dependent reporter gene to verify the appropriate stereotaxic coordinates to use for viral injection within the vicinity of the SCN, as well as examined the optimal AAV serotype necessary for adequate infection of SCN neurons. A follow-up experiment examined two alternative SCN projection sites, the anteroventral paraventricular nucleus (AVPV) and the intergeniculate leaflet (IGL). Finally, an experiment still in progress (due to laboratory shut-down by Covid-19 pandemic) examined an alternative strategy of targeting the subparaventricular zone (SPZ) for DREADD expression to functionally inhibit the primary neuronal output from the SCN.

Methods

Animals

Young adult male Sprague Dawley rats were obtained from a commercial vendor (Envigo). Rats were approximately 60 days old upon arrival and were housed in an accredited animal facility at the University of Colorado Boulder (Association for the Assessment and

Accreditation of Laboratory Animal Care, International). Rats were pair-housed in filter top

cages under the following conditions: temperature kept between 20º-22ºC, humidity kept

between 48-52%, and a 12:12 light dark cycle. Rats were provided with standard rat chow and

water *ad libitum*. Prior to surgery, rats were allowed to acclimate for at least one week to their

cages and light cycles. All procedures were conducted in accordance with the ethical treatment

of animals and were approved by the University of Colorado Institutional Animal Care and Use

Committee.

Microinjection Surgery

Ibuprofen drinking water (0.4 mg/mL) was started two days prior to microinjection

surgeries as an analgesic and anti-inflammatory treatment. Rats were administered vaporized

isoflurane anesthesia and positioned in a stereotaxic apparatus, where isoflurane was

administered continuously during surgery. Microinjections were performed with 10 μL Hamilton

syringes (Hamilton, Reno, NV) delivering a rate of 100 nL/min. Ibuprofen drinking water was

continued for three days after each surgery. Subcutaneous injections of both buprenorphine

hydrochloride (Par Pharmaceutical Inc., Endo International) and enrofloxacin (Baytril, Bayer

Corporation) were administered for analgesia and to prevent bacterial infection, respectively.

Intracardial Perfusion

Rats received an intraperitoneal (ip) injection of FatalPlus (Vortech Pharmaceuticals) at a

dose corresponding to their weight ((weight in grams ÷ 500) = mL administered). When reflexes

were no longer present (assessed with tail and paw pinch), a buffer solution of 0.1M PBS (pH

7.4) was injected in rats by securing a needle in their aortas before being switched to a 4%

paraformaldehyde solution in 0.1M PB (pH 7.4). Animals received 400 mL of each solution at a

rate of 0.8 mL/min, were then decapitated, and underwent brain extraction. Brains were placed in 4% paraformaldehyde solution for 24 hours before being transferred to a 30% sucrose solution. Once brains in the 30% sucrose solution had sunk to the bottom of their containers, indicating that brains have adjusted had fully absorbed the sucrose necessary for cryopreservation, brains were flash-frozen in isopentane kept between -30º and -35ºC for 90 seconds. Tissue was stored at -70ºC.

Tissue Collection

Brain tissue slice location and collection sites for regions of interest were determined by comparisons to the Rat Brain Atlas (Paxinos and Watson, 2013). Coronal slices from tissue in all experiments were sliced on a cryostat (Leica CM 1850) at a width of 35 µm between -19 to -23ºC. Tissue was stored in free-floating wells with cryoprotectant at -20ºC.

Microscope Imaging and Analysis

All experimental brain sections were mounted in 0.01M PB and coverslipped with ProLong™ Gold antifade reagent or ProLong™ Gold antifade reagent with DAPI (Thermo Fisher Scientific, USA). Fluorescent images were captured and digitized with a Zeiss microscope (AxioImager M1, Zeiss, Oberkochen, Germany) with an attached Axiocam 305 mono camera. All images were taken with a 10x objective. ZEN Blue (version 2.6) imaging software was used to process and stitch together tiled images.

Cell counts were done manually. Cre-positive cells were identified as bright fluorescent somas in the green channel setting (green fluorescent protein or AlexaFluor 488 detection). DREADD-expressing cells were identified as bright fluorescent soma in the red channel setting

(mCherry fluorescent protein detection). Co-localized cells were identified by a yellow-orange color in the soma after merging of the two fluorescent channel images.

Experiment 1: Use of intersectional viral strategy to attempt silencing of SCN neurons with inhibitory DREADDs

Methods. Rats weighing between 380 - 420g received unilateral microinjections of AAV retrograde (rg)-pmSyn1-eBFP-CRE (Addgene, Cambridge, MA) in the PVT (ML: ±0 mm; AP: -2.2 mm; DV: -5.3 mm; n = 3) or bilaterally in the PT (PT; ML: ±0.5 mm; AP: -1.10 mm; DV: -5.8 mm; n = 3) at a volume of 1000 nL per injection. Injection sites were determined by comparisons to the Rat Brain Atlas (Paxinos and Watson, 2013). These sites were chosen because they received strong innervation from the SCN and were thought to be far enough away from the SCN DREADD injection site to prevent unspecific DREADD expression. When completed, the needle was left in place for an additional 10 minutes before being slowly removed from the site. Rats were allowed to recover for one week. One week later, bilateral injections of AAV8-hsyn-hm4Di-DIO-mCherry (Addgene, Cambridge, MA) at a volume of 1000 nL each were given in the SCN (Angle = 8°; ML: ±1.2 mm; AP: -0.8 mm; DV: -8.9 mm; n = 6). Rats were left for three weeks to allow optimal transgene expression. Tissue was collected after intracardial perfusion. Slices from hypothalamic/thalamic regions spanning ~ 0.4 to 2.1 mm posterior to Bregma (Paxinos and Watson, 2013) were gathered to collect SCN and PT/PVT containing regions. *Results.* DREADD expression was localized to the PVT, even in brains that had targeted Cre in the PT. Both Cre groups did not have DREADD expression in the SCN (see Results Section and Figure 1 for full experimental results).

Experiment 1 Immunohistochemistry (IHC) for Cre expression

Brain sections were placed in free-floating wells containing 0.01M PBS buffer at room temperature. The wells were washed three times for five minutes in 0.01M PBS, with agitation. Sections were moved to solution containing 0.1% sodium borohydride in 0.01M PBS and shaken for 30 minutes. Tissue was washed in fresh 0.01M PBS and three times for five minutes. Sections were then placed in blocking buffer consisting of 0.3% Triton X-100 (Sigma Aldrich, USA), 5% normal goat serum (NGS), 1% bovine serum albumin (Sigma Aldrich, USA), and 0.01M PBS and shaken for 1.5 hours. Tissue sections were incubated on a shaker at 4°C in a solution of 0.3% Triton X-100, 2% NGS, and 0.01M PBS with a 1:1000 dilution of polyclonal mouse anti-CRE (Santa Cruz, USA) antibodies for 48 hours. Tissue was washed in 0.01M PBS for 5 minutes, before being transferred to more PBS and washed 2 times for 15 minutes each. Wells were covered in foil after this step to prevent fluorescent bleaching. Sections were incubated in solution containing 0.3% Triton X-100, 2% NGS, 0.01 M PBS, and a 1:750 dilution of Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (Thermo Fisher Scientific, catalog # A-11029, RRID AB_2534088). Tissue sections were washed in 0.01M PBS for 5 minutes. Two additional PBS washes at 15 minutes each occurred before sections were mounted onto microscope slides in 0.01 PB buffer. Sections were cover slipped in ProLong™ Gold antifade reagent with DAPI. *Results.* In Experiment 1, the IHC revealed Cre localized to the PVT/PT (Figure 1B).

Experiment 2: Validation of appropriate stereotaxic coordinates for targeting the SCN

Rationale. Due to insufficient DREADD expression in the SCN in experiment 1, a viral delivery of a fluorescent reporter (green fluorescent protein; GFP) that can be visualized without amplification by IHC was used to test the SCN stereotaxic coordinates. *Methods.* Six rats previously involved in fear conditioning and extinction experiments that weighed between 340g

– 380g received bilateral injections of AAVrg-pmSyn1-CRE-eGFP (Addgene, Cambridge, MA) in the preceding SCN coordinates (n = 6). A retrograde Cre virus was used to deliver a reporter gene due to prior experimental success in visualizing infection sites. Animals were left for two weeks to allow for optimal transgene expression. Rats were intracardially perfused as previously detailed. Tissue slices spanning the hypothalamic/thalamic regions were collected to visualize where the viral load for SCN coordinates was deposited. *Results.* There was minimal Cre expression in the SCN across all brains (see Results Section and Figure 2 for full experimental results). This may have been due to the viral serotype used to infect cells having poor efficacy in the SCN.

Experiment 3: Testing viral gene expression of differing serotypes of AAVs within the SCN

Rationale. Due to a lack of SCN DREADD transgene expression in Experiment 1, and minimal GFP expression in Experiment 2, a virus of a different serotype, AAV9 rather than AAV8 or AAVrg, was tested to deliver a transgene encoding the fluorescent reporter mCherry (red). *Methods.* Rats that weighed between 390-420g received bilateral injections of AAV9-psyn-mCherry (n = 4), a non-Cre dependent virus. Intracardial perfusions occurred three weeks after SCN injections to allow for optimal transgene expression. Brain tissue was collected as previously described. *Results.* The SCN had greater expression of mCherry reporter than rats injected with AAVrg or AAV8 (see Results Section and Figure 3 for full experimental results).

Experiment 4: Use of intersectional viral strategy to express inhibitory DREADDs in the SCN through retrograde virus delivered to the anteroventral periventricular nucleus (AVPV) or the intergeniculate leaflet (IGL)

Rationale. Due to increased confidence in SCN coordinates in experiment 2 and 3, it was determined that the Cre injection regions (PT/PVT) did not allow adequate Cre expression in the SCN. A new intersectional viral strategy for DREADD expression in the SCN was tested using different Cre regions. *Methods.* Rats that weighed between 370g - 440g received bilateral injections of AAV8-hsyn-DIO-hm4Di-mCherry (n = 8) in the SCN. During the same surgery, animals also received bilateral injections of pENN-AAVrg-hsyn-HI-eGFP-Cre (Addgene, James M. Wilson) in either the AVPV (AP: -0.1 mm, ML: ± 0.2 mm, DV: -8.4 mm; n = 4) or the IGL (AP: -4.5 mm, ML: ± 4.0 mm, DV: -5.2 mm; n = 4). Injection coordinates were determined from the Rat Brain Atlas (Paxinos and Watson, 2013). These areas were found to have extensive projections from the SCN (Watts et al., 1987) and were located at a sufficient distance from the SCN that Cre injection overlap with DREADD injections was not likely. Animals were left for three weeks to allow for optimal transgene expression before intracardial perfusion and tissue collection. *Results.* There was insufficient DREADD expression in the SCN in both the AVPV- and IGL-Cre treatment groups, but there was a good amount of DREADD expression in the subparaventricular zone (SPZ) (see Results Section and Figure 4 for full experimental results).

Experiment 5: Functional test of the use of intersectional viral strategy to silence the primary SCN relay nucleus, the subparaventricular zone of the hypothalamus, with inhibitory DREADDs

Rationale. Results from experiment 4 showed a limited amount of SCN DREADD expression. However, results showed consistent expression of DREADDs in the subparaventricular zone of the hypothalamus (SPZ), a region that receives the bulk of SCN efferents. The SPZ innervates a wide range of brain regions to relay circadian information for the SCN. Silencing the SPZ may therefore have the same expected outcome from silencing the SCN.

From Experiment 4 it was found that the median preoptic area (MnPO) highly expressed Cre

(see Figure 4B) and received a comparable amount of innervations from both the SCN and SPZ,

so it was chosen as the Cre retrograde virus injection site. This experiment also incorporated a

functional test of inhibitory DREADD activation immediately before rats were sacrificed. Rats

were exposed to a light-pulse during their dark phase, which has previously been shown to lead

to neuronal activation in the SCN (Highland et al, 2013) and the SPZ (Nakamura et al., 2008).

The functional component of this experiment will determine whether activation of inhibitory

DREADDs in the SPZ prior to the light pulse prevents SPZ neuronal activation (as assessed by

c-Fos, a neuronal activation marker).

   Methods. Animals weighing between 270g – 330 g received bilateral injections of either

AAV8-hsyn-DIO-hm4Di-mCherry (n = 12) or AAV8-hsyn-DIO-mCherry (Addgene,

Cambridge, MA) (n = 12) in the SPZ (AP: -1.3 mm, ML: ± 0.3 mm, DV = -9.3 mm). The non-

hm4Di virus variant served as a control virus for off-target CNO effects. In the same surgery,

animals received a unilateral injection of AAVrg-hsyn-HI-eGFP-Cre (n = 24) in the MnPO (AP:

+0.24 mm, DV = -7.8 mm). Animals were left for three weeks to allow for optimal transgene

expression before further manipulation.

   Rats were randomly assigned to receive a light pulse (n = 12) or to stay on their normal

L:D cycle (n = 12). Randomization was balanced such that all groups had equal numbers of rats

that received DREADD or control virus injections. Light pulses were given 4 hours after

initiation of the dark phase. All animals were given an ip injection of Clozapine-*N*-Oxide (CNO)

at a dose of 3 mg/kg. A 30-minute light pulse (or no light pulse for control rats) was given 60

minutes after CNO injection, after which all animals underwent rapid decapitation. CNO was

delivered 60 minutes prior to the light pulse to maximize its potential ligand binding to

DREADD receptors (Maclaren et al., 2016). Rapid decapitation was chosen as this allows for conducting *in situ* hybridization to facilitate viewing of *c-fos* and *Per1* mRNA in the SPZ. Trunk blood was collected for use in CORT and melatonin (two circadian-regulated hormones) assays. *c-fos* and *Per1* mRNA are shown to be transcribed in response to light pulses, with peak accumulation of mRNA occurring 20-30 minutes after a light pulse (Lu et al., 2001; Schwartz et al., 2011). Brains were extracted and placed in isopentane solution for 90 seconds, with isopentane kept between -30º to -40ºC.

Coronal slices from tissue in all experiments are to be sliced on a cryostat (Leica CM 1850) at a width of 12 µm between -19 to -23ºC. Tissue will be thaw mounted directly after slicing onto microscope slides. In situ hybridization will be performed on microscope slides for both *c-fos* and *Per1* mRNA by utilizing RNA probes with fluorescent reporters.

Results:

Experiment 1: Use of intersectional viral strategy to attempt silencing of SCN neurons with inhibitory DREADDs

This first experiment examined whether our initial choice of viruses and stereotaxic coordinates are effective in producing inhibitory DREADD expression selectively within the SCN of rats. Microinfusion of an AAV virus that expresses Cre-dependent inhibitory (hm4Di) DREADD with mCherry reporter protein was targeted to the SCN. Microinfusion of a retrograde AAV virus that expresses Cre was visualized with a green secondary antibody (AlexaFluor 488; AF488) and was targeted for two brain regions that are known SCN projection sites (PVT and PT). With this intersectional viral strategy, we will see mCherry expression only in neurons that were infected by both viruses. We found that there was indeed mCherry expression in the brains

of rats in this experiment, but only in those that received Cre in the PVT. Unexpectedly, we found that mCherry expression was primarily observed in brain regions outside the SCN (Fig. 1). mCherry was localized to the PVT, with a strong, bilateral profile of expression. Weaker expression was also found in the adjacent PT, with roughly equal expression in both hemispheres. Oddly, mCherry expressing cells did not co-localize with AF488 (indicating presence of Cre) positive cells. From this experiment, we were unable to determine if the choice of virus and stereotaxic coordinates were proper to achieve DREADD expression in the SCN. One possibility was that the two viruses had an overlapping zone of diffusion which then allowed for Cre-dependent DREADD expression, but that zone of overlap did include the SCN. Another possibility was that there was an intersection of viral expression via the retrograde transport of the AAV-Cre, but that the neuronal pathway involved did not include SCN projection neurons.
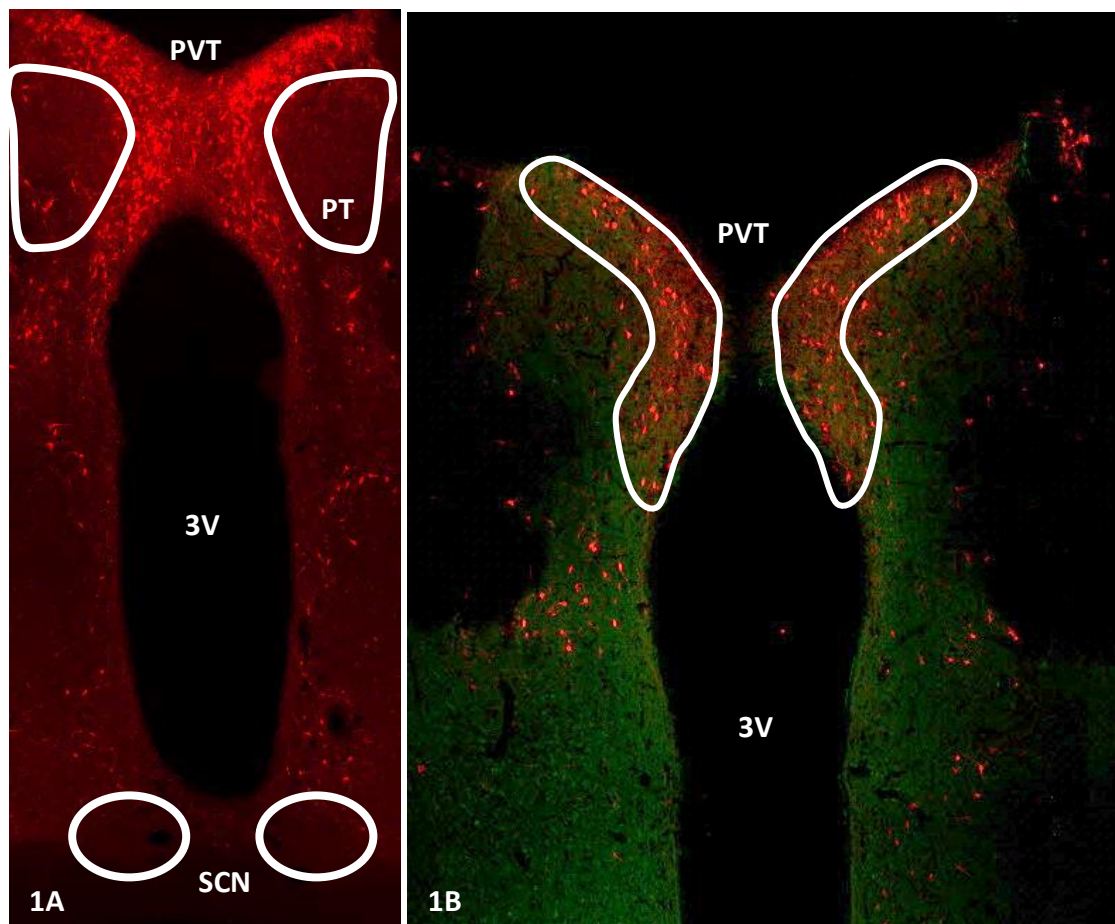
Figure 1:

Photomicrographs containing tissue at the level of the SCN, PT, and PVT. DREADD expression (mCherry reporter) is denoted by red fluorescence. mCherry was found in cells of the PVT (A-B), along with Cre, indicating DREADD expression in the PVT. The SCN did not have DREADD expression (A). PT-Cre brains did not express DREADD.

Experiment 2: Validation of appropriate stereotaxic coordinates for targeting the SCN

The second experiment addressed whether the choice of stereotaxic coordinates for targeting the SCN were correct. Microinfusion of an AAV virus that expresses enhanced green fluorescent protein (eGFP) as a reporter gene was utilized to determine where viral loads were being deposited and where expression was occurring. We found that eGFP expression did occur in the SCN (Fig. 2). However, expression of Cre occurred in only one brain and was localized in one hemisphere, with only a minority of the SCN displaying expression. None of the other brains expressed Cre. From this experiment, we saw that the SCN could be targeted, but we were still

unsure if the current stereotaxic coordinates for the SCN were the most appropriate. In addition, hypothesized that the low expression profile across all brains may indicate that the SCN is resistant to infection by certain viral serotypes.
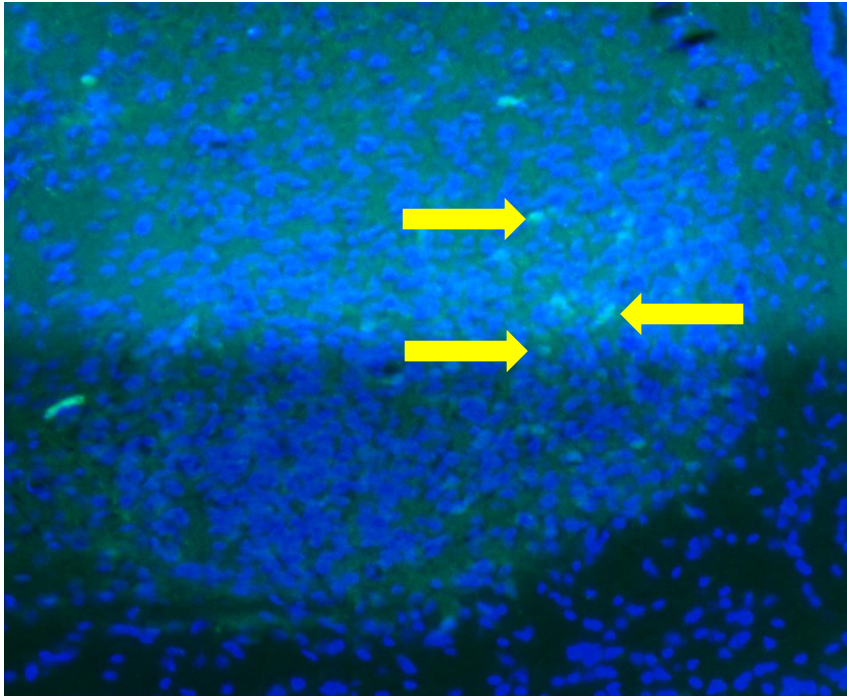

Figure 2:

Photomicrograph of tissue SCN of rats that received AAVrg-pmSyn1-CRE-eGFP co-stained with DAPI (blue) and expressing Cre (green). Arrows point at cell bodies with both GFP and DAPI.

Experiment 3: Testing DREADD expression using a different serotype of AAV within the SCN

The third experiment utilized a different viral serotype containing a non-Cre dependent transgene with only a fluorescent marker: mCherry. An AAV9 viral construct was used as it has demonstrated a higher range of cell type promiscuity than other AAV serotypes and may then be able to express its transgene in the SCN if the stereotaxic coordinates were correct. We found that expression of mCherry was much stronger in brains from this experiment. The SCN successfully expressed mCherry with a bilateral profile (Fig. 3). Other regions that expressed the

transgene included the medial preoptic area (MPOA), striatum, subparaventricular zone of the

hypothalamus (SPZ), and regions of the PT/PVT. From this experiment, we determined that the

stereotaxic coordinates to target the SCN were not the issue as we had mCherry expression. The

sites chosen for retrograde Cre may have been the underlying issue. However, this experiment

did illustrate that microinfusion of a virus in this region will diffuse to other brain regions outside

of the SCN, reinforcing the importance of optimizing our approach to restrict DREADD
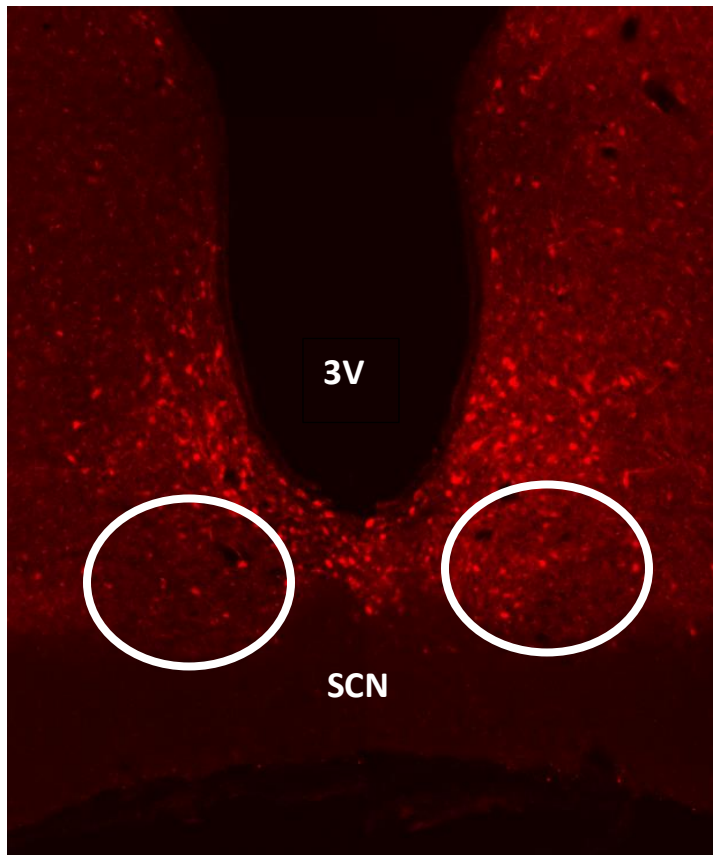
expression to the SCN.

Figure (Exp 3):

Photomicrograph showing tissue at level of SCN after injection
with AAV9-psyn-mCherry. Successful transgene expression is
denoted by red fluorescence. Both hemispheres of the SCN
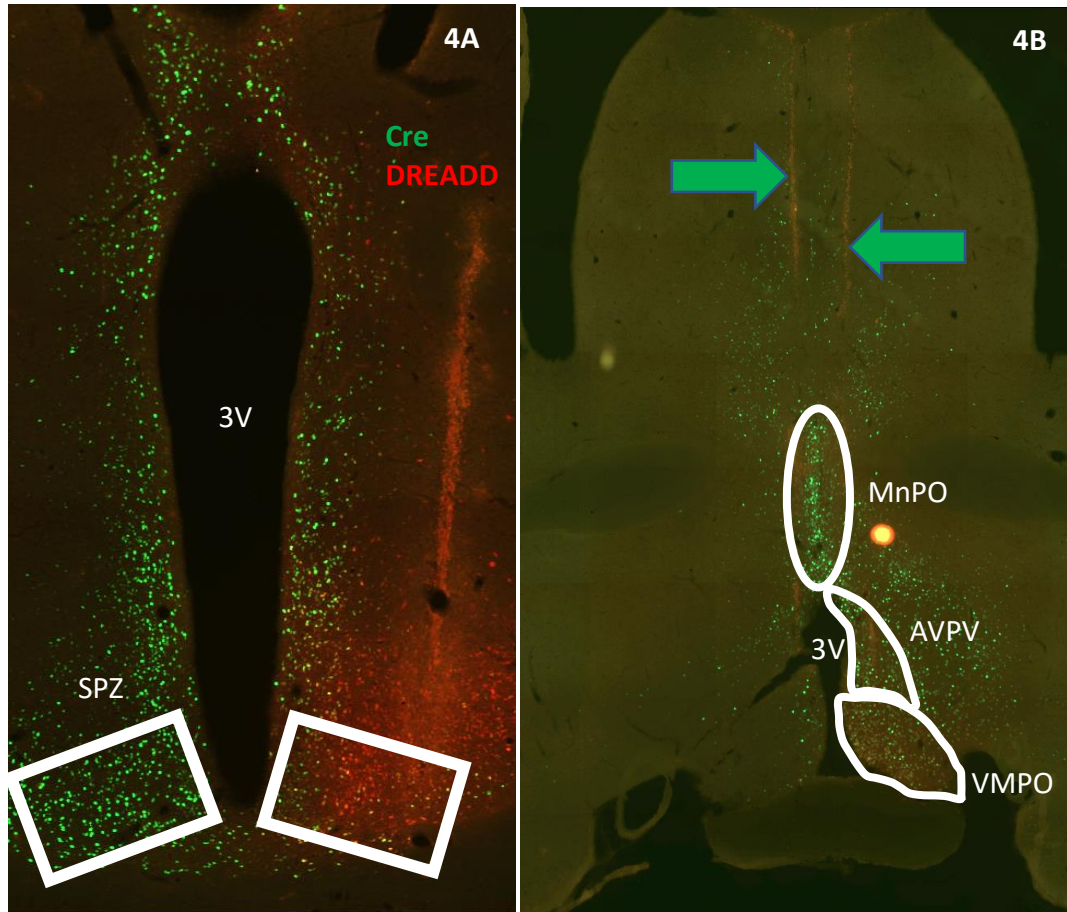indicate successful expression.

Figure 4:

Photomicrographs of brains that received AAVrg-Cre-eGFP in their AVPVs (A-B). Strong DREADD expression is found in the SPZ and indicated by red fluorescence (A). There is successful transgene expression of Cre in the AVPV, indicated by green fluorescence (B), along with expression in the MnPO and VMPO, regions that receive innervations from the SCN.

Experiment 4: **Use of intersectional viral strategy to express inhibitory DREADDs in the SCN through retrograde Cre-expressing virus delivered to the anteroventral periventricular nucleus (AVPV) or the intergeniculate leaflet (IGL)**

The fourth experiment re-explored the intersectional viral strategy approach. Microinfusions of AAV-Cre-dependent inhibitory DREADD used the same SCN stereotaxic coordinates as used in the first 3 experiments. Cre-containing AAVs were microinfused into the AVPV and IGL instead of the PVT and PT. Both regions have demonstrated SCN projections and were located farther away from the SCN (and each other) than the PT/PVT. We found that

Cre-dependent DREADD (mCherry reporter) expression did occur, but again was primarily observed in regions outside of the SCN. IGL brains did not display any fluorescence to indicate successful Cre and DREADD injection locations. AVPV brains demonstrated strong fluorescence of both Cre-GFP and DREADD-mCherry. eGFP was found primarily in regions of the AVPV, MnPO, and the ventral medial preoptic area (VMPO) (Fig. 4B). Of the AVPV brains, one brain demonstrated successful DREADD integration into the SCN. mCherry and GFP expression in this SCN was weak and only occurred in one hemisphere. Stronger DREADD-mCherry expression was observed in the surrounding tissue with expression in the following: the MPOA, medial preoptic nucleus (MPON), striatum, SPZ, PT/PVT, and the subfornical organ (SFO). mCherry expression occurred primarily in one hemisphere while the other hemisphere of the same tissue had extensive eGFP expression, which may have resulted from a misplaced DREADD injection in one hemisphere. From this experiment, we found that the retrograde AAV-Cre injection site for the AVPV was successful and allowed for Cre-dependent DREADD expression in areas surrounding the SCN, but not in the SCN. Of note, a prominent DREADD-mCherry expressing area is the SPZ (Fig. 4A), a region that receives the bulk of SCN innervations and correspondingly coordinates circadian information to widespread regions. This area was targeted in Experiment 5.

Experiment 5: Functional test of the use of intersectional viral strategy to silence the primary SCN relay nucleus, the subparaventricular zone of the hypothalamus, with inhibitory DREADDs

The fifth experiment was done to examine whether the intersectional virus approach for selective inhibitory DREADD expression can be developed for the SPZ instead of the SCN. If the SPZ receives the majority of SCN projections and relays circadian information, it should be

viable to inhibit the SPZ and expect results to be similar to inhibiting the SCN. Due to the COVID-19 pandemic, brains from this experiment have yet to be examined.

Discussion

In these experiments, I aimed to develop a method by which to selectively and temporarily inhibit the SCN. Past experiments targeting the SCN for lesioning have demonstrated that the SCN is missed entirely, partly lesioned, and/or involves extensive off target damage to other brain regions (Kriegsfield et al., 2004; Liu et al., 2012). As it is a permanent and irreversible manipulation, lesioning also does not allow temporal control in experiments to measure circadian behaviors or gene expression. To address these issues of lesions, an intersectional viral strategy for expression of inhibitory DREADD was used to inhibit the SCN. Microinfusing the PVT/PT brain regions with a retrograde AAV-Cre virus and targeting the SCN with an AAV-Cre-dependent DREADD virus yielded insufficient expression of DREADDs in the SCN. However, the PVT, but not the PT, expressed DREADDs. A possible explanation could be the PVT/PT's bi-directional connections with the SCN. The PVT/PT both receive innervations from and project to the SCN. Previous unpublished experiments in our lab have shown expression in areas that are not expected, given the intersectional target regions used. The virus may have the ability to travel transynaptically (Salegio et. al 2013), which would be sufficient to cause DREADD expression in the PT/PVT.

The mechanisms surrounding AAV serotype transport are not well defined and may exert different effects in differing brain regions (Aschauer et al., 2013). In Experiments 1 and 4, we used an AAV8 serotype that contained the Cre-dependent DREADD transgene. Another

possibility is the AAV-Cre-dependent DREADD virus diffused to the site of the retrograde AAV-Cre virus infusion site, including the possibility of traveling through the third ventricle due to its proximity to the SCN injection sites. Patterns of expression in brain tissue indicate that this is unlikely (e.g. expression of Cre or DREADD in only one hemisphere indicate some level of selective expression).

To validate the stereotaxic coordinates used for the SCN, a different viral vector was used that did not require intersection between two different viruses in order to see reporter gene expression. We chose to use a retrograde AAV-Cre virus that had a GFP reporter that is readily visible without need for amplification via IHC. Although we did see expression in the SCN, this was not sufficiently expressed for a functional manipulation in the SCN. This is in line with previous results displaying the difficulty of targeting the SCN (Watts et al., 1987; Kriegsfield et al. 2004). The virus used in the second experiment did express some Cre in the SCN but was inconsistent. Expression may have been weaker in this experiment due to waiting only two weeks after surgery for optimal transgene expression. Visualization of Cre may be better accomplished if immunohistochemistry against Cre was used in these brains to amplify Cre signal

In the third experiment, animals that received AAV9-psyn-mCherry targeting the SCN had good mCherry expression in the SCN and surrounding regions. The AAV9 serotype has been shown to successfully infect a variety of cell types that other serotypes (e.g. AAV8) do not (Dayton et al., 2012). Of its 10,000 neurons, the SCN contains neurons that signal with GABA, glutamate, arginine vasopressin (AVP), and vasoactive intestinal peptide (VIP), among others (Welsh et al., 2010). This make-up of the SCN may confer differential cell membrane structure and prevent certain serotypes from optimal infection. We saw better viral expression with

AAV9, therefore using an inhibitory DREADD in this viral construct may be better in targeting similar regions in future experiments.

As the SCN stereotaxic coordinates appeared to be sufficient to obtain viral infection and gene expression in the SCN, it shifted credence to the likelihood that the PVT/PT regions were not adequate regions to target with retrograde AAV-Cre in order to produce Cre-dependent DREADD expression in the SCN. Thus, Experiment 4 tested whether the AVPV and IGL could be used instead as they also demonstrated significant SCN projections without the possible bi-directional properties of the PVT/PT (Watts et al., 1987; Vujovic et al., 2015). However, rats in this experiment did not demonstrate sufficient DREADD expression in the SCN in either group. Instead, there were off-target sites restricted to the hypothalamus that expressed DREADDs in the AVPV group. Of interest to our lab was that one consistent site was the SPZ. The SPZ receives the bulk of SCN efferents and relays circadian information to the septal region, thalamus, hypothalamus (including the paraventricular nucleus), and brainstem, among other brain regions (Leak and Moore 2001). It may be that the SPZ, as the relay, is receiving the retrograde Cre in place of the SCN. The SPZ projects to the same regions that the SCN targets, and all of the regions that received Cre in these experiments were areas that the SPZ projected to densely. Instead of avoiding this region by trying different Cre and DREADD virus injection coordinates for the SCN, we may target SPZ with inhibitory intersectional DREADDs. The viability of this region is greater than the SCN as the SPZ offers a larger size to target and has a similar role to the SCN. From these results and supporting evidence that the SPZ is the main circadian relay of the SCN, the SPZ is a prime target for future experiments focused on utilizing intersectional DREADDs to inhibit circadian output. Moreover, the critical role of the SPZ as a major functional relay of SCN circadian information has not been previously tested. Therefore,

these future studies would provide important anatomical information about SCN efferent function.

If subsequent experiments targeting the SPZ do not work, another avenue to consider would be using adjusted promoter sequences. Most SCN and SPZ neurons are GABAergic, so a promoter targeting GABA (e.g. GAD) may be able to express in more cells (Todd et al., 2018). In the case of insufficient DREADD expression in the SPZ using the MnPO as the AAV-Cre site, targeting multiple brain regions with AAV-Cre that receive strong innervations may bolster DREADD expression. Transgenic animals expressing Cre in SCN-specific cell types (e.g. VIP-expressing neurons) also present a viable option to express inhibitory Cre-dependent DREADDs in the SCN (Jones et al., 2018).

Conclusion

The SCN is considered the "master pacemaker" of circadian rhythms due to its role in aligning molecular clocks throughout the body to the perceived time-of-day from light information. However, it is unknown how the SCN communicates this information globally. A systemic hormone, CORT, is hypothesized to contribute to this alignment. Abnormalities in either the CORT system or the SCN can lead to circadian misalignment. Those diagnosed with a mental illness like anxiety or depression often have misalignment to their circadian rhythms. Anxiety is attributed to circadian dysregulation in areas like the PFC, and CORT is shown to have heightened secretion in these individuals. Research examining molecular clocks of the brain and their relation to mental illnesses have shown disruption to several brain regions associated to mental illness. Furthermore, some medications for mental illnesses are able to decrease the

severity of misalignment and improve symptomology. Manipulating the SCN using DREADDs offers a technique that may afford answers to how alignment of molecular clocks occurs in relation to CORT (particularly in the brain) and may identify new treatment avenues for mental illnesses.

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